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THESIS

MOLECULAR EPIDEMIOLOGY OF *B.BOVIS* AND *B.BIGEMINA* INFECTIONS OF CATTLE AND BUFFALOES IN THAILAND

PACHARATHON SIMKING

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Bovine babesiosis is an important tick – borne disease that has an impact on economic losses and animal health due to increase mortality and production losses. This disease in cattle is caused by *Babesia bovis* and *B.bigemina* and transmitted by tropical cattle tick, *Rhipicephalus microplus*. The objectives of this study were to investigate the prevalence and factors associated with *B.bovis and B.bigemina* infection of cattle and buffaloes, and to analyze genetic diversity and phylogenetic of *B.bovis* and *B.bigemina* distributed in all areas of Thailand. A total of 2,685 blood samples were collected from dairy and beef cattle, and water buffaloes in the high populated areas, and screened by nested PCR (nPCR) using *B.bovis* SBP 2 gene and *B. bigemina rap1* - α gene. The risk factors associated with *Babesia* infection including provinces, regions, herd and age were statistical analyzed. The overall infection of *B.bovis and B.bigemina* infection were 7.6% (204/2,685) and 11.2% (300/2,685) respectively. Beef cattle had the highest infection for both organisms and the host speciation showed significant relationship in both *Babesia* spp. ($p<0.01$). In addition, the high prevalence of *Babesia* spp infection was found in animals under 1year (31%, 35/113) and this factor characterized as one of the risk factor of *Babesia* infection in the animals. The phylogenetic analysis of *B.bigemina* using *rap – 1* α gene and *B.bovis* using SBP2 gene showed the cluster of *Babesia* isolation in Thailand were grouped within the host species. Additional analysis of B cell epitope prediction of MSA – 2b gene of *B.bovis* indicated that the polymorphism of this major surface antigen might influence the *Babesia* infection.

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Student's signature Thesis Advisor's signature

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Pacharathon Simking

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LIST OF ABBREVIATIONS

°C	=	Degree(s) Celsius
μg	=	Microgram(s)
μl	=	Microliter(s)
μm	=	Micrometer(s)
bp	=	Base pair(s)
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
DW	=	Distilled water
EDTA	=	Ethylenediamine tetraacetic acid
ELISA	=	Enzyme linked-immunosorbent assay
et al	=	*et alli*
g	=	Gram(s)
IFAT	=	Indirect Fluorescence Antibody Test
kg(s)	=	Kilogram(s)
L (l)	=	Liter(s)
M	=	Mole
mg	=	Milligram(s)
min	=	Minute(s)
ml	=	Milliliter(s)
mm	=	Millimeter(s)
mM	=	Millimolar(s)

LIST OF ABBREVIATIONS (Continued)

MW	=	Molecular weight
PCR	=	Polymerase chain reaction
pH	=	Negative logarithm of hydrogen ion activity
RNA	=	Ribonucleic acid
rpm	=	Round(s) per minute
TBE	=	Tris – boric acid - EDTA
U/μl	=	Unit(s) per microliter
U/g	=	Unit(s) per gram
UDW	=	Ultrapure distilled water
V	=	Volts

MOLECULAR EPIDEMIOLOGY OF *B.BOVIS* AND *B.BIGEMINA* INFECTION OF CATTLE AND BUFFALOES IN THAILAND

INTRODUCTION

Tick- borne diseases (TBD) are an important disease of cattle, transmitted by cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*, widespread in tropical and subtropical regions in the world. The direct losses of tick infestations on animals are shown by weight losses, low productions, damaged hide and increasing mortality. The cattle tick is a biological vector of a few pathogens including rickettsia (*Anaplasma marginale*) and piroplasm (*Theileria* spp., *Babesia bovis* and *B. bigemina*). These pathogens are also worldwide importance due to their severe clinical signs such as anemia, icterus, fever and death. TBDs have the economic impact in many developing countries including Thailand.

As one of major TBDs, babesiosis caused by *Babesia* spp. (*B. bigemina, B. bovis, B. divergen* and *B. major*) is the most economically important of cattle in tropical and subtropical regions of the world. Both *B. bovis* and *B. bigemina* were reported as the major effect on cattle health and productivity in Southeast Asian countries (Aboulaila *et al*., 2010).

