DETECTION AND STUDY OF GENETIC DIVERSITY OF SELECTED VECTOR-BORNE PROTOZOA AND RICKETTSIA IN BUFFALOES IN THAILAND BY BIOMOLECULAR METHOD

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การตรวจหาและการศึกษาความหลากหลายทางพันธุกรรมของเชื้อโปรโตซัวและริกเก็ตเซียที่มีพาหะนำโรคในกระบือในประเทศไทยด้วยวิธีการทางอณูชีววิทยา

น.ส.หวง ลาน อัน เหงียน

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Thesis Title	DETECTION AND STUDY OF GENETIC DIVERSITY OF SELECTED VECTOR-BORNE PROTOZOA AND RICKETTSIA IN BUFFALOES IN THAILAND BY BIOMOLECULAR METHOD
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การตรวจหาและการศึกษาความหลากหลายทางพันธุกรรมของเชื้อโปรโตซัวและริกเก็ตเซียที่มีพาหะนำโรคในกระบือในประเทศไทยด้วยวิธีการทางอณูชีววิทยา. (DETECTION AND STUDY OF GENETIC DIVERSITY OF SELECTED VECTOR-BORNE PROTOZOA AND RICKETTSIA IN BUFFALOES IN THAILAND BY BIOMOLECULAR METHOD) อ.ที่ปรึกษาหลัก : มรกต แก้วธรรมสอน, อ.ที่ปรึกษาร่วม : สนธยา เตียวศิริทรัพย์

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบการเกิดขึ้น การกระจายตัวทางภูมิศาสตร์และความหลากหลายทางพันธุกรรมของโปรโตซัวและริคเก็ตเซียที่มีเห็บและยุงเป็นพาหะในกระบือด้วยปฏิกิริยาลูกโซ่พอลิเมอเรส (PCR) และการหาลำดับดีเอ็นเอ (DNA sequencing) เก็บตัวอย่างเลือดได้จำนวนทั้งสิ้น 456 ตัวอย่าง จาก 8 จังหวัดของประเทศไทย ได้แก่ มุกดาหาร อุทัยธานี ลำปาง อำนาจเจริญ หนองบัวลำภู พัทลุง สุรินทร์ และฉะเชิงเทรา ตัวอย่างเลือดได้ถูกนำมาสกัดดีเอ็นเอและตรวจหาการมีอยู่ของเชื้อ ตามด้วยการหาลำดับดีเอ็นเอ จากผลการศึกษาพบว่าความชุกรวมของริคเก็ตเซีย โปรโตซัวและมาลาเรียคิดเป็นร้อยละ 41, 25.4 และ 12.9 ตามลำดับ พบการติดเชื้อสองชนิดและสามชนิดร่วมกันอยู่ที่ร้อยละ 19.1 จากตัวอย่างทั้งหมด พบความสัมพันธ์อย่างมีนัยสำคัญทางสถิติระหว่างการติดเชื้อ *A. marginale* กับเพศของกระบือด้วย Pearson's Chi-squared test เมทริกซ์เอกลักษณ์นิวคลีโอไทด์ (nucleotide identity matrix) และแผนภูมิวิวัฒนาการ (phylogenetic tree) ของ *A. marginale* และ *T. orientalis* แสดงให้เห็นว่าเชื้อทั้ง 2 ชนิดมีความคล้ายคลึงกันในระดับสปีชี่ส์และมีความสัมพันธ์ใกล้ชิดกันทางวิวัฒนาการ ค่าความหลากหลายของนิวคลีโอไทด์แสดงให้เห็นว่าความหลากหลายทางพันธุกรรมของ *A. marginale* และ *T. orientalis* อยู่ในระดับต่ำ เนื่องจากคุณสมบัติการอนุรักษ์ของยีน อย่างไรก็ตามยีน cytochrome b ในประชากรของเชื้อ *P. bubalis* มีตำแหน่งที่แตกต่างและพบความหลากหลายของนิวคลีโอไทด์อย่างมาก การศึกษานี้ยังเผยให้เห็นการติดเชื้อ *A. platys* ในกระบือเป็นครั้งแรกและพบถิ่นของการระบาด (endemic area) ของมาลาเรียในจังหวัดอื่นๆ ที่ยังไม่เคยทำการศึกษาอีกด้วย.

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Hoang Lan Anh Nguyen : DETECTION AND STUDY OF GENETIC DIVERSITY OF SELECTED VECTOR-BORNE PROTOZOA AND RICKETTSIA IN BUFFALOES IN THAILAND BY BIOMOLECULAR METHOD. Advisor: Assoc. Prof. MORAKOT KAEWTHAMASORN, Ph.D. Co-advisor: Assoc. Prof. SONTHAYA TIAWSIRISUP, Ph.D.

This study aimed to determine the occurrence, geographical distribution and genetic diversity of tick and mosquito-borne protozoa and rickettsia in buffaloes by the PCR – sequencing approach. A total of 456 blood samples were collected in provinces of Thailand including Mukdahan, Uthai Thani, Lampang, Amnat Charoen, Nong Bua Lamphu, Phatthalung, Surin and Chachoengsao. DNA was extracted from buffalo blood and screened for the presence of pathogens, followed by sequencing. The results indicated that the overall prevalence of rickettsia, protozoa, and malaria were 41%, 25.4%, and 12.9% respectively. Double and triple infection of buffaloes with pathogens were observed in 19.1% of the samples. A significant association between *A. marginale* infection and the buffalo gender was seen by Pearson's Chi-squared test. Nucleotide identity matrixes and phylogenetic trees of *A. marginale* and *T. orientalis* indicated that those isolates had high similarity and close relationships with each other. Nucleotide diversity values suggested that low levels of polymorphism and genetic diversity of *A. marginale* and *T. orientalis* were observed between different isolates owing to the highly conserved property. However, cytochrome b in *P. bubalis* population showed a higher number of segregating sites and nucleotide diversity. The present study revealed the infection of *A. platys* in buffaloes for the first time and extended the endemic area of malaria parasites to other unexplored provinces.

Field of Study: Veterinary Science and technology — Student's Signature .....

Academic Year: 2019 — Advisor's Signature .....

Co-advisor's Signature .....

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Hoang Lan Anh Nguyen

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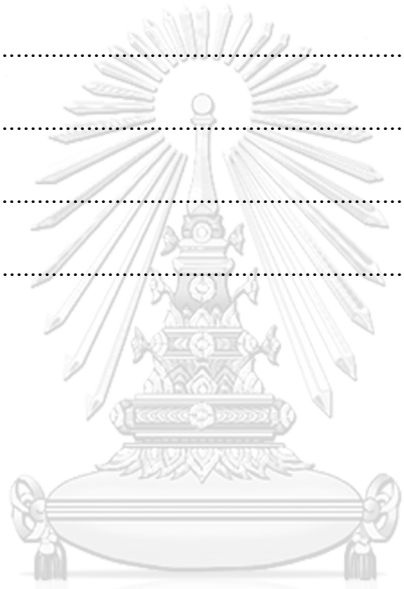

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CHAPTER 1

INTRODUCTION

The Asian water buffalo (*Bubalus bubalis*) was originally domesticated in China and India from wild water buffalo (*Bubalus arnee*) 4 to 5 millennia ago and widely distributed in the present day (Yang et al., 2008). The native *B. bubalis* was believed to originate from South Asia, Southeast Asia and China. Presently, it is also raised in Europe, South America, and some African countries. The global water buffalo population in 2011 was approximately 172 million heads with more than 97% was found in Asia (Borghese, 2011). In Thailand, the number of water buffalo declined from more than 3.3 million heads to less than 1.24 million within the 16-year period between 1996 and 2011 (Uriyapongson, 2013). However, buffaloes still sustain integral roles in Thai culture and society, not only for economic value but also for social development. In the past, buffaloes were used as power for ploughing and drafting on dry or wet land. Approximately 30–50% of bovine in Asia are employed for draft operations in agriculture (Parikh, 1988). River buffaloes have higher lactation yield than swamp buffaloes and are raised mainly for meat and milk. Generally, it was documented that buffalo's meat and milk were healthier for human consumption than cow's products. Buffalo meat contains lower level of saturated fat, less cholesterol and calories, more protein and minerals than beef. It was also reported that buffalo milk has higher fat, protein, lactose and ash in comparison to cow and goat milk. Besides food-providing role, buffalo's dung can be used as fertilizer or as fuel for cooking, heating and marking biogas. Furthermore, buffaloes have contribution to Thai's entertainment including annual racing festival in Chonburi and agricultural-style tourism in Chiang Mai (Nanda and Nakao, 2003). Despite the fact that buffaloes have great benefits for human society, they are also known to be a potential reservoir for

many diseases including bovine babesiosis, theileriosis, rickettsioses and malaria that resulted in significant economic losses (Kocan et al., 2010; Templeton et al., 2016; Wang et al., 2018). At present, the rising need of buffaloes for its meat and milk has led to a growing demand of disease-free buffaloes.

Anaplasmosis is one of the most common tick-transmitted diseases which causes high morbidity, mortality and economic losses in both wild and domestic ruminants all over the world. The causative organisms are obligatory intraerythrocytic rickettsia namely *Anaplasma marginale* and *A. centrale*. Two new species of *Anaplasma* i.e. *A. phagocytophilum* and *A. bovis* were discovered in 2001 in rodent reservoir (Dumler et al., 2001). They have been reported to infect cattle, but do not cause clinical disease (Hofmann-Lehmann et al., 2004; Dreher et al., 2005). *A. marginale*-infected cattle were manifested by progressive anemia, jaundice, fever, loss of appetite and decreased milk production (Kuttler, 1984). *A. marginale* has been also found in other species such as swamp buffaloes in northeast Thailand (Saetiew et al., 2013), white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*Odorcoileus hemionus columbianus*), mule deer (*Odocoileus hemionus*), American bison (*Bison bison*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) (Rymaszewska and Grenda, 2008). Besides *A. marginale*, *A. centrale* has capability to induce moderate anemia but its clinical signs seem to be asymptomatic. *A. centrale* is utilized to produce a live vaccine for cattle in Australia, Africa, Israel and South America (de la Fuente et al., 2005).

Bovine babesiosis is one of the deadliest and most economically important diseases in ruminants in tropical countries. The causative agent is an intraerythrocytic protozoan parasite in the genus *Babesia*. Five *Babesia* species, namely *Babesia bovis* (Asiatic red water), *B. bigemina* (African red water), *B. orientalis, B. major*, and *B. ovata*,

have been identified in cattle worldwide, which are usually transmitted by the one-host ticks *Rhipicephalus decoloratus* and *R. microplus* (Bock et al., 2004). *B. bovis* is known to be the most virulent and lethal species in cattle. Unlike most of the other animal parasites in which they are host specific, *B. bovis* appears to be capable of infecting the other ruminant species including water buffalo. Terkawi and colleagues investigated about the molecular prevalence of *B. bovis* and *B. bigemina* in water buffaloes in northeast regions of Thailand by nested PCR targeting the spherical body protein 4 and rhoptry-associated protein 1 genes, respectively (Terkawi et al., 2011a).

Theileriosis is a common tick-borne disease found in a wide range of ruminants comprising cattle and buffaloes. It is caused by an intracellular protozoan in the genus *Theileria* spp. *Theileria* is phylogenetically closely related to *Babesia*, an obligatory intracellular protozoan that invades only erythrocytes while *Theileria* has capability to invade both erythrocytes and leukocytes of ruminants. Theileriosis is an economically important disease since ruminants are usually asymptomatic carriers which result in production losses (Bishop et al., 2004). *T. parva* and *T. annulata* were believed to be the most pathogenic species causing lymphoproliferative disease and high mortality rates for cattle (Onuma et al., 1998). Other *Theileria* species including *T. sergenti*, *T. buffeli* and *T. orientalis* are described as benign pathogenic parasites in cattle and small ruminants. According to research study of Altangerel and colleagues, there were 7 genotypes of *T. orientalis* in water buffaloes in northeast regions of Thailand consisting of genotype 1, 3, 4, 5, 7, N2 and N3 based on analysis of major piroplasms surface protein (MPSP) gene. Among that, genotype 7 was predominantly detected in all locations surveyed (Altangerel et al., 2011).

Malaria – an infectious mosquito-borne disease, is one of the most significant diseases that has attracted considerable concerns from humans for a long time. The

causative agent of malaria is an intracellular protozoan parasite of the genus *Plasmodium*. Malaria parasites of even-toed hoofed mammals were first reported in 1913 from duiker antelope in Malawi (*Plasmodium cephalophi*) (Bruce et al., 1913), followed by a second report in grey duikers (*P. brucei*) and additional descriptions in water buffalo (*P. bubalis*), goat (*P. caprae*), mouse deer (*P. traguli*), and North American white-tailed deer (*P. odocoilei*) (Sheather, 1919; Mello and Paes, 1923; Garnham and Edeson, 1962; Garnham and Kuttler, 1980; Perkins and Schaer, 2016; Templeton et al., 2016). Until now, there have been very limited number of research studies in ungulate malaria parasites using molecular biology approach. Recently, studies led by Templeton et al. (2016) and Kaewthamasorn et al. (2018) confirmed the findings of malaria in water buffaloes and goats in Thailand and later followed by the two reports in pamper deer by Asada et al (2018) and dos Santos et al (2018) in Brazil.

In Thailand, the prevalence, dispersal and genetic variety of anaplasmosis, piroplasmosis, babesiosis and malaria in the indigenous water buffaloes and in the insect vectors remain largely unexplored and outdated. Most of the studies in the country mainly focused on dairy and beef cattle. The present study, therefore, aims to determine the occurrence, geographical distribution and genetic diversity of vector-borne protozoa and rickettsia in buffaloes by molecular biology approach.

Objectives of Study

1. To address the occurrence of *Ehrlichia* spp., *Anaplasma* spp.; *Plasmodium* spp., *Babesia* spp. and *Theileria* spp. in buffaloes in different geographic locations of Thailand using PCR-based detection.

2. To differentiate and genetically characterize *Ehrlichia* spp., *Anaplasma* spp., *Plasmodium* spp., *Babesia* spp., and *Theileria* spp. based on DNA sequences.

Keywords (Thai): เลือด กระบือ การตรวจหา ความหลากหลายทางพันธุกรรม โปรโตซัว ริกเก็ตเซีย

Keywords (English): Blood, Buffaloes, Detection, Genetic diversity, Protozoa, Rickettsia

Hypothesis

The occurrence of tick and mosquito borne infections of the following parasites: *Ehrlichia* spp., *Anaplasma* spp.; *Plasmodium* spp., *Babesia* spp. and *Theileria* spp. in buffaloes are dynamic. Geographical distribution and genetic diversity of pathogens mentioned before are unique to buffaloes in Thailand.

CHAPTER 2

LITERATURE REVIEW

Tick-borne infections are only the second to mosquito-borne diseases in terms of human and animal health concerns in all over the world. Bovine anaplasmosis, babesiosis and theileriosis emerge as the most important arthropod-borne pathogens that have a negative influence on humans and animal health owing to considerable morbidity, mortality and economic damages in livestock in the tropical and subtropical countries (Anwar, 1974; Uilenberg, 1995; Vidotto et al., 1998; Kocan et al., 2010). Asian water buffalo is known as a reservoir host for various tick-borne protozoa and rickettsia which are usually asymptomatic, however when transmitting to susceptible animals, are known to induce severe disease (Andrew and Norval, 1989; Allsopp et al., 1999; Kocan et al., 2010). Buffalo is a reservoir host for *Anaplasma*, *Theileria* and *Babesia* species, causing anaplasmosis, piroplasmosis or severe hemolytic disease (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2010).

2.1 Anaplasmosis

2.1.1 Taxonomic classification

Anaplasma is a genus of bacteria belonging to the alpha proteobacteria, order Rickettsiales, family Anaplasmataceae, a family of compulsory intracellular organisms that are found solely within membrane-bound vacuoles in the host cell cytoplasm (Kocan et al., 2005). All members of the family are obligatory intracellular bacteria that share similar morphological features and can cause the same clinical manifestations when they are pathogenic. The *Anaplasma* genus has been expanded to consist of three species transferred from the genus *Ehrlichia*: (i) *Anaplasma phagocytophylum* (previously known as *Ehrlichia phagocytophyla*, *E. equi* and human granulocytic

ehrlichiosis agents (HGE), which infect numerous mammal species such as domestic and wild ruminants (Walls et al., 1998) and humans (Foley et al., 1999); ii) ruminant pathogens *A. marginale*, *A. centrale* (previously *A. marginale* subspecies *centrale*) and *A. bovis* (previously *Ehrlichia bovis*) and (iii) canine pathogen *A. platys* (previously *Ehrlichia platys*) (Dumler et al., 2001).

Clinical bovine anaplasmosis results from *A. marginale* infection. It is known to be pathogenic to cattle and usually produces non-apparent infection in other species (Kuttler, 1984); however, it does not cause disease in humans (Aubry and Geale, 2011). *A. centrale*, a less pathogenic species for cattle than *A. marginale*, was initially isolated in 1911 (Potgieter, 1979) and has been extensively used as a live vaccine to prevent anaplasmosis in Australia (Rogers, 1979), South Africa (Potgieter, 1979) and Israel (Pipano et al., 1986). Furthermore, *A. bovis* is mainly detected in cattle, causing monocytic anaplasmosis and clinical signs are usually found in calves (Uilenberg, 1997; Santos and Carvalho, 2006). It is also observed in other small mammals including rabbits (Goethert and Telford, 2003) and domestic cats (Sasaki et al., 2012; Oliveira et al., 2018). *A. bovis* has been discovered in Brazil (Santos and Carvalho, 2006), Africa (Belkahia et al., 2015) and Japan (Kawahara et al., 2006; Ooshiro et al., 2008; Yoshimoto et al., 2010). In addition, *A. ovis* is a pathogen of sheep and goat without clinical signs (Splitter et al., 1956) but infection has been described to cause severe disease in bighorn sheep in North America (Tibbitts et al., 1992).

2.1.2 Geographical distribution

Bovine anaplasmosis is circulated in tropical and subtropical regions throughout the world including Africa, America, southern Europe, Australia, Asia, and Caribbean (Kocan and de la Fuente, 2003). The raising transport of cattle with successive mechanical or biological transmission from asymptomatic persistently infected animals

to susceptible ones leads to wide distribution of bovine anaplasmosis (Kocan et al., 2010). *Anaplasma centrale* was first described by Sir Arnold Theiler in 1911 from South Africa as *A. marginale* subspecies *centrale* and later is utilized as live vaccine against *A. marginale* for cattle in Australia, Africa, Israel and South America. Bovine anaplasmosis has been reported as enzootic in the southern Atlantic states, Gulf Coast states, and several of the Midwestern and Western states in the USA. It is endemic in Central and South America, Caribbean Islands, Mexico, and in all Latin American countries, except for mountain and desert areas (Guglielmone, 1995). Bovine anaplasmosis is also endemic in many parts of South Africa and Asia including Thailand (Saetiew et al., 2013), China (Qiu et al., 2016), India (Kumar et al., 2019), Vietnam (Chien et al., 2019) and Malaysia (Koh et al., 2018).

2.1.3 Host occurrence

Anaplasma spp. are known to infect domestic ruminants only. Cattle are naturally prone to *A. marginale* and *A. centrale* and sheep to *A. ovis*. There is little evidence or published research that wild ruminants are susceptible to *Anaplasma* infection. Most of all wild ruminants can be persistent carriers of *A. marginale* in the laboratory but they rarely come to be infected under natural conditions (Kuttler, 1984) with the exception of two reports of acute anaplasmosis in giraffes (Williams and Barker, 2008).

There are wildlife reservoirs for bovine anaplasmosis that are vulnerable to experimental *A. marginale* infection including pronghorn antelope (*Antelocapra americana*), bighorn sheep (*Ovis canadensis*), elk (*Cervus elaphus*) and bison (*Bison bison*); however, naturally occurring infections have not been confirmed yet in these species (Williams and Barker, 2008). In the USA, black-tailed deer (*Odocoileus hemionus culumbianus*) seems to be the most susceptible to *A. marginale* while white-tailed

deer (*Odocoileus virginianus*) is doubtful to be reservoir of *A. marginale* (Kuttler, 1984). In North America, mule deer (*Odocoileus hemionus*) can be naturally infected with *A. marginale*, *A. ovis* (de la Fuente et al., 2007) or *A. phagocytophilum* (Yabsley et al., 2005).

2.1.4 Life cycle

After primary infection and incubation period of 7-60 days, *A. marginale* invades erythrocytes and experiences replication cycles in the ruminant. There are two main tick species responsible for transmitting bovine anaplamosis comprising *Dermacentor* spp. and *Rhipicephalus* spp. Tick gut cells become infected with *A. marginale* after the tick sucks infected erythrocytes from the cattle via blood meal, followed by the infection of many other tick tissues comprising the salivary glands, from where the rickettsia are spread to vertebrates during feeding (Kocan, 1986; Kocan et al., 1992a; Kocan et al., 1992b). At each site of infection in ticks, *A. marginale* matures within membrane-bound vacuoles or colonies. There are two forms of *A. marginale* within the colonies: reticulated (vegetative) form and dense (infective) form. The first form is then divided by binary fission, forming large colonies that may comprise hundreds of organisms to become dense form. The second form is infective and can live outside the host cells. Cattle become infected with *A. marginale* when the dense form is passed on during tick feeding via the salivary glands (Kocan et al., 2003).

Bovine anaplasmosis is characterized by anaemia, jaundice without haemoglobinemia and haemoglobinuria, weight loss, lethargy, fever, abortion and death. *A. marginale* infection can be occurred in cattle of all ages but the brutality of disease is age-dependent. The sickness is rarely seen in young cattle under 6 month-old. Between 6 months and 1 year-old, cattle usually develop mild disease. Animals from 1 to 2 years of age get acute but hardly fatal disease. However, anaemia, fever,

icterus, abortion, lethargy and sudden death are often notable in animals over 2 year-old (Kocan et al., 2003). After recovery from acute infection, cattle develop persistent infections manifested by low level of rickettsemia (Kieser et al., 1990; French et al., 1998; French et al., 1999) and remain carriers for a lifetime. Persistently infected or carrier cattle have a lifelong immunity to clinical anaplasmosis but they serve as a supply of infection for others including buffaloes.

2.1.5 Transmission

Anapalsma marginale can be transmitted from infected cattle to other ones by three means of transport: biological, mechanical and transplacental. Biological transmission by ticks is the most efficient way owing to replication and persistence capabilities within tick's gut and salivary glands (Kocan et al., 1992a; Kocan et al., 1992b). There are approximately 20 different tick species possible to transmit *A. marginale* worldwide including *Ixodes ricinus*, *Dermacentor* spp., and *Rhipicephalus* spp. In North America, *Dermacentor* tick species consisting of the three-host ticks *D. andersoni* (Rocky Mountain wood tick), *D. occidentalis* (Pacific Coast tick – not present in Canada), *D. variabilis* (American dog tick) and the one-host tick *D. albipictus* (winter of moose tick) are known as vectors for *A. marginale* transmission (Kocan et al., 2005). The tropical cattle tick, *Rhipicephalus microplus* is responsible for *A. marginale* transmission in Asia and Africa.

Arthropods, the mechanical transmission of *A. marginale* can occur in places where tick populations are limited. At least 12 species of biting flies are experimentally reported to transmit *A. marginale* including stable flies (*Stomoxys calcitrans*), eight species of tabanids (horse or deer flies) and three species of midges (Culicidae) (Potgieter et al., 1981). Another way of mechanical transmission is via blood-contaminated fomites comprising contaminated needles, nose tongs, tattooing

instruments, ear-tagging devices and dehorning, castration or spaying equipment (Kocan et al., 2010).

Transplacental is another mode of anaplasmosis transmission. In a South African study, 77 calves were born chronically infected with 15.6% rate of in utero transmission of *A. marginale* or *A. centrale*. Another evidence can be seen in a study reporting that 32 out of 37 calves born by cows which had been acquired clinical anaplasmosis within the last 2 months of gestation (Salabarria and Pino, 1988).

2.1.6 Control methods

Different geographic location may have different control measures for bovine anaplasmosis depending on the availability, cost and feasibility of application. The general control and prevention methods consist of (i) vector control; (ii) maintenance of *Anaplasma*-free herds through movement and import control, checking and elimination of carrier cattle; (iii) antibiotic administration; (iv) prevention of iatrogenic transmission and (v) vaccination (preimmunization with live vaccines and immunization with killed vaccines) (Kocan et al., 2000).

In South Africa, acaricides are used to control both ticks and biting flies vector of cattle in endemic areas, however, this method appears to be expensive and labour-intensive. Moreover, repeated application of acaricides can result in the development of resistant tick and fly populations and it also poses an influence on the environment. Although tick control is extensively implemented in Africa but it is seldom used in the United State (Kocan et al., 2000).

All animals in the herd should be tested to ensure that they are *A. marginale*-free, as well as any new addition to the herd. *Anaplasma* – postitive cattle need to be eliminated since they serve as a source of infection for other animals. Carriers could

be treated and tested to confirm disease – free after treatment period. Antimicrobial therapy for bovine anaplasmosis comprises the use of tetracycline drugs (tetracycline hydrochloride, oxytetracycline, chlortetracycline, and doxycycline), gloxazone, and imidocarb (Richey and Howard, 1981). Chemotherapy targets to prevent clinical anaplasmosis but cattle are not protected from becoming persistently infected. Furthermore, cattle receiving antibiotic remedy may not be empty of infection (Kocan et al., 2000).

Vaccination seems to be the most effective control measure against anaplasmosis throughout the world since animals are capable to develop long-term immunity after receiving the vaccine. There are two types of vaccine including live (attenuated) and killed (inactivated) vaccines. Both live and killed vaccines have relied on the usage of infected blood as the source of infection or antigen to cause protective immunity or to avoid clinical disease (Kocan et al., 2000). However, these vaccines cannot protect cattle from becoming persistently infected. Cattle with persistent *A. marginale* infection serve as a reservoir for mechanical transmission or source of infection for ticks (Kocan and de la Fuente, 2003). Most significantly, geographical isolates of vaccine do not cross protect other ones, therefore, vaccine use may be limited to a geographical region (Kuttler, 1984).

2.2 Ehrlichiosis

Ehrlichia is an obligatory intracellular Gram-negative bacterium of the family Anaplasmataceae that has been associated with infections in both human and various animal species. The major pathogenic *Ehrlichia* species are *E. ruminantium, E. ewingii, E. canis*, *E. chaffeensis,* and *E. muris*. Among that, *E. ewingii* and *E. chaffeensis* were reported to cause human infections known as human monocytic ehrlichiosis and

human ewingii ehrlichiosis, respectively (Dumler et al., 2007). Human monocytic ehrlichiosis occurred across the south-central, south-eastern, and mid-Atlantic states of America, where both the lone star ticks (*Amblyomma americanum*) and the white-tailed deer (*Odocoileus virginianus*) thrive. Having the same tick vector as *E. chaffeensis*, *E. ewingii* was thought as another *E. canis* strain. *Ehrlichia canis* is frequently detected in lymphocytes and monocytes; however, this new strain was discovered in granulocytes. Therefore, this strain was called Canine Granulocytic Ehrlichia (CGE). *E. canis*, a species that infects dogs worldwide, has been reported in Chiang Mai with two genogroups, the US and Taiwanese genogroups based on phylogenetic analysis of gp36 gene (Nambooppha et al., 2018).

Another species of *Ehrlichia*, *E. ruminantium* (formerly *Cowdria ruminantium*) causes heartwater disease or cowdriosis in cattle, sheep, goats and some wild ruminants (Dumler et al., 2001). Heartwater occurs in almost the sub-Saharan countries of Africa and Caribbean. It is transmitted by three-host ticks belonging to the genus *Amblyomma*, primarily *A. variegatum* and *A. hebraeum*. There are eight different genotypes of *E. ruminantium* based on phylogenetic analysis of srRNA gene of African buffaloes in South Africa (Allsopp, 2010). The most important *Ehrlichia* species infecting ruminants, *E. ruminantium*, circumscribed to Africa and some Caribbean islands (Zhang et al., 2015). In China, *Ehrlichia* spp. were found to infect domestic ruminants; moreover, there might also be *A. platys*, *A. phagocytophilum* or *Anaplasma* spp. (Qiu et al., 2016).

2.3 Babesiosis

2.3.1 Taxonomic classification

Babesia is a genus of haemoprotozoan parasite belonging to the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, suborder Piroplasmorina and family Babesiidae which causes a disease called bovine babesiosis. According to its 18S rRNA gene and phylogenetic analyses, piroplasmids were divided into 5 clades: (i) a group comprised of mainly *Babesia* species from ungulates: *B. bovis*, *B. bigemina*, *B. caballi*, *B. ovis*, and *Babesia* spp. from cow (proposed name: Ungulibabesids); (ii) a group of *Babesia* species comprising *B. gibsoni* and *B. canis* from canids together with *B. divergens* and *B. odocoilei* (proposed name: Babesids); (iii) *B. microti* group, with *B. leo, B. rodhaini*, *B. microti*, *B. felis*, , and *T. annae* (proposed name: Archaeopiroplasmids); (iv) *Theileria* group, containing all *Theileria* species from Bovinae (proposed name: Theilerids); (v) Western USA Theilerid-like group (proposed name: Prototheilerids) (Criado-Fornelio et al., 2003). Among 5 genera of *Babesia* spp. in cattle, *B. bovis* and *B. bigemina* are considered as major causative agents of babesiosis in tropical countries including China (Yin et al., 1997), northern Thailand (Iseki et al., 2010; Terkawi et al., 2011a) and Brazil (Canever et al., 2014).

2.3.2 Geographical distribution

Bovine babesiosis can be found throughout the world wherever the tick vectors exist but it presents the most common in tropical and subtropical regions. *B. bovis* and *B. bigemina* are of major importance in Asia, Africa, Australia, Central and South America while *B. divergens* is economically critical in some parts of Europe. *B. bigemina* is much more prevalent than other *Babesia* species because of wider vector range. Therefore, it can be found more prevalent than *B. bovis* in South Africa (Mtshali and Mtshali, 2013). The suitable habitat for the tick vectors of *B. bovis* and *B. bigemina* were formerly endemic in southern U.S. However, *B. bigemina* has been eradicated

from the United States of America and now is found only in a quarantine buffer zone along the Mexican border (Anon, 2009).