The clinical signs of babesiosis are characterized by fever, anemia, icterus and haemoglobinuria in the infected host (de Vos and Potgieter, 1994). Babesiosis caused by *B. bovis* is more severe than that by *B. bigemina* (Ristic, 1981). The clinical signs caused by *B. bovis* are shown as anemia, sensorial depression and high mortality. Anemia and microvasculature alteration are resulting of the consequence of specific modification provoked by *B. bovis* to the erythrocytes, causing sequestration to the endothelial cell and hemolysis (Kuttler, 1988; Homer *et al*., 2000). The clinical signs of *B. bigemina* were usually found as high fever (106.7°F), anorexia, haemoblobinuria and ruminal atony (Taylor *et al*., 2007). The anemia is caused by enormous erythrocytes destroyed (≥75%) led to severe haemoglobinemia and haemoglobinuria.

Mortalitiy of *B.bigemina* infection is variable and may reach 50% or higher but in the absence of undue stress, some animals might survive (Taylor *et al*., 2007).

The diagnosis of bovine babesiosis is relied on clinical signs of disease, microscopic examination, serological test, and molecular technique. The microscopic examination is still the most practical method for the diagnosis of acute babesiosis for veterinarious in the fields. Due to its low sensitivity and time consuming, this method is practical in epidemiological study. Several serological methods such as ELISA and IFAT have been developed and extensively employed in the epidemiology studies because of their high sensitivity. However, the drawback of this technique is the cross – reaction between *B. bovis* and *B. bigemina* and the lack of discrimination between previous exposure and current infection (Wagner *et al*., 1992; Passos *et al*., 1998). To identify carriers is important for the assessment risks of dairy farms since they might serve as a reservoir host of infection for ticks and for other naive animals. The molecular technique including polymerase chain reaction (PCR) was more specificity to detect and differentiate *Babesia* spp. since the efficient PCR has been verified as a useful tool in epidemiological studies (Aboulaila *et al*., 2010).

The molecular evolution of *Babesia* spp. in recent year provide a molecular data that support the phylogenic framework and currently integrating in term of biodiversity (Morrison. 2009). For example, the result of phylogenic studies can reveal the unidentified *Babesia* spp. that found in Turkey were similar to *B. bigemina* and *B. major* (Altay *et al*., 2008). Furthermore, the evolutionary studies also show the conserve region of *Babesia* DNA sequences as a genetic marker or candidate vaccine antigen (He *et al*., 2009).

Bovine babesiosis in Thailand has been reported for more than 4 decades, and the distribution of this disease affected the economic losses in livestock production including meat, milk and raw hides. Recently, the serological studies in water buffaloes in Northeast provinces of Thailand demonstrated the prevalence ranged 3.6 – 16.8% (Terkawi *et al*., 2011a). The prevalent vector, the cattle tick (*Rhipicephalus microplus*), is the important factor of the disease's distribution in the endemic areas.

However, no complete molecular information regarding *B.bigemina* and *B. bovis* infections associated with livestock in Thailand are existed. Therefore, this study has the objectives to (1) to determine the prevalence of *Babesia* spp. infections in ruminant host (buffaloes dairy and beef cattle) in Thailand, (2) to analyze factors associated with *Babesia* spp. infection in ruminant host and (3)to analyze genetic variation of *B.bovis* and *B.bigemina* among regions and provinces of Thailand.

OBJECTIVES

1. To determine the prevalence of *Babesi*a spp. infections in ruminant host (dairy cattle, beef cattle and buffaloes) in Thailand.

2. To analyze factors associated with *Babesia* spp. infection in ruminant host

3. To analyze genetic variation of *B.bovis* and *B.bigemina* among regions and provinces of Thailand

LITERATURE REVIEW

1. Morphology

All *Babesia* is belonged to the family Babesiidae, order Piroplasmida, and the phylum Apicomplexa. (Levine *et al*., 1980). Bovine *Babesia* (such as *B. bovis* and *B. bigemina*) is a large *Babesia* (2 – 5 µm in erythrocyte) (Meholhorn and Schein, 1984; Homer *et al*., 2000).