Other species of *Babesia*, *B. major* has been isolated in cattle from regions of Europe, Asia and Africa, and *B. ovata* from parts of Asia (Heyman et al., 2010). *B. occultans* has been found in cattle in Italy (Decaro et al., 2013), eastern Poland (Staniec et al., 2018), North Africa (Amaia et al., 2011) and Turkey (Aktas and Ozubek, 2015) and in grey kangaroos in Australia (Dawood et al., 2013). New *Babesia* sp. in white yaks was reported to have similar characteristics to *B. venatorum*, an organism known to infect humans in China (Liu et al., 2017).

2.3.3 Host occurrence

The main reservoir hosts for *B. bovis* and *B. bigemina* are cattle. They are also detected in water buffalo (*Bubalus bubalis*), wild African buffalo (*Syncerus caffer*), American bison (*Bison bison*), wild white-tailed deer (*Odocoileus virginianus*), nilgai antelope (*Boselaphus tragocamelus*), pampas deer (*Ozotoceros bezoarticus*), horses, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), yaks (*Bos grunniens*), impala (*Aepyceros melampus*) and a greater kudu (*Tragelaphus strepsiceros*) (Anon, 2009). *B. divergens* is apparently restricted in Europe and North Africa cattle due to the presence of three-host tick vector *Ixodes ricinus* (Bouattour and Darghouth, 1996). It was also reported to cause human babesiosis in a splenectomized man in Portugal (Centeno-Lima et al., 2003). *B. ovata, B. major*, and *B. occultans* are also found in cattle. Nucleic acids of *B. occultans* were reported in African buffalo (Eygelaar et al., 2015) and *B. major* can induce clinical symptoms in experimentally infected American bison (Findlay and Begg, 1977).

2.3.4 Life cycle

There are three stages for *Babesia* species to complete their life cycle: (i) gamogony (male and female gametes fusion and zygote formation in the tick gut); (ii) sporogony (asexual reproduction in the tick's salivary gland); and (iii) merogony (asexual reproduction in the vertebrate host). The life cycle of *Babesia* species begins after the cattle are infected by feeding ticks which inoculates sporozoites. *B. bovis* sporozoite invades an erythrocyte and transforms into a trophozoite. Each trophozoite undergoes the asexual reproduction by binary fission to form two merozoites. The mature merozoites separate before escaping the erythrocyte. After releasing from the erythrocyte, some merozoites are ingested by adult ticks to continue their life cycle in the invertebrate host while the others invade new erythrocytes and develop into trophozoites. Following a tick blood meal, gametocytes develop in the tick gut, where the male and female gametes fuse to form diploid zygote. The zygote develops into an infective stage and invade the intestinal cells then undergoes many multiplications before becoming haploid kinetes. The kinetes migrate to other organs consisting of the ovaries where further division occurs. This infection is transmitted to the next generation of ticks or it is called transovarial transmission. When the female tick lays eggs and after egg hatching, the kinetes move to salivary gland and form a sporoblast. Each sporoblast contains thousands of sporozoites and those sporozoites will be ingested by other ticks to continue the spread of infection (Mosqueda et al., 2012).

Clinical manifestations of this disease in cattle are anemia, fever, hemoglobinuria, marked splenomegaly and occasionally death. These infections lead to economic damages to the livestock industry especially from mortality, reduced meat and milk production, control measure expenses and also have an influence on international cattle trade. Cattle recovered from acute infections, either naturally or

experimentally, remain persistently infected, therefore, become a reservoir for transmission and also pose a threat for future outbreaks in farms that do not have a proper disease management (Bock et al., 2004; Terkawi et al., 2011b).

On the one hand, *B. bovis* causes more severe disease in susceptible cattle than *B. bigemina* due to its capability to dramatically alter the structure and function of infected erythrocytes leading to massive destruction of these cells (Gohil et al., 2010). This is accompanied by the accumulation of parasitized red blood cells in the microvasculature of many organs such as brain and lungs, and subsequent development of fatal clinical complications including cerebral babesiosis, respiratory distress and multiorgan failure (Gohil et al., 2013). On the other hand, *B. bigemina* causes milder disease, resulting in anemia with higher level of parasitemia (Bock et al., 2004; Terkawi et al., 2011b).

Experimental buffaloes infected with cryopreserved *B. bovis* and fresh parasites inoculated by tick have got the same clinical signs as in cattle including continuous pyrexia, anemia, jaundice and hemoglobinuria (Yao et al., 1997). However, in another recent study, naturally *B. bovis*-infected water buffaloes showed less severe clinical signs of infection compared to cattle. These clinical signs were in the form of fever, loss of appetite, pale conjunctival and vaginal mucus membrane but icterus, hemoglobinuria and nervous manifestations were not observed in the affected buffaloes (Mahmmod, 2013).

2.3.5 Transmission

Ixodid ticks play an important role for babesial parasites transmission. All *Babesia* species are naturally transmitted between animals through the bite of ticks and within ticks. *Babesia* can be transmitted transovarially (transmission of infection from mother ticks to the next generation via eggs) or transstadially (transmission of

infection from egg to larvae to nymph to adult) (Ghosh et al., 2007). Ticks vector for *B. bigemina* transmission are *Rhipicephalus microplus* (formerly *Boophilus microplus)*, *Haemaphysalis*, *Hyalomma* and *Ixodes* while those for *B. bovis* transmission are *Boophilus* and *Ixodes*. Evidence of *B. bigemina* transmitted by *R. microplus* ticks was reported early in Indian (Wadhwa et al., 2008). In a study carried out in 2008, the highest prevalence of tick vector is *Hyalomma* spp., followed by *Boophilus*, *Haemaphysalis* and *Rhipicephalus* spp. (Durrani et al., 2008). *Ixodes ricinus* is the vector responsible for *B. divergens* transmission. Moreover, *B. occultans* is supposed to be transmitted by *Hyalomma marginatum, Hy. rufipes* while the vector for *B. major* is *Haemaphysalis punctata.*

2.3.6 Control methods

Three main measures are conducted to control bovine babesiosis including vector control, chemoprophylaxis and immunization. In order to obtain the maximal result, three methods should be combined to create the most cost effective use of each and also to exploit breed resistance and the growth and conservation of enzootic stability (Demessie and Derso, 2015). Tick control by using acaricides in areas of high infection challenge can reduce animals' exposure to *Babesia*, however, development of ticks' resistance to acaricides is also a big concern.

Cattle can be vaccinated against babesiosis by using live attenuated vaccines containing selected strains of *B. bovis* and *B. bigemina.* These vaccines are administered to calves from 6-12 month-old because at that age, they are the most susceptible to piroplamosis. Adult animals should be monitored after vaccination and treated if any clinical symptoms develop. Attenuated vaccines contain K strain of *B. bovis* and G strain of *B. bigemina* from Australia have been used effectively to immunize cattle in Africa, South America, Southeast Asia, islands of the Caribbean (Bock

et al., 2004). Vaccination against *B. divergens* has been proven effectively for cattle under one year of age since they still have natural resistance to babesiosis (Zintl et al., 2003).

Several compounds have been utilized for chemical control of bovine babesiosis including imidocarb dipropionate and diminazene aceturate. Chemoprophylaxis was not a lasting alternative for effective immunization so imidocarb and diminazene were used to protect cattle for several months. At dosage of 3 mg/kg imidocarb can prevent cattle from *B. bovis* infection at least four weeks and *B. bigemina* infection at least eight weeks (Kahn, 2010).

2.4 Theileriosis

2.4.1 Taxonomic classification

Theileriosis is another intracellular hemoprotozoan tick-transmitted disease of livestock caused by parasite belonging to the Kingdom of Protista, Subkingdom: Protozoa, Phylum: Apicomplexa, Class: Sporozea, Subclass: Piroplasmia, Order: Piroplasmida, Family: Theileriidae and Genus: *Theileria*. It is closely related to *Babesia* and can be found in cattle (*Bos taurus*), zebu (*Bos indicus*) and Asian water buffalo (*Bubalus bubalis*). *Theileria* parasites that infects different animals are divided into two groups including host-cell transforming and non-transforming species based on the ability of parasite to transform host leukocytes in a way that enables the infected cells to proliferate indefinitely along with the parasites occupying them. The first group is host-cell transforming *Theileria* consisting of *T. annulata*, *T. parva*, *T. taurotragi* and *T. lestoquardi* (Dobbelaere and Küenzi, 2004), which causes malignant lymphoproliferative theileriosis, also known as tropical theileriosis and East Coast fever respectively in cattle while the taxonomy classifications of second group (non-

transforming *Theileria*) have not been well-defined yet, cause non-lymphoproliferative bovine theileriosis. Members of the second group are *T. mutans*, *T. velifera*, *T. cervi*, *T. sergenti* in Japan, *T. buffeli* in Australia and *T. orientalis* in elsewhere. Therefore, these parasites are often known as the *T. sergenti/ buffeli/ orientalis* group (Fujisaki et al., 1994) or common name benign *Theileria* species of cattle (Sarataphan et al., 2003).

2.4.2 Geographic distribution

Bovine theileriosis is widely distributed in most continents including Asia (Altay et al., 2008; Hassan et al., 2018), Africa (Gebrekidan et al., 2014), north America, Europe and Oceania (Lawrence et al., 2018). The geographic distribution of tropical theileriosis is determined by different species of *Theileria* and its tick vector. More specifically, *Theileria annulata* – a causative agent of tropical theileriosis occurs from southern Europe and the Mediterranean coast through the Middle East, North Africa and some parts of Asia (Spickler and Roth, 2006). This disease is also prevalent in the South Eastern Europe, Southern Europe, Middle East, Central Asia, India and China (Gaafar, 2008). East Coast fever, a disease caused by *Theileria parva*, is discovered in sub-Saharan Africa and in Southern, Central and Eastern Africa (Tarimo, 2013). *Theileria mutans* has been reported in the U.S., African and on some Caribbean islands while *T. velifera* and *T. taurotragi* occur in Africa only (Spickler and Roth, 2006). *Theileria orientalis/buffeli* is widespread throughout the world, especially from Palaearctic regions to the Near and Far East regions and to Australia (Kreier, 2013).

2.4.3 Host occurrence

Theileria species infect a wide variety of both wild and domestic animals. The African buffalo (*Syncerus caffer*) is known as the natural host of *T. parva* and *T. parva lawrencei* (Kreier, 2013). *T. parva* is also detected in cattle, Indian water buffalo (*Bubalus bubalis*) and waterbuck (*Kobus ellipsiprymnus*) but clinical symptoms are

commonly seen merely in cattle and water buffalo (Spickler and Roth, 2006). *T. parva* is highly virulent for European dairy cattle, however, the indigenous cattle breeds and African buffaloes have natural resistance in endemic regions (Radostits et al., 2006). *Theileria annulata* occurs in cattle (*Bos taurus*), zebu (*Bos indicus*), water buffalo (*Bubalus bubalis*), American bison (*Bison bison*), domestic yak (*Bos grunniens*), and camels (Pieszko, 2015). Since the infection produces no symptoms in the water buffalo, this species is considered to be the most adapted host (Kreier, 2013). The African buffalo and domestic cattle are also the natural host for *T. mutans* and *T. velifera* while *T. taurotragi* is known as nonpathogenic species and found in elands (*Taurotragus oryx*) and domestic cattle. *T. orientalis/T. sergenti/T. buffeli* is responsible for non-transforming theileriosis and known to cause disease in cattle (Watts et al., 2016). Other species of *Theileria*, *T. lestoquardi*, *T. separata* and *T. ovis* are likely to infected in sheep and goats (Kreier, 2013).

2.4.4 Life cycle

The life cycle of *Theileria* parasite is complicated and characterized by schizonts in lymphoid cells and piroplasms in erythrocytes of the vertebrate host. Ticks are infected when feeding on an infected host whose erythrocytes harbour *Theileria* spp. piroplasms. After entering the bovine host, *Theileria* sporozoites promptly enter mononuclear leukocytes, where they mature into macroschizonts and continue multiplying by merogony in host cells. Microschizonts gradually develop into macroschizonts and further into merozoites, then they are released from leukocytes. These merozoites invade erythrocytes and become piroplasms (Abdela and Bekele, 2016). Next, larval or nymphal stage of ticks ingest piroplasms and the released piroplasms differentiate into gametocytes and fertilization (syngamy) occurs in the gut lumen to form a zygote. The zygote then divides into motile kinetes which infect the

tick gut epithelial cells and migrate to haemolymph and consequently infect the salivary glands. After moulting and commencement of feeding by the tick, sporogony lead to the proliferation of sporozoites in the salivary gland before injection into the feeding site by nymphs or adult ticks (Mans et al., 2015).

Clinical symptoms of theileriosis vary depending on the parasite species, severity of the parasite strain and host's susceptibility. *Theileria annulata* infection (tropical theileriosis) is described by high pyrexia, weakness, anemia, weight loss, conjunctival petechial, loss of appetite, and enlarged lymph nodes. At later stages of infection, lateral recumbency, diarrhea and dysentery are also seen (Radostits et al., 2006). East Coast fever, a disease caused by *T. parva* results in immuno-depression, secondary bacterial infection of the upper respiratory tract, fever, anorexia and frequently leading to death (Muhanguzi et al., 2014). The pathogenicity of *T. orientalis* is lower than that of *T. annulata* and *T. parva* but disease caused by *T. orientalis* has been often described as a subclinical condition, although the clinical manifestations may include anemia and other nonspecific signs (Kawazu et al., 1999). Nevertheless, it may consequently lead to severe economic losses in endemic areas (Kim et al., 2004).

2.4.5 Transmission

Theileria species causing economically important losses in cattle and small ruminants are transmitted by ixodid ticks of the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis*. The most significant vector of *T. parva* is the three-host tick *Rhipicephalus appendiculatus*, however, *R. zambesiensis* and *R. duttoni* are also able to produce and transmit this organism in parts of Africa (Spickler and Roth, 2006). *Theileria annulata* is transmitted by ticks in the genus *Hyalomma*. The main vectors around the Mediterranean coast are *H. anatolicum* and *H. detritum* while *H. lusitanicum* is in Europe and *H. impeltatum* in Nigeria (Kreier, 2013). The main tick

vector responsible for transmitting *T. mutans* and *T. velifera* is *Amblyomma variegatum* but *A. hebraeum* and *A. gemma* are also suitable tick hosts. The same as tick vector transmitting *T. parva*, *Rhipicephalus appendiculatus and Rhipicephalus* spp. spread *T. taurotragi* (Spickler and Roth, 2006). Vectors of *T. orientalis* belong to the genus *Haemaphysalis*: *H. punctata* and *H. longicornis*, which are responsible for theileriosis transmission in Europe and East Asia, respectively (Kreier, 2013).

2.4.6 Control methods

Measures for prevention and control of bovine theileriosis are similar to those for babesiosis including tick control, immunization by vaccines and chemotherapy. Tick control serves as an important role in determining the occurrence and existence of bovine theileriosis. There are many ways to apply acaricides to kill ticks such as spraying, spotting-on or dipping and this method results in some efficiency. However, tick control practice is not always fully effective for many reasons comprising development of acaricides resistance of tick after long-lasting use, high cost of acaricides, poor management of tick control. Therefore, immunization by vaccine seems to be an alternative method for theileriosis prevention and control. The first successful vaccination against *T. annulata* and *T. parva* has been attained by using attenuated live parasitized cell lines but it can be only used locally within a country. Later, another vaccination for *T. parva* known as method of infection and treatment was developed. In this method, cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting oxytetracycline to delay the parasite development. After receiving the sporozoites and antibiotic, cattle develop a mild or transient infection followed by recovery. Recovered cattle have a strong long-lasting protection to large doses of *T. parva* used for immunization (Morrison, 2015).

Chemotherapy is also beneficial for control of theileriosis during the acute phase of the disease. Several compounds including tetracyclines, buparvaquone, parvaquone, and halofuginone are available to treat *T. parva* and *T. annulata* infections. Tetracyclines was the first chemotherapeutic compound used against *T. parva*, however, it is effective only at the early phases of the infection (Gachohi et al., 2012). Until now, the greatest theilericidal drugs belong to the hydroxynaphtoquinones family: parvaquone and buparvaquone (Gharbi and Darghouth, 2015). Besides theileriosis treatment role, they can also be used as a remarkable prophylactic method against the disease (Qayyum et al., 2010). Last but not least, good management such as restriction of livestock movement, implementation of quarantine measures to keep the tick free, diseased cattle separately from infected animals also contribute to control bovine theileriosis.

2.5 Ungulate malaria

2.5.1 Taxonomic classification

The Plasmodiidae is one of the four families in the Order Haemosporida, Class Aconoidasida, Phylum Apicomplexa that is an intraerythrocytic parasitic alveolate in mammalians. Plasmodiidae comprises the genus Plasmodium, is known to cause malaria in both humans and wide range of animals. Ungulate malaria is found in both domestic and wild even-toed hoofed mammals. In domestic animals such as water buffaloes, malaria is caused by *Plasmodium bubalis* and was first observed by Sheather in India (1919) and followed by many researchers (Edwards, 1923; Cooper, 1926; Sen, 1932; Rao, 1938; Shastri et al., 1985). Other Haemosporida parasites of wild ungulates have been described including *Plasmodium traguli* and *Hepatocystis feldi* in the Asian mouse deer (Garnham and Kuttler, 1980), a *Hepatocystis* parasite in the hippopotamus

(Garnham, 1958), *Plasmodium limnotragi* in the African marshbuck in 1937, and *Plasmodium caprae* in African goats in 1923.

2.5.2 Geographic distribution

Ungulate malaria parasite is globally distributed ranging from North and South America to Africa and Asia. *Plasmodium odocoilei* was detected in while-tailed deer in North America (Guggisberg et al., 2018). In another study, South American pampas deer was proven to harbour *Plasmodium* spp. that was closely related to *P. odocoilei* clade 2 from North American white-tailed deer (Asada et al., 2018). Another species of *Plasmodium*, presumably *P. caprae* was found in domestic goat from Sudan and Kenya in Africa, Iran in West Asia, Myanmar and Thailand in Southeast Asia (Kaewthamasorn et al., 2018). *Plasmodium cephalophi* and *Plasmodium brucei* were detected in duiker antelope (*Sylvicapra grimmia*) in Central Africa (Boundenga et al., 2016).

2.5.3 Host occurrence

Malaria is mainly occurred in humans and most deaths are caused by *P. falciparum* since *P. vivax*, *P. ovale*, and *P. malariae* generally result in a milder form of malaria (Caraballo and King, 2014). Besides human infecting species, *Plasmodium* species those are capable of infecting ungulates are seen in duiker antelope (*Sylvicapra grimmia*) (Bruce et al., 1913), water buffalo (*Bubalus bubalis*) (Sheather, 1919), African marshbuck (*Tragelaphus spekii*) (1937), domestic goat (*Capra aegagrus hircus*) (1923), mouse deer (family Tragulidae) (1963), white-tailed deer (1980) and pampas deer (*Ozotoceros bezoarticus*) (Asada et al., 2018).

2.5.4 Life cycle

All *Plasmodium* species have similar life cycle which requires vertebrate (animals) and invertebrate (mosquito) hosts. The vertebrate phase of infection initiates

with the bite of an infected mosquito. Sporozoites invade the liver of vertebrate host and experience an initial phase of progress and asexual reproduction called merogony. At this point, some *Plasmodium* species of primates (*P. ovale* and *P. vivax*) can form a dormant stage called a hypnozoite, where the parasite maintains inactive for more than a year (Markus, 2011; Vaughan and Kappe, 2017). After releasing from the liver, they enter the circulation where they invade erythrocytes. Following invasion of erythrocytes, merozoites transform firstly into ring-shaped form and a larger form called trophozoites. Trophozoites then mature to schizonts which divide several times to produce new merozoites. Next, most merozoites continue their replication cycle while some develop into sexual form (male microgametocytes, female macrogametocytes). Gametocytes are then ingested by another mosquito host taking a blood meal and move to the mosquito's midgut. Here, male and female gametes fertilize each other to form a zygote. Zygote then matures to a motile form called an ookinete, and further develop into an oocyst. After oocyst sporulation or division, large numbers of sporozoites are produced. Finally, these sporozoites migrate to the salivary gland of the mosquito and continue to be spread to new vertebrate host by the mosquito bites via a blood meal (Kreier, 2013). Malaria in water buffalo was characterized by anemia, pale mucous membrane, hyperthermia, inappetence and reduced rumen motility (Sundar et al., 2004).

2.5.5 Transmission

Principle means of transmission of malaria is by the bites of female mosquito but what mosquito vector depends on different vertebrate host. Mosquito vector for malaria transmission is well-studied in human and primates than other wild and domestic animals. In human, malaria was known to be transmitted by *Anopheles* mosquito. Among more than 480 species of *Anopheles*, approximately 50 species

transmit malaria, with every continent having its own species of these mosquitoes: *An. gambiae*, *An. arabiensis* and *An. funestus* in Africa; *An. quadrimaculatus*, *An. albimanus* and *An. darling*i in Americas; *An. atroparvus* and *An. messeae* in Europe and Middle East; *An. dirus* and *An. minimus* complexes across much of Southeast Asia (Sinka et al., 2012). *An. leucosphyrus*, *An. latens*, *An. cracens*, *An. hackeri*, *An. dirus* have been identified as the vectors for the transmission of *P. knowlesi* (Vythilingam et al., 2008). Transmission was greater during the rainy season, when mosquito populations thrive, and was also higher at mid-height than on the ground (Molina-Cruz and Barillas-Mury, 2016). Although there were many studies about the distribution of the dominant vectors of human malaria, however, vectors for ungulate malaria, especially malaria in water buffalo remain unidentified until now.

2.5.6 Control methods

Malaria is a serious and lethal disease occurred in both human and animals, therefore, it has attracted considerable concerns from the whole society with the aim as elimination of malaria. Feasible measures can be applied for humans including mosquito control, malarial vaccine and antimalarial drugs, however, they are still limited for applying in animals. Human can protect themselves by personal protection against mosquito bites such as closure of windows and doors to prevent entry, using bed nets (insecticide treated) and mosquito repellent leading to reduction in mosquito eggs and hence mosquito population. People can also prevent the source of mosquitos' egg laying by avoiding water logging, destroying unwanted water collections and keeping the water containers closed. Furthermore, insecticides or biological larvicides (Guppy or Gambusia fish or bacteria or fungii) can be utilized on breeding grounds to destroy the developing larvae and pupae.

Many kinds of malarial vaccine targeting the different phases of the parasite's life cycle has been achieved but they still cannot totally protect human against malaria. Currently, scientists had set out a strategic goal to develop a malaria vaccine by 2025 that would have a protective efficacy of more than 80% against clinical disease and would provide protection for more than 4 years (Moorthy et al., 2009). Chemotherapy treatment by using antimalarial drugs has been effective against malaria for a long time. Antimalarial drugs can be classified according to antimalarial activity or structure. However, in general, several typical compounds are Artemisinin derivatives, Chloroquine, Amodiaquine, Quinine, Quinidine (cinchona alkaloids), Mefloquine, Halofantrine, Sulphones, Sulphonamides, Proguanil, Chloroproguanil, Pyrimethamine, Primaquine. Some antimicrobials including Tetracycline, Doxycycline, Clindamycin, Azithromycin, Fluoroquinolones are also capable of using as short-term prophylaxis for malaria (White, 1996).

2.6 Genetic variability

All animal species are specified by their own genotypes and phenotypes. Genotypes are complete sets of genes carried by an individual. Phenotypes are observable properties or external traits of an organism produced by the genotypes in conjunction with the environment (Klose, 1999). Genetic variability is the formation of differences in genotype of individuals. This is in contrast to environmentally induced differences which cause only temporary, non-heritable changes of the phenotype. There are many factors resulting in genetic variability including homologous recombination, genetic drift and mutation. Immigration, emigration, and translocation of animals in the specific population may contribute to genetic diversity of next generation if they randomly reproduce (Ehrich and Jorde, 2005). Genetic variation is

beneficial to a population because it allows some individuals to adapt themselves to the changing environment while sustaining the survival in the population.

2.7 Blood parasite examination

Diagnosis of tick-borne protozoa and rickettsia can be performed by demonstration of Giemsa's stained thin-blood smears from clinically infected animals during the acute phase of infection. This method is rapid and cheap; however, it is unreliable for detecting infection in pre-symptomatic or carrier animals. The conventional diagnosis method for identification of blood parasites relying on blood smear is inconclusive on many occasions owing to its low sensitivity and inability to differentiate between species and its closely related pathogens. Molecular diagnosis provides much better sensitivity and specificity for pathogen detection but remain time-consuming and unaffordable to most of developing countries. Currently, DNA amplification has become a routine diagnostic assay for detection of wide variety of pathogens as it can improve the detection limit, provide better accuracy and shorten the diagnostic procedure. In molecular-approach for parasite diagnosis technique, small subunit ribosomal RNA gene is frequently used as the target for PCR amplification because it is highly conserved among different species of organism (Coenye and Vandamme, 2003; Meyer et al., 2010). Besides, cytochrome b gene (cytb) in mitochondria DNA is also widely used as a target for amplification since it contains many copies in the parasite (Polley et al., 2010; Calzetta et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

Buffalo blood samples were collected in eight provinces of Thailand including Mukdahan, Uthai Thani, Lampang, Amnat Charoen, Nong Bua Lamphu, Phatthalung, Surin and Chachoengsao from January 2015 to June 2018 (Table 1 and Figure 1). Decision on choosing locations of buffaloes was made based on support and cooperation between the local veterinary authorities and the farm owners. Buffaloes were restrained in metal squeeze pens and blood samples were aseptically taken from the jugular vein. Approximately 8 ml of blood was drawn into BD Vacutainer® containing 1.5 ml of anticoagulant Acid Citrate Dextrose Solution (ACD) (BD Franklin Lakes, NJ, USA). Buffaloes are mostly indigenous breeds except for Murrah in Chachoengsao Province. There are no preferences for animal gender, weight, and age for blood sampling. A total of 456 blood samples distributing across locations in Thailand were achieved for the investigation. Blood samples were then kept at 4°C and transferred to a laboratory at Faculty of Veterinary Science, Chulalongkorn University within 72 hours, except for the samples taken from Phatthalung. The Phatthalung samples were stored at -20°C at the Veterinary Research and Development Center in Nakhon Si Thammarat Province for weeks due to its distant location. Shipping of these samples were made to the Veterinary Parasitology Laboratory at Chulalongkorn University. Aliquots of 1.5 ml of whole blood were kept at -80°C for DNA extraction.

This study was carried out with the consent of the farm owners and was approved by the Chulalongkorn University Animal Care and Use Committee. The

project was reviewed and approved by the Institutional Biosafety Committee in accordance with the faculty regulations and policies governing biosafety procedures (Animal Use Protocol Number 1931027).

Table 1. Sampling location and time

Site number	Sampling site	Sampling time	Number of samples
1.	Mukdahan	January 2015	88
		January 2016	61
		December 2017	36
2.	Uthai Thani	May 2015	8
3.	Lampang	June 2016	60
4.	Amnat Charoen	December 2016	21
5.	Nong Bua Lamphu	December 2016	50
6.	Phatthalung	April 2017	37
7.	Surin	March 2018	13
8.	Chachoengsao	June 2018	82
	Total		**456**

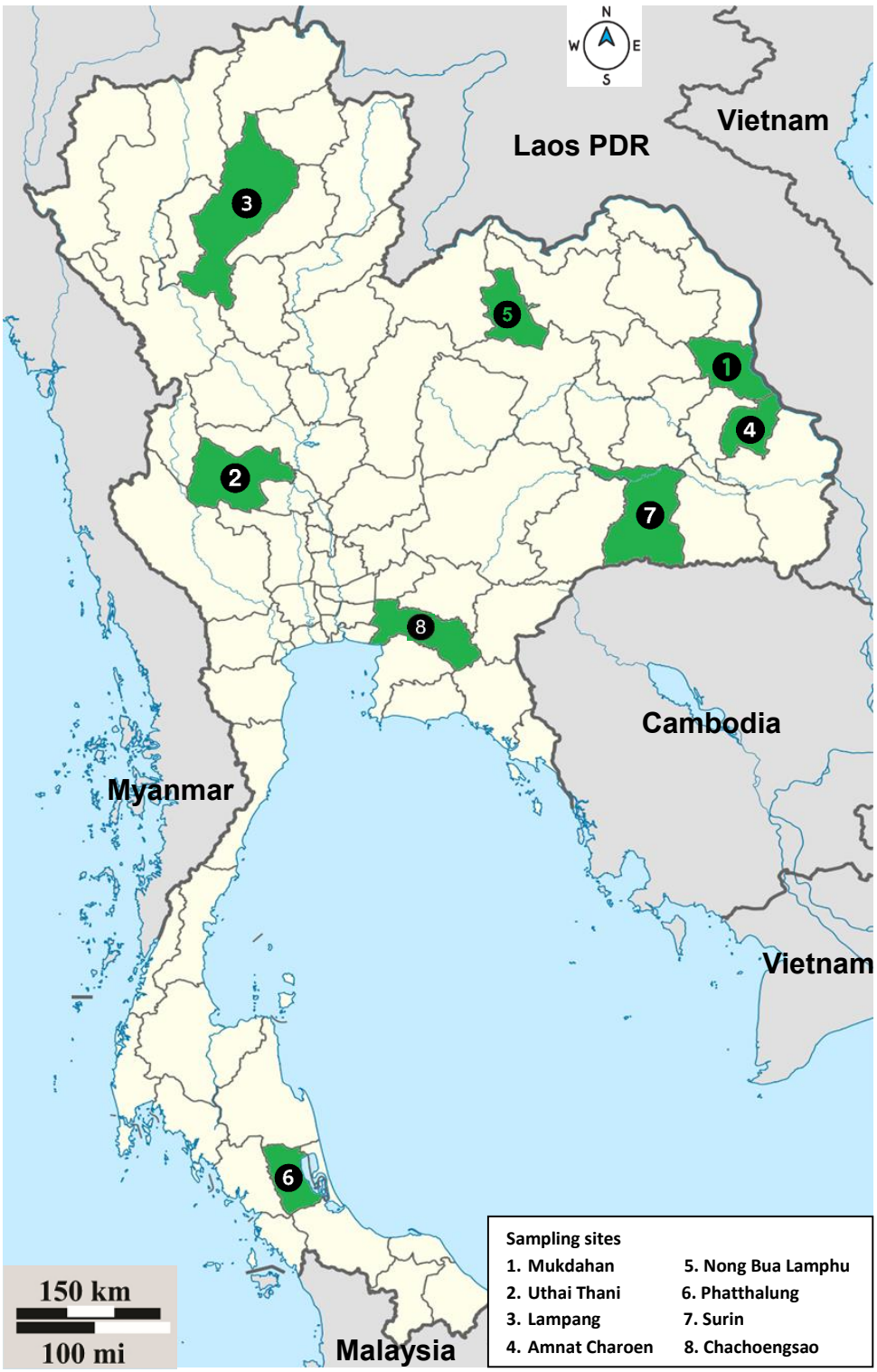

Figure 1. Map of Thailand depicting blood sampling sites of buffaloes

3.2 DNA extraction

Frozen blood samples were thawed at room temperature for approximately 15 min and centrifuged at 14,100 rpm for 5 min in miniSpin Plus (Eppendorf, Germany) then the supernatant was discarded. The pellets were re-suspended in 1 ml of ice-cold 0.15% (w/v) saponin (Sigma, Germany) in 1X PBS for a few minutes in order to facilitate further hemolysis. Samples were then centrifuged at 14,100 rpm for 5 min, discarded the supernatants and repeatedly washed with 1X PBS until the color become clear. The pellets were then subjected to DNA extraction kit using a NucleoSpin® Blood (Macherry-Nagel, Germany) following the manufacturer's instructions, except for the final elution buffer volume was reduced to 50 µl. Genomic DNA of buffalo blood samples were stored at -20°C for later use.