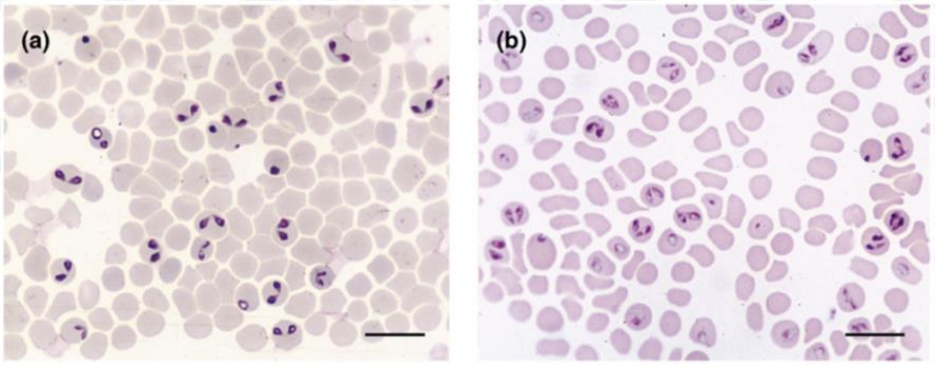


Figure 1 Blood smears from (a) *B. bovis* and (b) *B. bigemina* (scale bars = 10 mm)

Source: Shkap *et al*. 2007

Four pathogenic *Babesia* spp. including *B. bovis*, *B.bigemina*, *B. divergens* and *B. major* are reported in the cattle worldwide. The *Babesia* spp. found in domestic animals vectors and distribution was shown in table1.

Table 1 *Babesia* species of domestic animals, vectors and geographic distribution

Species	Domestic hosts	Vector	Distribution
B. beliceri	Cattle	*Hyalomma*	Russia
B. bigemina	Cattle, buffaloes	*Boophilus, Rhipicephalus*	Africa, America, Asia, Australia, Europe
B. bovis	Cattle, buffaloes	*Boophilus, Rhipicephalus*	Africa, America, Asia, Australia, Europe

Table 1 (Continued)

Species	Domestic hosts	Vector	Distribution
B. divergens	Cattle	*Ixodes*	Europe
B. jakimovi	Cattle, reindeer	*Ixodes??*	Siberia
B. major	Cattle	*Haemaphysalis*	Europe
B. occultans	Cattle	*Hyalomma*	Africa
B. ovata	Cattle	*Haemaphysalis*	Asia
B. orientalis	Buffalo	*Rhipicephalus*	Asia
B. crassa	Sheep, goats	Unknow	Asia
B. matasi	Sheep, goats	*Haemaphysalis*	Africa, Asia, Europe
B. ovis	Sheep, goats	*Rhipicephalus*	Africa, Asia, Europe
B. caballi	Horse, donkey, mule	*Dermacenter*, *Hyalomma*, *Rhipicephalus*	Africa, America, Asia, Europe
B. perroncitoi	Pig	Unknow	Africa, Europe
B. trautmanni	Pig	*Rhipicephalus*	Africa, Europe
B. canis	Dog, cat?	*Dermacenter*	Europe
B. rossi	Dog	*Haemaphysalis*	Africa
B. vogeli	Dog	*Rhipicephalus*	Africa, America, Asia, Australia, Europe
Babesia spp.	Dog	?	USA
B. gibsoni and other small piroplasm of dogs	Dog	*Haemaphysalis*, *Rhipicephalus*	Africa, America, Asia, Europe
B. (*canis*) *presentii*	Cat	Unknow	Asia (Israel)
B. felis	Cat	Unknow	Africa, Europe?

Source: Uilenberg. (2006)

2. *Babesia* in ruminant

2.1 Cattle.

Babesia species commonly found in cattle including *B. bovis, B. bigemina*, *B. divergence* and *B. major*. *B. ovata* (closely related to *B. major*) were reported in Japan and *B. occultans* was found in South Africa (Gray and De Vos, 1981). In Russia, *B. beliceri* and *B. jakimon* were reported with less information (Uilenberg, 2006). Recently, *B. beliceri* and *B. occultans* were reported in China and *Hyalomma* was the potential vector with *B. bigemina* and *B. bovis* worldwide (Luo *et al*., 2002).

2.2 Water Buffalo

Water buffaloes can be also infected. In China, *B. orientalis* has been found as a causing agent of the disease (Liu *et al*., 1997). In addition, the recently report also demonstrated the prevalence of *B.bovis* and *B.bigemina* in water buffaloes host in northeast region of Thailand (Terkawi *et al*., 2011a).

3. Life cycle of bovine babesiosis

Life cycle of babesiosis contain 2 stages including a development in the vertebrate host and the development in the tick vector as shown in figure 2.

3.1. The development in the vertebrate host

The infective stage (sporozoites) is infected into the host through the saliva of the tick during feeding and directly penetrated into host's red blood cells and the sporozoites transform to piroplasms. The piroplasm's multiplication generally yield in two daughter cells, which leave the infected cell and then invades another red blood cell. The multiplication of piroplasm are usually persisted until the burst of the host cell, or until the immune system of the host resist the multiplication of the parasite (Uilenberg, 2006).