3.3 Primer designs

Sequence alignments of each known groups of rickettsia and piroplasms and infecting buffaloes were made using MUSCLE software freely available at www.ebi.ac.uk. Sequences sharing among groups of pathogens mentioned before were chosen for primer binding regions. GenBank accession number of sequences included in our alignment were as follow: AF414868, AF414869, AF283007, AY262124, AB211163, M60313, KT264188, CR767821 and FJ169957 for rickettsia; KY581623, JN121382, AY077621, AF478129, AY044161, KX987394, KU585938, KU585922, KU585930, KU585944, MH716434 and MH716431 for *Anaplasma platys* confirmation; L19077, AY66151, AF236094, AB520955, X59604 and AY072925 for piroplasms. Universal primers targeting hemosporidian parasites as previously described by Liu and colleagues (2010) and Templeton et al. (2016) were used for *P. bubalis* malaria parasite detection.

Table 2. Primers used for PCR amplification and sequencing in this study

Primer	Target gene (amplicon length)	Sequence (5' to 3')	Reference
Ehrlianaplas 16SR5U	16S rRNA	CAAACTTGAGAGTTTGATCCTGG	This study
Ehrlianaplas 16SR3U	(1520 bp)	ACGATAAGAAAGCCTAAAAGGAGG	This study
Ehrlianaplas 16FWD	16S rRNA	TGGCAGACGGGTGAGTAATG	This study
Ehrlianaplas 16REV	(1286 bp)	TAAGCCAATTCCCATGGCGT	This study
Aplatys-groEL-F	groEL	GAAGAGTATTAAGCCTGAGGAACCGC	This study
Aplatys-groEL-R	(825 bp)	GTCGTTGTGTCCTTAGTGATGCGAAC	This study
Pan Piro F1	18S rRNA	GCAAATTACCCAATCCTGACACAGG	This study
Pan Piro R1	(1221 bp)	CCGAATAATTCACCGGATCACTCG	This study
DW2	cytb	TAATGCCTAGACGTATTCCTGATTATCCAG	(Liu et al., 2010)
DW4	(1254 bp)	TGTTTGCTTGGGAGCTGTAATCATAATGTG	(Liu et al., 2010)
NCYBINF	cytb	TAAGAGAATTATGGAGTGGATGGTG	(Templeton et al., 2016)
NCYBINR	(822 bp)	CTTGTGGTAATTGACATCCAATCC	(Templeton et al., 2016)

3.4 PCR amplification and sequencing

Genomic DNA of individual blood sample was screened for the presence of DNA of rickettsia, piroplasms and malaria parasite using primers listed in Tables 2. Each PCR reaction was performed in a 12.5 µl volume containing 6.25 µl of 2X PCR buffer KOD FX Neo, 2.5 µl of dNTPs (0.4 mM each), 0.375 µl of each primer (10 pmol/ µl), 0.25 µl of KOD FX Neo DNA polymerase (Toyobo, Japan), 1 µl of the extracted DNA template and 1.75 µl of sterile distilled water. PCR mixtures containing distilled water were used as the negative controls. The thermocycling conditions for the PCR to screen for rickettsia, piroplasms and *Plasmodium* spp. were set as described in Table 3. In each cycling condition, denaturation, annealing and extension steps were repeated for 40 cycles. Genomic DNA of *A. marginale* isolate AmCU01 (accession no. KT264188), *T. orientalis* isolate ToCU01 (accession no. KT264187) and *P. bubalis* isolate MUK2014-69 (accession no. LC090213) were used as the positive controls. Sterile distilled water was used as negative control. After the first round of PCR amplification, the primary PCR products were diluted with distilled water at the ratio 1:10 and used as DNA templates for nested PCR. The nested PCR amplifications were carried out under the same cycling conditions as primary PCR. All PCR reactions were carried out in an Axygen® MaxyGene II Thermal Cycler (Life Sciences, USA). Gel electrophoresis was set at 100 volts and 400 mA of electricity and run for 45 min in 1.5% agarose gel with 0.5X TAE buffer. The gel was stained with ethidium bromide and PCR products was visualized under a UV transilluminator.

Table 3. PCR condition for pathogen detection

PCR condition	Pathogen		
	Rickettsia	*Plasmodium* spp.	Piroplasms
Initial denaturation	94°C / 2 min	94°C / 2 min	94°C / 2 min
Denaturation	98°C / 10 sec	98°C / 10 sec	98°C / 10 sec
Annealing	55°C / 30 sec	62°C / 3 min	60°C / 30 sec
Extension	68°C / 90 sec		68°C / 90 sec
Final extension	68°C / 5 min	68°C / 5 min	68°C / 5 min
Stored	12°C / indefinite	12°C / indefinite	12°C / indefinite

Positive samples were scaled up to 25 µl of PCR product before further prepared for sequencing. PCR products without nonspecific bands were treated with a 10-time dilution of ExoSAP-IT™ (USB Corporation, USA) according to the manufacturer's instruction to digest the remaining single strand DNA. The PCR cleanup protocol was set as follows: 37°C for 30 min to degrade remaining primers and nucleotides and then 80°C for 15 min to inactivate ExoSAP-IT™ reagent. ExoSAP-IT™ treated PCR products were directly sequenced in both directions using corresponding primers mentioned previously. Nucleotide sequences were then manually edited and assembled in BioEdit software which is freely available at www.mbio.ncsu.edu. Singleton mutation was confirmed with sequencing results of at least 2 independent PCR products. Ambiguous sequences were excluded from further analysis. Sequences were initially assessed to identify the best fit using the BLASTn program on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 Sequence and statistical analyses

Chromatogram obtaining from the sequencing results was visually inspected and manually edited where necessary using BioEdit 7.0.5.3 software. Ambiguous sequences or mixed infections were excluded from further analyses. The chi-square test was used to evaluate significant differences ($p < 0.05$) of disease prevalence in eight provinces. The interaction between two pathogens was analyzed using Pearson correlation coefficient and Fisher's exact test in SPSS software version 22. Genetic diversity of the nucleotide sequence data was assessed in DnaSP software version 6.12.01 online available at www.ub.edu/dnasp. The best-fit models for phylogenic construction were calculated using Find Best DNA/Protein Model implemented in MEGA X. The software is freely available at www.megasoftware.net. Phylogenetic trees were constructed based on the lowest Bayesian Information Criterion score. Internal branch confidence was assessed by the bootstrapping method using 1000 bootstrap replicates. Reference sequences of the corresponding pathogens i.e. *Anaplasma* spp. 16S rRNA genes, *Plasmodium* spp. cytb gene and *Theileria* spp. 18S rRNA genes were retrieved from GenBank database.

Table 4. Reference sequences used for phylogenetic trees in this study

***Anaplasma* spp. 16S rRNA gene**			
Accession no.	**Taxonomic classification**	**Host**	**Country**
DQ341370	*A. marginale*	Macheng buffalo	China
HM538192	*A. marginale*	Buffalo	China
HM538193	*A. marginale*	Buffalo	China
KU686794	*A. marginale*	Cattle	Uganda
JQ839012	*A. marginale*	*Boophilus microplus*	Philippines

Accession no.	Taxonomic classification	Host	Country
AF311303	*A. marginale*	NA	Southeastern U.S.A.
KT264188	*A. marginale*	Cattle	Thailand
MH020201	*A. marginale*	Dog	Hungary
KX987330	*A. marginale*	*Boophilus microplus*	China
EF520690	*A. centrale*	Cattle	Italy
AF414869	*A. centrale*	*Rhipicephalus simus*	South Africa
AF283007	*A. centrale*	NA	Japan
FJ169957	*A. bovis*	Cattle	China
HQ872464	*A. phagocytophilum*	Goat	China
AB196721	*A. phagocytophilum*	Wild deer	Japan
AY527214	*A. phagocytophilum*	Horse	Sweden
KM215228	*A. phagocytophilum*	*Ixodes ricinus*	Slovenia
AF399917	*A. platys*	Dog	Venezuela
MH686048	*A. platys*	Cattle	Vietnam
MH762081	*A. platys*	*Rhipicephalus simus*	China
EF139459	*A. platys*	Dog	Thailand
L36217	*Rickettsia rickettsii*	NA	France
U11021	*Rickettsia rickettsii*	*Dermacentor andersonii*	U.S.A.

***Plasmodium* spp. cytb gene**			
Accession no.	**Taxonomic classification**	**Host**	**Country**
LC090213	*P. bubalis*	Water buffalo	Thailand
LC090214	*Plasmodium* sp.	Water buffalo	Thailand
LC090215	*Plasmodium* sp.	Goat	Zambia

Accession no.	Taxonomic classification	Host	Country
LC326032	*Plasmodium* sp.	Goat	Thailand
AB564276	*P. gallinaceum*	Chicken	Japan
KP025675	*P. gallinaceum*	Chicken	Thailand
AF069612	*P. gallinaceum*	Monkey	NA
AY099029	*P. gallinaceum*	Chicken	Vietnam
AF155926	*P. falciparum*	Human	Thailand
AJ298787	*P. falciparum*	Human	India
KM527175	*P. falciparum*	Human	Central African Republic: Bayanga
KP293840	*P. falciparum*	Human	Kenya
AF069609	*P. falciparum*	Primates	China
GU815518	*P. ovale*	Chimpanzee	Cote d'Ivoire
AF069625	*P. ovale*	Primates	Africa
GQ231520	*P. ovale*	Human	Africa
GU723543	*P. ovale*	Human	Africa
AF069624	*P. malariae*	Primates	Uganda
GU815516	*P. malariae*	Chimpanzee	Cote d'Ivoire
GU815517	*P. malariae*	Chimpanzee	Cote d'Ivoire
AY099051	*P. yoelii*	Rodents	NA
DQ414660	*P. yoelii yoelii*	Rodents	Central African Republic
KM527161	*P. vivax*	Gorilla	Central African Republic: DSPA
AF069619	*P. vivax*	Primates	Brazil
KC750255	*P. vivax*	Great apes	Africa
AB302215	*Leucocytozoon caulleryi*	Chicken	Japan
NC015304	*Leucocytozoon caulleryi*	Chicken	Japan
AB299369	*Leucocytozoon sabrazesi*	Chicken	Malaysia
NC009336	*Leucocytozoon sabrazesi*	Chicken	Malaysia
DQ630004	*Haemoproteus pallidus*	Bird	Europe

Accession no.	Taxonomic classification	Host	Country
DQ630014	*Haemoproteus balmorali*	Bird	Lithuania
GU256261	*Parahaemoproteus bird* sp.	Bird	NA
GQ141585	*Parahaemoproteus* sp.	Bird	NA
GQ141597	*Parahaemoproteus* sp.	Bird	NA

***Theileria* spp. 18S rRNA gene**

Accession no.	Taxonomic classification	Host	Country
MG599089	*T. orientalis*	Cattle	China
MG599099	*T. orientalis*	Cattle	Pakistan
AB668373	*T. orientalis*	Cattle	Japan
AB520958	*T. orientalis*	Cattle	Australia
JF719834	*T. sergenti*	NA	China
AY661515	*T. sergenti*	Cattle	Japan
AY661512	*T. buffeli*	Cattle	U.S.A.
EU277003	*T. sinensis*	Domestic yak	China
JX469515	*T. luwenshuni*	Goat	China
HQ184406	*T. cervi*	Sika deer	China
AY726011	*T. capreoli*	European roe deer	Spain
AY262116	*T. uilenbergi*	Sheep	China
AY260172	*T. ovis*	Sheep	Turkey
FJ426369	*T. annulata*	Cattle	Italy
L02366	*T. parva*	Cattle	NA
L19082	*T. taurotragi*	NA	NA
U97047	*Theileria* sp. type A	Cattle	Korea, Japan, Kenya
U97048	*Theileria* sp. type B	Cattle	Korea, Japan
U97049	*Theileria* sp. type B1	Cattle	Korea, U.S.A.

Accession no.	Taxonomic classification	Host	Country
U97051	*Theileria* sp. type C	Cattle	Korea
U97052	*Theileria* sp. type D	Cattle	Korea, U.S.A.
U97053	*Theileria* sp. type E	Cattle	Korea
U97054	*Theileria* sp. type F	White-tailed deer	U.S.A.
U97055	*Theileria* sp. type G	Elk	Canada
U97050	*Theileria* sp. type H	Cattle	Korea
AY262118	*Theileria* sp. China 1	Sheep	China
FJ595120	*Theileria* sp. HAN1	Deer	South Korea
FJ599640	*Theileria* sp. HAN2	Deer	South Korea
AB012201	*Theileria* sp. CC3A	Serow	Japan
AB012194	*Theileria* sp. CNY1A	Yezo sika deer	Japan
AB012198	*Theileria* sp. CNY3B	Yezo sika deer	Japan
DQ866842	*Theileria* sp. 3185/02	Roe deer	Spain
AY421708	*Theileria* sp. 3185/02	Red deer	Germany
L19080	*Cytauxzoon felis*	NA	NA

NA: Not Available

CHAPTER 4

RESULTS

4.1 Buffaloes' gender, age and pregnancy status

A total of 456 blood samples were collected from buffaloes in 8 provinces of Thailand between January 2015 and June 2018 via 10 surveys: In Mukdahan Province, there were 3 collection periods in 3 districts including (i) 88 samples in January 2015, (ii) 61 samples in January 2016 and (iii) 36 samples in December 2017; (iv) 8 samples from Uthai Thani Province in May 2015; (v) 60 samples from Lampang Province in June 2016; (vi) 21 samples from Amnat Charoen Province in December 2016; (vii) 50 samples from Nong Bua Lamphu Province in December 2016; (viii) 37 samples from Phatthalung Province in April 2017; (ix) 13 samples from Surin Province in March 2018 and (x) 82 samples from Chachoengsao Province in June 2018. Among these, 223 buffaloes were recorded as 47 males and 176 females. Furthermore, the number of buffaloes aged 2 years-old or younger was 87 individuals while 136 buffaloes were over 2 years. There were 3 pregnant buffaloes aged older than 3 years and 1 pregnant at the age of 2. Information regarding sex, age and pregnancy status of other remaining buffaloes were unavailable.

4.2 Occurrence of selected vector-borne protozoa and rickettsia in buffaloes in Thailand

In the present study, the overall prevalence of *Anaplasma* spp.; *Plasmodium* spp. and *Theileria* spp. in buffaloes in 8 provinces of Thailand were 41%, 12.9% and 25.4%, respectively. *Ehrlichia* spp. and *Babesia* spp. were not detected in any sequenced samples in this study. Among 3 pathogens detected, *Anaplasma* spp. was

the highest prevalence (41%), followed by *Theileria* spp. (25.4%). *Plasmodium* spp. was recorded less dominant than other two pathogens with 12.9% (Table 4). Anaplasmosis and theileriosis were widely distributed in all investigated provinces while malaria parasite was found in 6 out of 8 provinces, except for Uthai Thani and Surin. Buffaloes in Phatthalung Province had the highest occurrence of *Anaplama* spp. and *Theileria* spp. while Chachoengsao Province took the lead in *Plasmodium* parasite with more than one third of buffaloes got malaria. Another species of *Anaplasma*, *A. platys* was detected in 3 out of 456 (0.66%) buffalo blood samples. The *A. platys* positive samples was further confirmed by PCR and DNA sequencing with primers targeting groEL gene (Table 2) that specifically amplify *A. platys*.

Table 5. Prevalence of selected vector-borne protozoa and rickettsia in buffalo in 8 provinces of Thailand

Sampling site	**Sampling time**	**Tested sample**	**No. of positive samples**		
			***Anaplasma /Ehrlichia* spp. (%)**	***Plasmodium* spp. (%)**	***Babesia /Theileria* spp. (%)**
Mukdahan	Jan 2015	88	38 (43.2)	10 (11.4)	18 (20.5)
	Jan 2016	61	40 (65.6)	4 (6.6)	11 (18.0)
	Dec 2017	36	7 (19.4)	2 (5.6)	0
Uthai Thani	May 2015	8	4 (50)	0	2 (25)
Lampang	June 2016	60	28 (46.7)	3 (5)	4 (6.7)
Amnat Charoen	Dec 2016	21	5 (23.8)	1 (4.8)	7 (33.3)
Nong Bua Lamphu	Dec 2016	50	3 (6)	3 (6)	3 (6)

Sampling site	**Sampling time**	**Tested sample**	***Anaplasma* /*Ehrlichia* spp. (%)**	***Plasmodium* spp. (%)**	***Babesia* /*Theileria* spp. (%)**
Phatthalung	April 2017	37	25 (67.6)	7 (18.9)	30 (81.1)
Surin	March 2018	13	2 (15.4)	0	1 (7.7)
Chachoengsao	June 2018	82	35 (42.7)	29 (35.4)	40 (48.8)
Total		**456**	**187 (41)**	**59 (12.9)**	**116 (25.4)**

4.3 Association between pathogens and the host's age and gender

Table 6 illustrated the factors associated with the pathogen infection**.** Two age groups of buffaloes were detected the same prevalence of *A. marginale* infection (32 positive out of 87 buffaloes ≤2 years-old, 50 positive out of 136 buffaloes >2 years) and equivalent prevalence of *P. bubalis* infection (7 positive out of 87 buffaloes ≤2 years-old, 11 positive out of 136 buffaloes >2 years), indicating that pathogen infection proportions were independent with the host's age (odds ratios were 0.999 and 1.006 respectively); and no statistically significant difference was observed ($p > 0.05$). Regarding *T. orientalis*, a higher prevalence in buffaloes with the age ≤2 was observed but there was statistically insignificant difference with $p > 0.05$.

Additionally, female buffaloes were more frequently infected with *A. marginale* and *P. bubalis* than male; however, statistically significant difference was only observed in *A. marginale* with $p < 0.05$, except for malaria parasite. This result conflicted with the finding in Pakistan (Farooqi et al., 2018). In contrast, the prevalence of piroplasms was higher recorded in male than female (8 positive out of 47 male, 24 positive out of 176 female); nevertheless, odds ratio showed that there was negatively correlated between *T. orientalis* infection and gender with $p > 0.05$.

Table 6. Factors associated with the pathogen infection

(a) Age

Pathogens		Age		Odds ratio	95% confidential interval	p-value
		≤2	>2			
A. marginale	Positive	32	50	0.999	0.572 – 1.746	1
	Negative	55	86			
P. bubalis	Positive	7	11	1.006	0.374 – 2.702	1
	Negative	80	125			
T. orientalis	Positive	16	16	0.592	0.279 – 1.256	0.176
	Negative	71	120			

(b) Gender

Pathogens		Gender		Odds ratio	95% confidential interval	p-value
		Male	Female			
A. marginale	Positive	11	71	2.213	1.057 – 4.635	**0.041**
	Negative	36	105			
P. bubalis	Positive	2	16	2.250	0.499 – 10.152	0.376
	Negative	45	160			
T. orientalis	Positive	8	24	0.770	0.321 – 1.845	0.639
	Negative	39	152			

4.4 Interplay between *P. bubalis* and other pathogens co-infected in buffaloes

Because the buffalo malaria parasite, *P. bubalis* was detected in 86 samples out of 331 samples in the previous study (Templeton et al., 2016), possible effect of a specific pathogen infection against the other pathogens was evaluated in this study. A correlation coefficient value (R_{ij}) was calculated for each two-pathogen interaction. Co-infection of pathogens in Thai buffaloes are summarized in Table 7.

Table 7. Co-infection of pathogens in buffaloes from 8 provinces of Thailand

Pathogens	**Positive numbers (%)**
Single infection	
Rickettsia	110 (24.1)
Plasmodium spp.	18 (3.9)
Piroplasms	46 (10.1)
Double infection	
Rickettsia & *Plasmodium* spp.	17 (3.7)
Rickettsia & Piroplasms	46 (10.1)
Plasmodium spp. & Piroplasms	10 (2.2)
Triple infection	
Rickettsia & *Plasmodium* spp. & Piroplasms	14 (3.1)

The correlation coefficient value (R_{ij}) between *A. marginale* and *P. bubalis* was negative, indicating that there was a negative correlation between them. Other two correlation coefficient values among *A. marginale* versus *T. orientalis*; and *P. bubalis* versus *T. orientalis* were positive, indicating that there were positive associations among them. However, no statistically significant differences were observed with $p > 0.05$ (Table 8).

Table 8. Fisher's exact test for the coinfection of two pathogens

Pathogen	*A. marginale*	*P. bubalis*	*T. orientalis*
A. marginale	–		
P. bubalis	-0.021 (0.754)	–	
T. orientalis	0.086 (0.202)	0.067 (0.323)	–

Pearson correlation coefficient between two pathogens (R_{ij}) are shown with p-value in the bracket; p-value were calculated by Fisher's exact test.

4.5 Genetic relationships and phylogenetic analysis of *A. marginale*, *P. bubalis* and *T. orientalis* from buffalo in Thailand

According to DNA sequencing result, two species of *Anaplasma* including *Anaplasma marginale* and *A. platys* were detected in this study. *A. platys* was re-confirmed by conventional PCR using different pair of primers targeting heat shock protein gene (groEL) that specifically amplified *A. platys* DNA, followed by sequencing. Sequencing result of groEL gene showed that our three samples had percentage of identity to *A. platys* ranging from 98.36 to 99.62% based on basic local alignment search tool (BLAST) in GenBank. Regarding piroplasms and malaria parasites, *P. bubalis* and *T. orientalis* were found in all sequencing samples and percentage of similarity to GenBank database was shown in Table 9.

Table 9. Species identification based on DNA sequencing

No.	Pathogen	No. of sequencing samples	Sequences producing significant alignments		% Similarity to GenBank
1.	Rickettsia	40	*Anaplasma marginale*:	37/40	95.05 – 99.83%
			Anaplasma platys:	3/40	99.53 – 99.76%
2.	*Plasmodium* spp.	12	*Plasmodium bubalis*:	12/12	94.76 – 100%
3.	Piroplasms	22	*Theileria orientalis*:	22/22	98.88 – 99.91%

In order to determine the genetic relationships of vector-borne protozoa and rickettsia among different buffaloes in Thai population and other countries, pairwise nucleotide identity analyses were conducted. For anaplasmosis, *A. marginale* nucleotide sequences of the 16S rRNA gene showed percentage of identity ranging from 97.2 to 100% (Table 10a) while *A. platys* sequences among three Thai buffalo isolates depicted an identical level of 100%. When comparing the *A. platys* sequences in buffaloes in Thailand with other countries, these sequences were more similar to *A. platys* sequences in China, South Africa and Thailand (99.7%) than those in Venezuela

(99.5%) and Vietnam (99.3%) (Table 10b). Pairwise nucleotide identity of *P. bubalis* based on cytb gene was less similar, from 94.2 to 100% (Table 11). However, in case of *T. orientalis*, sequences of the 18S rRNA gene indicated a high degree of percentage identity between 97.2 and 100% (Table 12).

Table 10. (a) Pairwise nucleotide identity matrix of *A. marginale* from Thai buffaloes based on 16S rRNA gene

		Identity (%)																												
	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1.	Thailand (KT264188)	100.0																													
2.	North China (HM538192)	100.0	100.0																												
3.	Central China (AJ633048)	99.8	99.8	100.0																											
4.	South China (DQ341370)	100.0	100.0	99.8	100.0																										
5.	Southwest China (HM538193)	99.0	99.0	98.8	99.0	100.0																									
6.	Southeastern U.S.A. (AF311303)	100.0	100.0	99.8	100.0	99.0	100.0																								
7.	MT27 Uganda (KU686794)	99.9	99.9	99.7	99.9	98.9	99.9	100.0																							
8.	Central Philippines(JQ839012)	100.0	100.0	99.8	100.0	99.0	100.0	99.9	100.0																						
9.	Thailand B15-14	100.0	100.0	99.8	100.0	99.0	100.0	99.9	100.0	100.0																					
10.	Thailand B15-24	100.0	100.0	99.8	100.0	99.0	100.0	99.9	100.0	100.0	100.0																				
11.	Thailand B15-35	99.0	99.0	98.8	99.0	98.0	99.0	98.9	99.0	99.0	99.0	100.0																			
12.	Thailand B15-48	99.4	99.4	99.2	99.4	98.4	99.4	99.5	99.4	99.4	99.4	99.0	100.0																		
13.	Thailand B15-49	99.8	99.8	99.6	99.8	98.8	99.8	99.7	99.8	99.8	99.8	98.9	99.3	100.0																	
14.	Thailand B15-51	99.0	99.0	98.8	99.0	98.0	99.0	99.0	99.0	99.0	99.0	99.0	99.5	98.9	100.0																
15.	Thailand B15-52	99.6	99.6	99.4	99.6	98.6	99.6	99.7	99.6	99.6	99.6	98.6	99.6	99.4	99.1	100.0															
16.	Thailand B15-55	99.9	99.9	99.7	99.9	98.9	99.9	99.8	99.9	99.9	99.9	99.0	99.3	99.7	99.0	99.5	100.0														
17.	Thailand B15-56	99.2	99.2	99.0	99.2	98.2	99.2	99.1	99.2	99.2	99.2	99.1	99.0	99.0	99.5	98.9	99.3	100.0													
18.	Thailand B15-69	99.8	99.8	99.6	99.8	98.8	99.8	99.7	99.8	99.8	99.8	99.0	99.6	99.6	99.1	99.6	99.7	99.2	100.0												
19.	Thailand B15-87	99.9	99.9	99.7	99.9	98.9	99.9	99.8	99.9	99.9	99.9	98.9	99.3	99.7	98.9	99.5	99.8	99.1	99.7	100.0											
20.	Thailand B16-9	99.4	99.4	99.2	99.4	98.4	99.4	99.5	99.4	99.4	99.4	98.5	99.4	99.2	99.0	99.6	99.3	98.7	99.4	99.3	100.0										
21.	Thailand B16-17	99.7	99.7	99.5	99.7	98.7	99.7	99.8	99.7	99.7	99.7	98.7	99.7	99.5	99.2	99.9	99.6	99.0	99.7	99.6	99.7	100.0									
22.	Thailand B16-24	100.0	100.0	99.8	100.0	99.0	100.0	99.9	100.0	100.0	100.0	99.0	99.4	99.8	99.0	99.6	99.9	99.2	99.8	99.9	99.4	99.7	100.0								
23.	Thailand B17-500	99.8	99.8	99.6	99.8	98.8	99.8	99.7	99.8	99.8	99.8	98.8	99.2	99.6	98.8	99.4	99.7	99.0	99.6	99.9	99.2	99.5	99.8	100.0							
24.	Thailand B17-503	99.9	99.9	99.7	99.9	98.9	99.9	99.8	99.9	99.9	99.9	98.9	99.3	99.7	98.9	99.5	99.8	99.1	99.7	100.0	99.3	99.6	99.9	99.9	100.0						
25.	Thailand B17-510	98.8	98.8	98.6	98.8	97.8	98.8	98.7	98.8	98.8	98.8	98.9	98.6	98.7	98.5	98.5	98.7	98.8	98.8	98.9	98.2	98.5	98.8	98.8	98.9	100.0					
26.	Thailand B17-512	98.1	98.1	98.0	98.1	97.2	98.1	98.1	98.1	98.1	98.1	98.7	98.0	98.2	98.2	97.9	98.1	98.5	98.1	98.2	97.7	97.9	98.1	98.1	98.2	99.3	100.0				
27.	Thailand B17-527	98.4	98.4	98.2	98.4	97.4	98.4	98.3	98.4	98.4	98.4	98.6	98.2	98.3	98.1	98.1	98.3	98.3	98.4	98.5	97.9	98.1	98.4	98.4	98.5	99.2	99.3	100.0			
28.	Thailand B17-528	99.3	99.3	99.1	99.3	98.3	99.3	99.2	99.3	99.3	99.3	98.9	99.0	99.2	98.5	99.0	99.2	98.7	99.1	99.2	98.8	99.0	99.3	99.1	99.2	99.1	98.7	99.0	100.0		
29.	Thailand B17-531	98.9	98.9	98.7	98.9	97.9	98.9	98.8	98.9	98.9	98.9	98.9	98.7	98.8	98.4	98.6	98.8	98.6	98.7	98.8	98.4	98.6	98.9	98.7	98.8	99.0	99.0	99.0	99.2	100.0	
30.	Thailand B18-49	99.7	99.7	99.5	99.7	98.7	99.7	99.8	99.7	99.7	99.7	98.7	99.7	99.5	99.2	99.9	99.6	99.0	99.7	99.6	99.7	100.0	99.7	99.5	99.6	98.5	97.9	98.1	99.0	98.6	100.0

(b) Pairwise nucleotide identity matrix of *A. platys* from Thai buffaloes based on 16S rRNA gene

	Identity (%)							
Isolate	1	2	3	4	5	6	7	8
1. China (MH762081)	100.0							
2. Vietnam (MH686048)	99.5	100.0						
3. Venezuela (AF399917)	99.7	99.3	100.0					
4. Thailand (EF139459)	100.0	99.5	99.7	100.0				
5. South Africa (MK814449)	100.0	99.5	99.7	100.0	100.0			
6. Thailand B16-72	99.7	99.3	99.5	99.7	99.7	100.0		
7. Thailand B16-83	99.7	99.3	99.5	99.7	99.7	100.0	100.0	
8. Thailand B16-85	99.7	99.3	99.5	99.7	99.7	100.0	100.0	100.0