3.2: The development in the tick vector

The tick was infected by *Babesia* when ingesting infected blood cells, which should probably be refered as gametocytes. They develop into male and female gametes in the tick gut. A microgamete fuse with a macrogamete to form a motile zygote (Mehlhorn and Schein, 1984). The zygotes multiply and turn to "vermicules" which consequently invade numerous organs of the tick, including the ovaries. Thus, vermicules passes through the ovary and egg to the next tick generation resulted "transovarian transmission". Regularly, the female ticks become infected and their sporogony takes place in the salivary glands of larval, nymphal, and/or adult

ticks of the next generation. When the tick attaches to a new host, the maturation of the sporozoites is complete and the new host will be infected by sporozoites within saliva of ticks (Uilenberg, 2006).

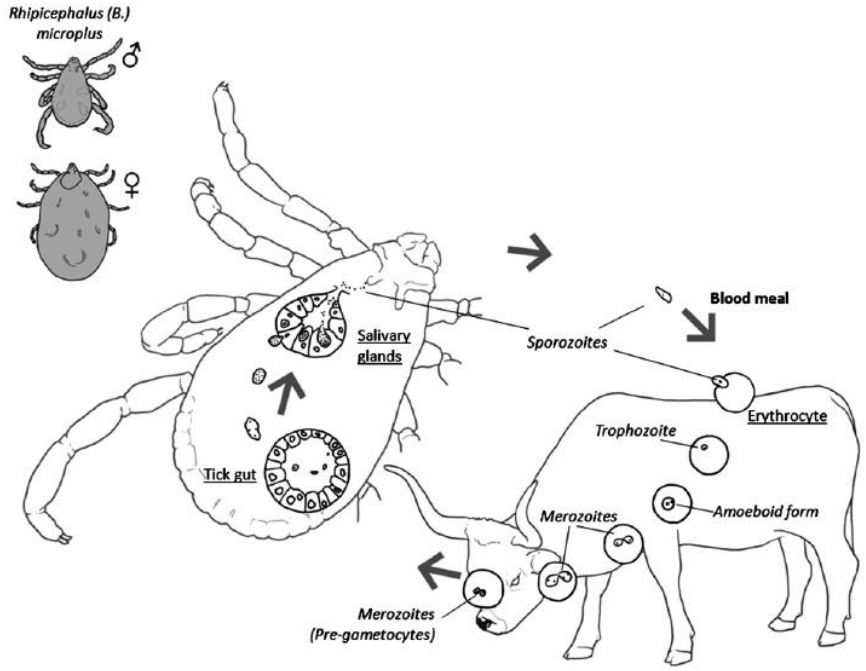


Figure 2 The life cycle of *Babesia bovis*. Infected *Rhipicephalus* (*Boophilus*) *microplus* ticks inject the infective sporozoite into the mammalian host and the parasites enter erythrocytes where they multiply by binary fission and undergo several changes (trophozoite, amoeboid form) until they become merozoites. After multiplication, merozoites break cell and invade other erythrocytes.

Source: Marcelino *et al*. (2012)

4. Clinical signs of bovine babesiosis

Although *B.bigemina* and *B. bovis* are transmitted by the same tick vector, their clinical signs are substantially differ. In acute *B. bigemina* infection, when the parasitemia become as high as 30%, the clinical sign will show and characterize as the

high fever, hemoglobinuria and anemia. For *B. bovis*, the infection is caused cerebral or nervous signs with the low parasitemia (7 - 10%) (Shkap *et al*., 2005).

In addition, hemolytic anemia caused by the destruction of infected and non – infected red blood cells, is usually one of the most critical features of the disease. In *B. bigemina* infection, the organism is completely related to a rapid and massive intravascular hemolysis (Bock *et al*., 2004). For *B. bovis*, this infection is also causing hemolysis, alteration in red cell deformability and rigidity, and damaging endothelial cells leading to pulmonary edema and cerebral dysfunction (Góes *et al*., 2007). *B. bovis* infection is usually resulting more severe and effect on mortality among susceptible cattle. This is probably depend on the ability of parasitized erythrocytes to attach and accumulate in microcapillaries of the kidney, lungs, and brain (Fig.3) led to organ failure, systemic shock, and death (Ristic, 1981; Uilenberg, 2006).

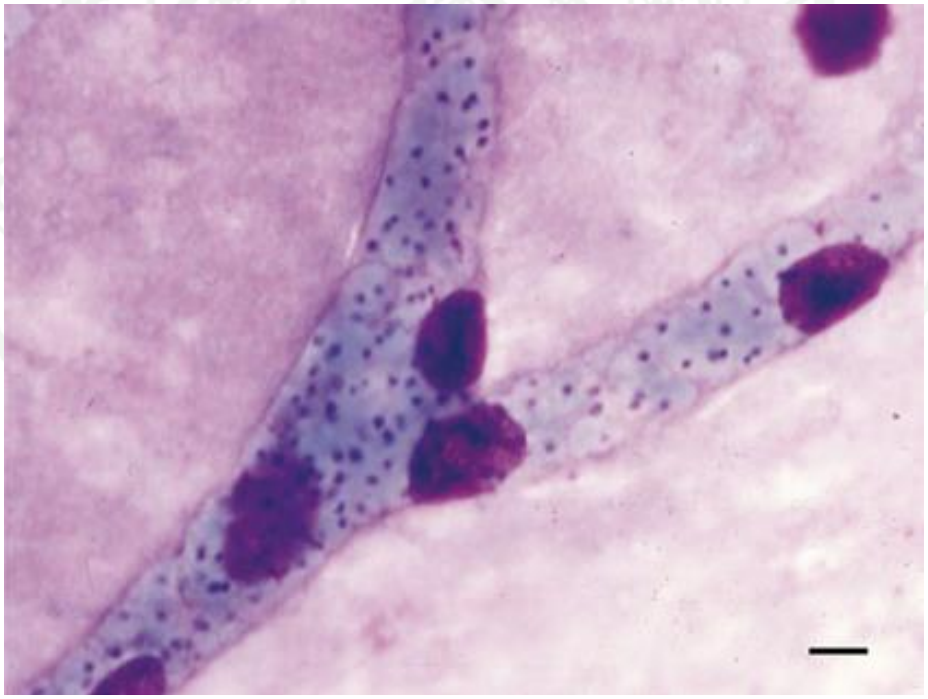


Figure 3 *B. bovis* in a brain capillary smear by Giemsa staining (scale bars = 10 mm).

Source: Shkap *et al*. 2007

5. Diagnosis of bovine babesiosis

Microscopic examination (ME) using Giemsa staining of cattle blood smear is mostly practically used as a confirmative diagnosis during the acute phase of the babesiosis, according to the clinical symptom. However, this method is not able to discriminate among *Babesia spp.* and is not useful for large scale screening test in the epidemiological study (Silva *et al*., 2010). Additionally, the low sensitivity and specificity of ME can not use to identify carrier animals (Almeria *et al*., 2001).

Generally, a detection of antibody is used to test for past or present exposure to the causative agent and, hence, widely used in epidemiological studies to estimate the seroprevalence of the disease, usually in the absence of clinical disease, such as in the subclinically infected carrier cattle (Silva *et al*., 2010). However, the hindrance of the serological test of bovine babesiosis is the occurrence of cross – reactions between *B. bovis* and *B. bigemina* (Passos *et al*., 1998) and the failure of discrimination between previous exposure and current infection (Wagner *et al*., 1992).

Advances in the molecular biology were conducted to the development of highly both sensitive and specific test for infectious diseases. With the advent of the polymerase chain reaction (PCR), small quantities of DNA can be greatly amplified resulting in a highly specific test. The efficiency of the PCR has been developed and verified in the use of epidemiological studies for babeisosis (Fahrimal *et al*., 1992; Figureoa *et al*., 1993; Smeenk *et al*., 2000; Almeria *et al*., 2001; Gayo *et al*., 2003; Oliveira – Sequeira *et al*., 2005; Costa – Júnior *et al*., 2006).

6. Control of bovine babesiosis

6.1 Babecidal Drug

The chemical elimination of *Babesia* infections in cattle or horses might play an important role to control the clinical signs for more than several decades. When drugs are therapeutically used in endemic regions, the aim is to help clinical

recovery to prevent losses. To allow a few parasites to survive, this will start reestablishing preimmunization in animal herds. Drugs commonly used in treatment of acute ovine or porcine babesiosis are quinuronium sulfate, imidocarb, and diminazene, which have sufficient effect against severe clinical signs. Infections of *Babesia spp*. in sheep, goat, and swine must be treated with higher doses than those normally recommended in cattle. Repeated administration of drugs was necessary to completely control *Babesia* spp. infections (Melhorne, 2001).