Table 11. Pairwise nucleotide identity matrix of *P. bubalis* from buffaloes based on cytb gene

	Identity (%)													
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Thailand (LC090213)	100.0													
2. Thailand (LC090214)	94.2	100.0												
3. Thailand B15-61	94.3	99.8	100.0											
4. Thailand B15-83	100.0	94.2	94.3	100.0										
5. Thailand B15-89	100.0	94.2	94.3	100.0	100.0									
6. Thailand B16-25	96.0	97.8	97.9	96.0	96.0	100.0								
7. Thailand B16-59	100.0	94.2	94.3	100.0	100.0	96.0	100.0							
8. Thailand B16-158	94.3	99.8	100.0	94.3	94.3	97.9	94.3	100.0						
9. Thailand B16-164	94.3	99.8	100.0	94.3	94.3	97.9	94.3	100.0	100.0					
10. Thailand B18-5	100.0	94.2	94.3	100.0	100.0	96.0	100.0	94.3	94.3	100.0				
11. Thailand B18-22	100.0	94.2	94.3	100.0	100.0	96.0	100.0	94.3	94.3	100.0	100.0			
12. Thailand B18-47	100.0	94.2	94.3	100.0	100.0	96.0	100.0	94.3	94.3	100.0	100.0	100.0		
13. Thailand B18-75	100.0	94.2	94.3	100.0	100.0	96.0	100.0	94.3	94.3	100.0	100.0	100.0	100.0	
14. Thailand B18-79	100.0	94.2	94.3	100.0	100.0	96.0	100.0	94.3	94.3	100.0	100.0	100.0	100.0	100.0

Table 12. Pairwise nucleotide identity matrix of *T. orientalis* from Thai buffaloes based on 18S rRNA gene

	Identity (%)																			
Isolate	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1. China (MG599089)	100.0																				
2. Kagawa Japan (AB668373)	98.0	100.0																			
3. Pakistan (MG599099)	98.6	99.1	100.0																		
4. Australia (AB520958)	97.7	98.8	98.9	100.0																	
5. Northern Japan (U97048)	97.6	98.7	98.8	99.9	100.0																
6. North Korea (U97050)	97.8	99.0	99.0	98.8	98.7	100.0															
7. South Korea (U97051)	98.7	99.2	99.9	99.0	98.9	99.1	100.0														
8. South Korea (U97053)	97.4	98.4	98.5	99.2	99.1	98.2	98.6	100.0													
9. South Central U.S.A. (AY661512)	98.0	100.0	99.1	98.8	98.7	99.0	99.2	98.4	100.0												
10. Thailand B16-10	97.7	98.6	98.9	98.7	98.6	98.5	99.0	98.3	98.6	100.0											
11. Thailand B117-38	97.6	98.5	98.8	98.6	98.5	98.6	98.9	98.2	98.5	99.8	100.0										
12. Thailand B17-250	97.3	98.3	98.4	98.2	98.2	98.2	98.5	97.9	98.3	98.8	99.0	100.0									
13. Thailand B17-254	97.5	98.4	98.7	98.5	98.4	98.3	98.8	98.2	98.4	99.8	99.6	98.7	100.0								
14. Thailand B17-257	97.2	98.4	98.3	98.2	98.1	98.0	98.4	97.8	98.4	98.8	98.8	99.5	98.7	100.0							
15. Thailand B17-266	97.6	98.5	98.8	98.6	98.5	98.4	98.9	98.2	98.5	99.9	99.7	98.9	99.7	98.9	100.0						
16. Thailand B17-271	97.3	98.3	98.4	98.2	98.2	98.2	98.5	97.9	98.3	98.8	98.9	99.9	98.7	99.4	98.9	100.0					
17. Thailand B17-275	97.5	98.6	98.7	98.5	98.4	98.3	98.8	98.2	98.6	99.1	99.0	99.6	99.0	99.5	99.1	99.7	100.0				
18. Thailand B18-21	97.4	98.2	98.5	98.2	98.1	98.3	98.6	97.8	98.2	99.4	99.4	98.9	99.6	98.7	99.3	98.9	99.0	100.0			
19. Thailand B18-32	97.4	98.2	98.5	98.3	98.2	98.3	98.6	98.0	98.2	99.6	99.6	98.9	99.6	98.7	99.5	98.9	99.0	99.8	100.0		
20. Thailand B18-51	97.4	98.2	98.5	98.3	98.2	98.2	98.6	98.0	98.2	99.6	99.4	98.5	99.8	98.5	99.5	98.5	98.8	99.4	99.4	100.0	
21. Thailand B18-71	97.4	98.5	98.6	98.4	98.3	98.4	98.7	98.1	98.5	99.0	99.1	99.7	98.9	99.4	99.1	99.8	99.7	98.9	98.9	98.7	100.0

After DNA sequencing, 37 sequences of the 16S rRNA gene of *A. marginale*, 3 sequences of the 16S rRNA gene of *A. platys*, 12 sequences of the cytb gene of *P. bubalis* and 22 sequences of the 18S rRNA gene of *T. orientalis* from buffaloes were successfully obtained. The phylogenetic trees were constructed by MEGA 10.0.5 software. Maximum Likelihood tree of *Anaplasma* spp. constructed with 16S rRNA sequences indicates that *A. marginale* and *A. platys* found in Thai buffalo isolates were clustered in 2 different clades (Figure 2a). *A. marginale* isolates were closely related within the Thai isolates and placed in a clade that was closely related to *A. marginale*

in buffalo in China (DQ341370, HM538192); cattle in Thailand (KT264188), Uganda (KU686794), China (AJ633048) and Italy (EF520690); tick in China (KX987330), the Philippines (JQ839012) and South Africa (AF414869); and dog in Hungary (MH020201). Another study conducted in the Philippines to detect tick-borne pathogens in water buffaloes by molecular method targeting groEL gene of *A. marginale* also showed that *A. marginale* sequences were identical to isolates from China and the Philippines (Galon et al., 2019). *A. platys* isolates found from buffalo in Thailand belonged to *A. platys* group. They were related to *A. phagocytophilum* group and placed separately with *A. marginale.* This result was in agreement with the finding in Mozambique, which reported that sequences for *A. platys* were related to *A. phagocytophilum* with genetic divergence of 0.8% (Machado et al., 2016). Phylogenetic tree of cytb sequences showed that twelve *P. bubalis* isolates in this study were grouped together in the same clade with strong bootstrap support of 94%. Furthermore, they were branched into 2 subclades. The first subclade including 8 sequences shown close relationships to *P. bubalis* (LC090213) detected in water buffaloes in Thailand before. The second one including 4 sequences indicated that there was a close relationship between *P. bubalis* in buffaloes and *P. caprae* in goat found in Thailand and Zambia (Figure 3). Our finding was totally matched with recently published phylogenetic tree of ungulate malaria parasites (Templeton et al., 2016). Regarding piroplasms, *T. orientalis* sequences in the present study were branched into 2 clades. First clade including thirteen sequences were clustered with non-virulent types of *T. orientalis* such as Type A, B, B1, C, E and H while another clade was not shared with any other known types of *T. orientalis* (Figure 4).

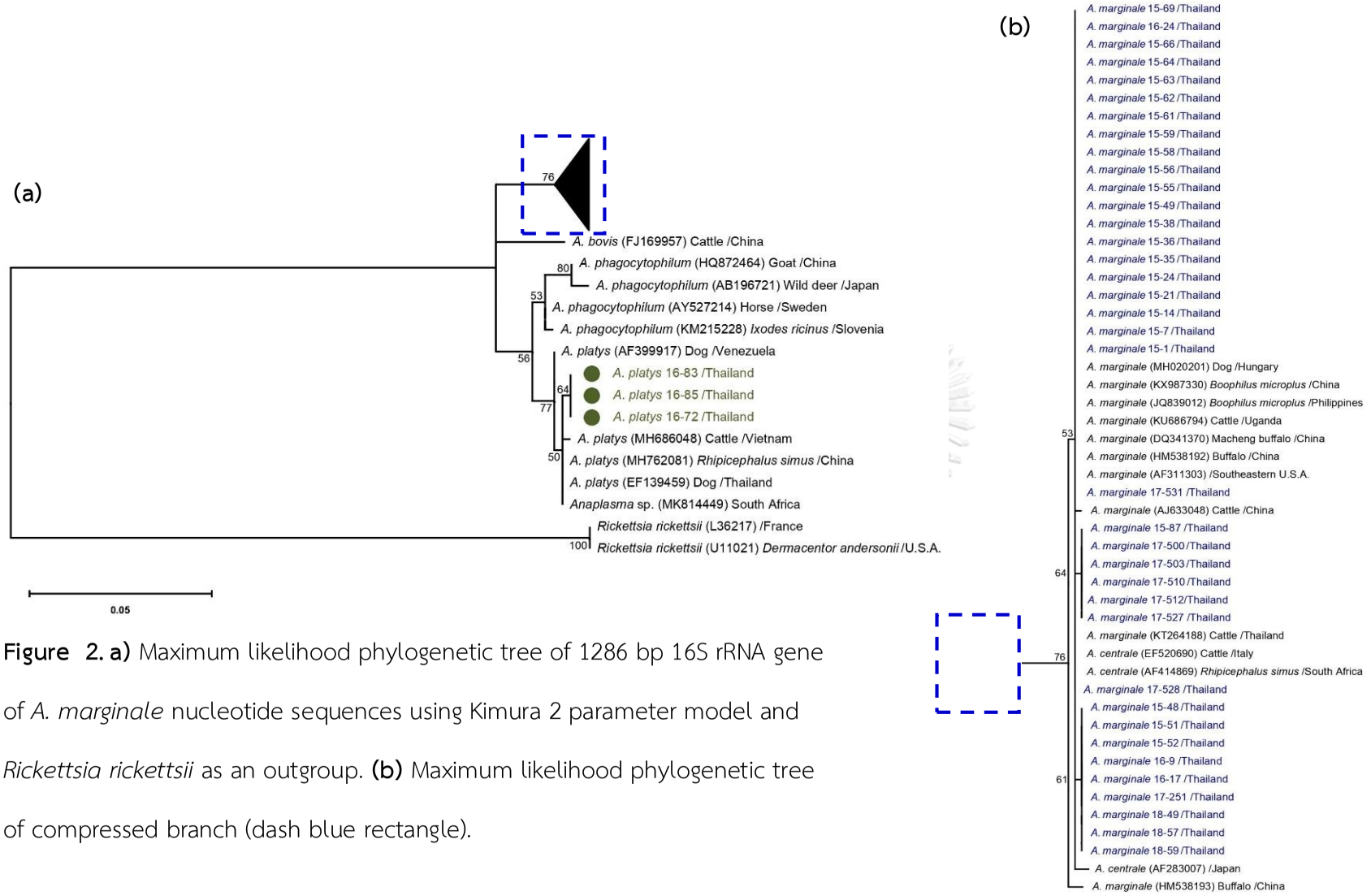

Figure 2. a) Maximum likelihood phylogenetic tree of 1286 bp 16S rRNA gene of *A. marginale* nucleotide sequences using Kimura 2 parameter model and *Rickettsia rickettsii* as an outgroup. **(b)** Maximum likelihood phylogenetic tree of compressed branch (dash blue rectangle).

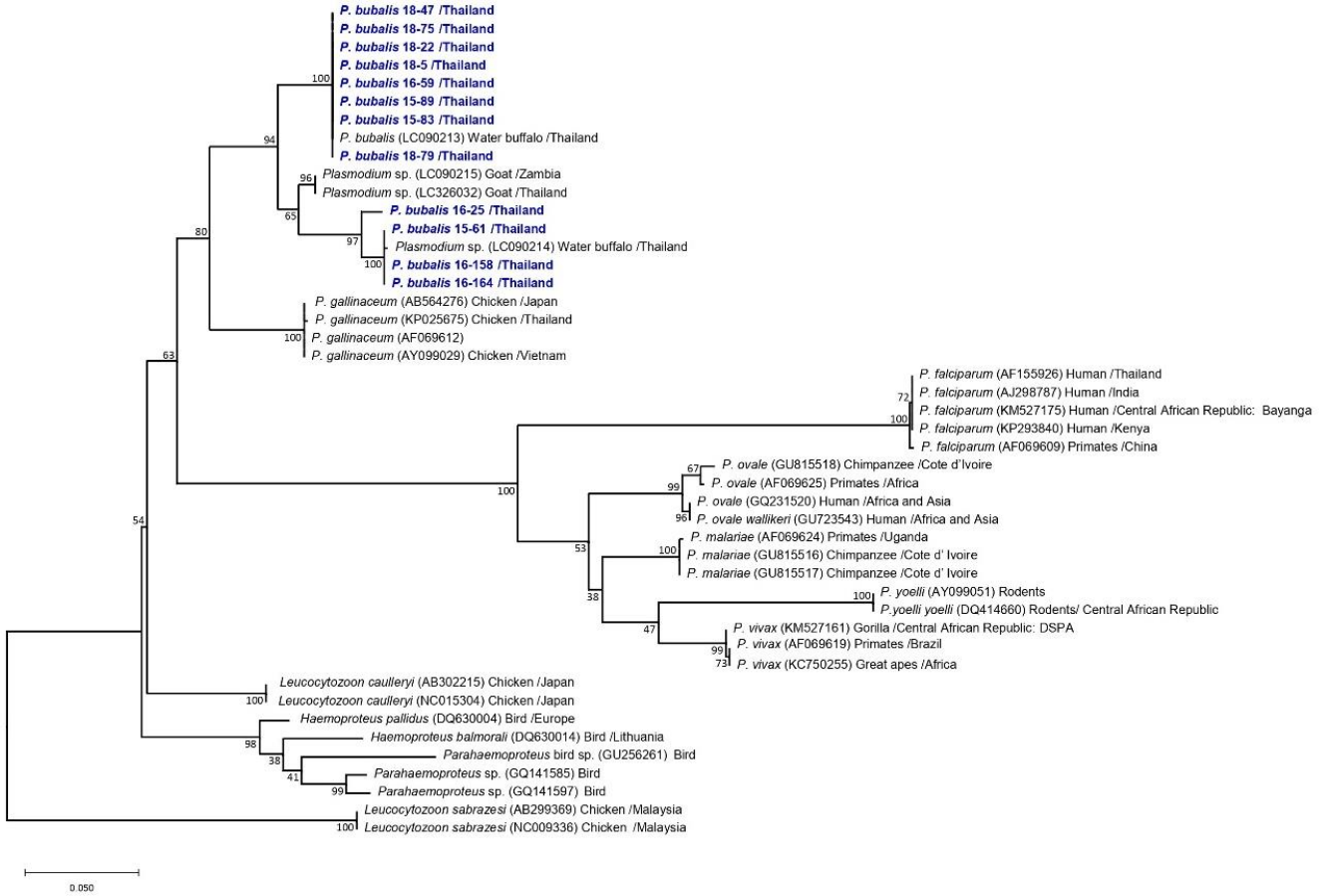

Figure 3. Maximum likelihood phylogenetic tree of 822 bp partial mitochondrial cytb gene of *P. bubalis* nucleotide sequences using General Time Reversible model and *Leucocytozoon sabrasezi* as an outgroup.

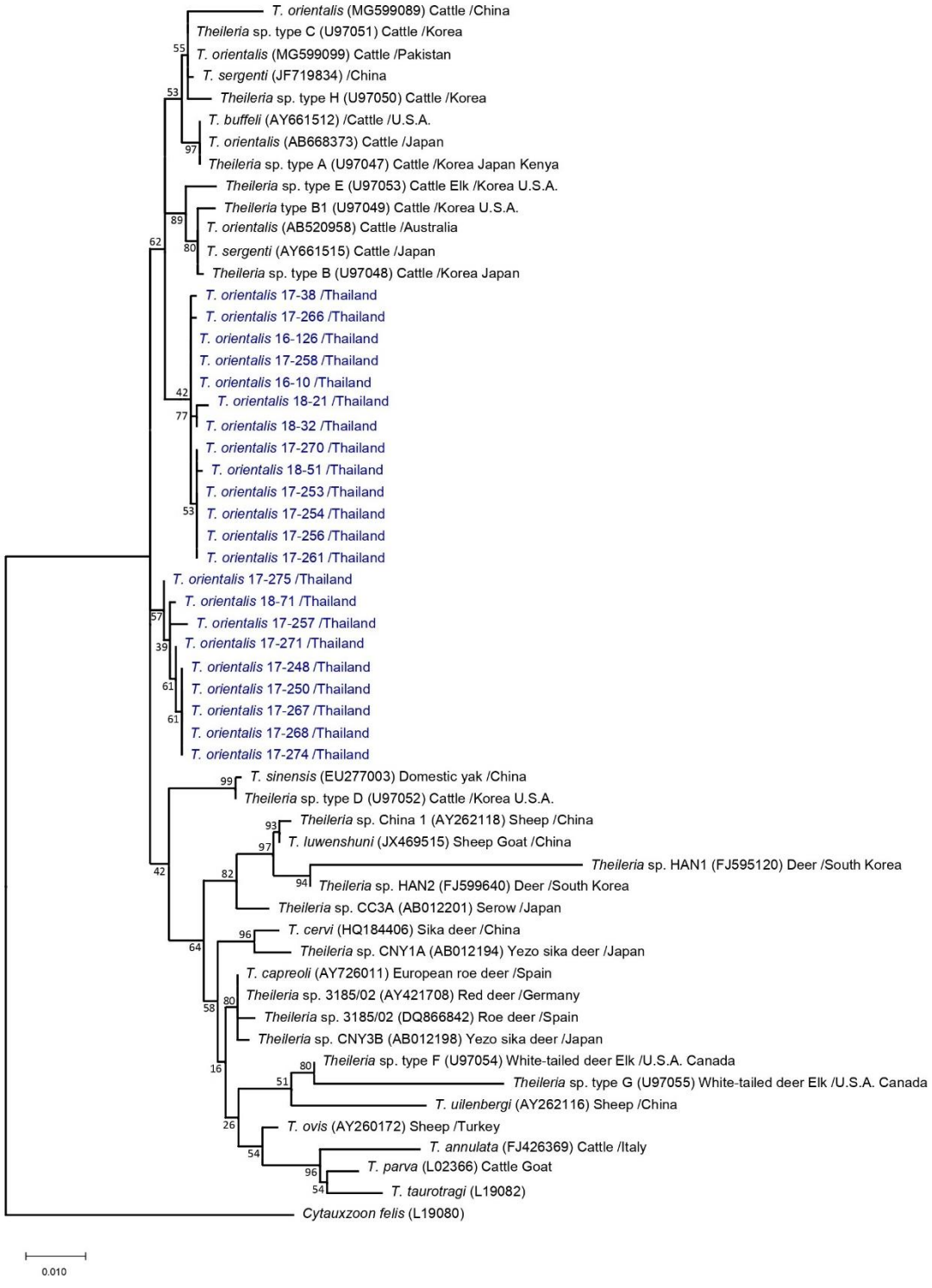

Figure 4. Maximum likelihood phylogenetic tree of 1221 bp 18S rRNA gene of *T. orientalis* nucleotide sequences using Kimura 2 parameter model and *Cytauxzoon felis* as an outgroup.

4.6 Nucleotide diversity among DNA sequences of *A. marginale*, *P. bubalis* and *T. orientalis* from buffaloes in Thailand

The nucleotide diversity of *A. marginale* 16S rRNA, *P. bubalis* cytb and *T. orientalis* 18S rRNA gene was shown in Table 13. Meanwhile, three *A. platys* isolates shown to be identical to each other in 1,124 bp aligned nucleotide positions and showed no segregating site and only 1 haplotype. Generally, in the present study, DNA sequences targeting ribosomal RNA gene (*A. marginale* 16S rRNA and *T. orientalis* 18S rRNA) showed limited polymorphic sites, lower nucleotide diversity but higher number of haplotypes and haplotype diversity than those of mitochondria gene (*P. bubalis* cytb).

Table 13. Nucleotide diversity of vector-borne protozoa and rickettsia in Thai buffaloes

Gene target	(bp)	N	S	H	Hd	π
16S rRNA (*A. marginale*)	1110	37	29	18	0.826	0.00586
cytb (*P. bubalis*)	796	12	46	3	0.530	0.02626
18S rRNA (*T. orientalis*)	1132	22	15	15	0.961	0.00626

N: number of sequences analyzed, S: number of polymorphic (segregated) sites, H: number of haplotypes, Hd: Haplotype diversity, π: nucleotide diversity (Pi)

CHAPTER 5

DISCUSSION

Buffaloes are susceptible to most cattle diseases; however, the effect is not as serious as that seen in infected cattle in the same ecosystem owing to their breed resistance (Rajput et al., 2005). Clinical anaplasmosis is most notable in cattle but water buffalo can become persistently infected and harbor the sub-clinical disease (Kuttler, 1984). Besides, pathogenic genotypes of *T. orientalis* (genotype Ikeda) were mainly limited to Japan, Australia, New Zealand and Southeastern U.S.A. (Izzo et al., 2010; Kamau et al., 2011; McFadden et al., 2011; Oakes et al., 2019) and have not been detected in Thailand yet. Although clinical anaplasmosis, malaria and piroplasmosis have rarely been detected in buffaloes in Thailand, the potential for these animals to serve as reservoir hosts for *Anaplasma*, *Plasmodium* and *Theileria* has not been investigated yet. Additionally, bovine anaplasmosis, theileriosis and ungulate malaria presenting in Thailand nowadays cause negative impacts on animal health and economic losses, they may have a risk to induce more severe diseases due to animal transportation among countries leading to transmission of exotic strains. Therefore, this study was carried out to investigate the occurrence and genetic diversity of rickettsia, malaria and piroplasms parasites. This study reported the detection and identification of *Anaplasma*, *Plasmodium* and *Theileria* DNA in the Thai buffaloes. The prevalence of *A. marginale* in buffaloes was different among eight provinces, ranging from 6 to 67.6% and the overall was 41%. The high prevalence of buffaloes showing positive to *A. marginale* was in the similar level with 2 studies conducted in Cuba (52%) (Obregón et al., 2018) and Mozambique (72.2%) (Machado et al., 2016). However, the present study was higher than those of previous studies in the Philippines (29%) (Galon et al., 2019), Malaysia (21.8%) (Koh et al., 2018), India (18.33%) (Kumar et al., 2019), South

Africa (17.3%) (Sisson et al., 2017), Pakistan (14.73%) (Farooqi et al., 2018), Columbia (13.1%) (Jaimes-Dueñez et al., 2018), and northeast Thailand (8%) (Saetiew et al., 2015). The different prevalence of *A. marginale* in buffaloes among provinces seems to be dependent on environmental factors including tick population, season and management system in each farm (Obregón et al., 2018). In addition, age of host also plays a role in disease susceptibility. Young animals are more likely susceptible to *A. marginale* infection in comparison with adult cattle because of their softer skin facilitating the mouth–part penetration of the vector, make them the preferred host of ticks (Kabir et al., 2011).

For malaria parasite, *P. bubalis* was detected in 6 out of 8 sampling provinces of Thailand with the malaria prevalence ranging from 4.8% to 35.4%. Among 6 provinces, river buffaloes in Chachoengsao Province showed the highest prevalence of malaria infection, matching with the finding of Templeton et al., 2016. Although study of buffalo malaria was still limited, there were some research studies on other ungulates. Elsewhere, a study regarding ungulate malaria was conducted in Southern Brazil but no positive sample was found owing to the cervid blood samples were collected at only single time point so parasitemia level may fluctuate over the parasite's life cycle (dos Santos et al., 2018). Another study proved that there was a close relationship between *Plasmodium* sequences detected from South American pampas deer (*Ozotoceros bezoarticus*) to *P. odocoilei* clade 2 in North American white-tailed deer (*Odocoileus virginianus*) (Asada et al., 2018).

For piroplasms, the overall prevalence of *T. orientalis* found in buffaloes in the present study was higher than the previous studies in the Northwest Thailand (9.4%) (Altangerel et al., 2011), Pakistan (6.1%) (Gebrekidan et al., 2017), and Brazil (4.2%) (Silveira et al., 2016). However, our result was matched with the findings of Khukhuu

et al. in Vietnam (25.6%) (Khukhuu et al., 2010) but another study conducted in Sri Lanka showed that *T. orientalis* was predominant in water buffaloes (82.5%) compared to *B. bovis* and *B. bigemina* (Sivakumar et al., 2014). A broad range of *T. orientalis* infection rates from 6.0 to 81.1% among different geographical locations indicated the wide distribution of parasites. These results suggested that the prevalence of vector-borne protozoa and rickettsia in buffaloes varied from region to region in Thailand. Factors associated with pathogen infection consisting of age, gender, breed of the host, tick and mosquito density according to season and animal husbandry or management of each farm might explain the inconsistency of the prevalence. *Ehrlichia* and *Babesia* spp. were not detected in any sequenced samples in this study. The present findings contradicted with the results obtaining in water buffaloes in Brazil (Da Silva et al., 2013) and northeast Thailand (Terkawi et al., 2011a), and beef cattle in northern and western regions of Thailand (Rittipornlertrak et al., 2017). On the one hand, the reasons may be because buffaloes are more resistant to babesiosis than cattle or different tick density and distribution between geographic regions of Thailand. On the other hand, only 10%, not all the piroplasms-positive samples were sequenced; therefore, the results may not be representative for the whole parasite population.

In the present study, majority of blood-borne pathogens were *A. marginale*, which was in agreement with previous reports from the Philippines (Galon et al., 2019), and Thailand (Saetiew et al., 2015). The remaining three samples were found to be *A. platys* based on sequence analysis of 16S rRNA and groEL genes. This finding was also observed in the studies in Vietnam (Chien et al., 2019), and Algeria (Dahmani et al., 2015). Another study also reported that *A. platys*–like was detected in Tunisian cattle, goats and sheep (Ben Said et al., 2017), and camels (Selmi et al., 2019). Despite the fact that *A. marginale* is mainly responsible for anaplasmosis in cattle and buffaloes,

A. platys is also known to cause disease in dogs, cattle and human (Maggi et al., 2013; Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014). Buffalo blood samples in this study were collected from buffalo farms, where dogs were seen around the buffalo stalls. Therefore, it was probable for brown dog ticks, *Rhipicephalus sanguineus,* which carry *A. platys*, to be transmitted from dogs to buffaloes. *P. bubalis* detected in the current study was in accordance with results of previous study in Thailand and Vietnam (Templeton et al., 2016). The ungulate malaria parasite clade evolved prior to the divergence of the bird, rodent, primate and human *Plasmodium* clades. In this analysis, *Haemoproteus* and *Parahaemoproteus* were branched out before divergence of the ungulate, bird, rodent and mammalian *Plasmodium* clades, which was consistent with a recent report (Borner et al., 2016). Pathogenic genotype of *T. orientalis*, genotype Ikeda, had caused clinical disease in cattle of Eastern Asia, Australia, New Zealand and Southeastern U.S.A. (Izzo et al., 2010; Kamau et al., 2011; McFadden et al., 2011; Oakes et al., 2019). but have not been detected in Thailand yet. Benign theileriosis in buffaloes caused by *T. orientalis* found in the present study was similar to findings in water buffaloes in Thailand previously (Altangerel et al., 2011; Jirapattharasate et al., 2016) and Sri Lanka (Sivakumar et al., 2014). In this study, tick-borne pathogens in Uthai Thani, Amnat Charoen, Nong Bua Lamphu, Phatthalung and Chachoengsao were surveyed for the first time.

16S rRNA and 18S rRNA genes are the most common targets for pathogen detection and identification then constructing the phylogenetic trees. However, the highly conserved property of these genes demonstrates the low level of polymorphism and genetic diversity between different isolates. The present study was successful in detecting the prevalence of rickettsia and protozoa in buffalo bloods. Nevertheless, the 16S rRNA and 18S rRNA gene sequences had a relatively low level of polymorphism

and genetic diversity when compared to other DNA targets such as outer membrane protein: *msp1α* (de Souza Ramos et al., 2019; Fedorina et al., 2019; Fernandes et al., 2019), *msp4* (Ramos et al., 2019), *msp5* and major piroplasm surface protein (MPSP) gene (Jirapattharasate et al., 2016). A previous study showed that although msp1α, *msp4* and *msp5* were found to be conserved genes, they were proved to be useful for phylogenetic analysis among geographic *A. marginale* strains (de la Fuente et al., 2005). Our results, consistent with previous observations, *A. marginale* 16S rRNA and *T. orientalis* 18S rRNA genes were very highly conserved in Thailand, as indicated by high nucleotide identity percentages, from 97.2% – 100%. These results suggest that ribosomal RNA genes may not be ideal targets for genetic diversity analysis; however, they are precious genes for detection and identification of rickettsia and protozoa in different geographic locations.

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS

6.1 Conclusions

A total of 456 buffalo blood samples were successfully examined for the occurrence of vector-borne protozoa and rickettsia. The results indicated that *A. marginale* and *T. orientalis* were widely distributed in all 8 investigated locations while *P. bubalis* presented in 6 out of 8 surveyed sites with the overall prevalence of 41%, 25.4% and 12.9% respectively. *Ehrlichia* spp. and *Babesia* spp. were not detected from any sequenced samples in this study. Nucleotide identity matrixes and phylogenetic trees of *A. marginale* and *T. orientalis* showed that those isolates had high similarity and close relationships with each other. Nucleotide diversity values suggested that low levels of polymorphism and genetic diversity of *A. marginale* and *T. orientalis* observed between different isolates owing to the highly conserved property of these genes. However, cytochrome b in *P. bubalis* population showed a higher number of segregating sites and nucleotide diversity.

6.2 Suggestions

This study was successful in detecting the prevalence and identifying the causative agents of anaplasmosis, theileriosis and ungulate malaria in buffaloes of 8 geographic sites in Thailand by PCR-based technique targeting 16S rRNA, 18S rRNA and cytb genes, respectively. Nevertheless, with the study purpose of genetic diversity analysis between different geographic locations, other gene targets including membrane surface protein (*msp*) genes, amino acid-coding genes, are necessary to be considered to better understand genotypes, pathogenicity, sequence variability and

mutation. Other factors that may influence the disease prevalence are tick and mosquito vectors. However, no ticks or mosquitos from these regions have been tested and incriminated as the vector of *T. orientalis* and *P. bubalis*. Hence, ticks and mosquitos should also be further investigated to better understand the role of parasites versus competent vectors regarding the disease prevalence and transmission.

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