The effect of an antibabesial drug might vary so that drug have to adjust depend on the severity of the disease, the dose, and the duration of treatment. Normally, the large *Babesia spp*. are distinctly more susceptible to chemotherapeutic agents than the small *Babesia* (Melhorne, 2001).

6.2 A development of a vaccine against bovine babesiosis

Live attenuated vaccine strains of *B. bovis* and *B. bigemina* have been used for many years in Australia, Argentina, Brazil, Uruguay, South Africa, and Israel, and it is also in progress in Colombia, Sri Lanka, Zimbabwe and Malawi (Shkap *et al*., 2007). Vaccines consisting of live, attenuated strains of *B. bovis*, *B. bigemina* or *B. divergens* are produced in several countries using the blood of infected donor animals or in-vitro culture. The vaccines are provided in frozen or chilled forms. Frozen vaccine has the advantage of allowing thorough post-production control of each batch, but has a much reduced post-thaw shelf life compared with chilled vaccine. The risk of contamination of this blood-derived vaccine makes thorough quality control essential, but this may be prohibitively expensive (Mangold *et al.,* 1996; Pipano, 1997; Bock *et al.,* 2004).

The recombinant vaccine of bovine babesiosis has been developed by using many candidate proteins. The immunization of native or recombinant rhoptry – associated protein (RAP – 1) from *B. bovis* and *B. bigemina* had an effect on the parasitemia level in infected cattle (Wright *et al*., 1992; Brown *et al*., 2006). However, there is no significant protection level after immunized the infected animal

with the candidate protein encoded from N-terminal region containing immuno-dominant T-cell epitopes of *B. bovis.* However, the specific RAP-1 immunoglobulin G and CD4+ T-cell had been applied to challenge infection (Norimine *et al*., 2003). Because immuno-dominant antigens eliciting strong cell – mediated immune responses failed to confer protective immunity, it has been suggested that identification of subdominant antigens might be important for an effective vaccine (Wright *et al*., 1992; Allred, 2001; Norimine *et al*., 2003; Brown *et al*., 2006). The information of *B. bovis* genomic is useful to identify the immunogenic proteins. Brown *et al* 2006) had proposed that the protective vaccine might be related with the multiple antigens. Moreover, the progress of vaccine against babesiosis required the better understanding of antigenic diversity, mechanisms to evade host immunity, and the heterogeneity of the major histocompatibility complex class II molecules that reacted to the host – parasite immune response (Wright *et al*., 1992; Allred and Al – Khedery, 2004; Brown *et al*., 2006; Carcy *et al*., 2006) The bioinformatics of major and potential gene of parasite will enhance the development of the effective, safe, and accessible vaccine (Gohil *et al*., 2010).

7. Epidemiological study of bovine babesiosis

The worldwide distribution of *B.bovis* and *B.bigemina* have been investigated in many tropical and subtropical countries. The distribution of babesiosis is depended on the available ixodid ticks as the vector (Gohil *et al*., 2010).

Recently, most epidemiological studies of bovine babesiosis were performed using molecular technique to identify strain, species and subspecies of parasite. These epidemiological studies help increasing the update information of disease distribution as shown in table 2.

Table 2 The worldwide reports of prevalence of bovine babesiosis during 2000 – 2012

Year	*Babesia* spp.	host	Place	Diagnosis technique	Prevalence (%)	References
2000	*B.bovis* and *B.bigemina*	Cattle	South America	ELISA	41.3% - 68.9%	Carique *et al*
2000	*B.bovis* and *B.bigemina*	Cattle	Eastern Bolivia	ELISA	75% – 78% and 24% - 57 %	Carrique *et al*
2001	*B.bovis* and *B.bigemina*	Cattle	Minorca (Balearic Island, Spain)	ME and PCR	6% and 0.75%	Almeria *et al*
2004	*B. divergens*	Cattle	Mid – east of France	PCR – RFLP and IFAT	7% - 20%	Devos and Geyson
2005	*B.bovis* and *B.bigemina*	Cattle and engorge female tick	Sâo Paulo State, Brazil	Thin blood smear and PCR	*B.bovis* (85.2%) *B.bigemina* (92.6%)	Oliveria *et al*
2007	*B.bovis* and *B.bigemina*	Cattle	China and Brazil	ELISA and IFAT	Depend on diagnosis method	Kim *et al*
2008	*B.bovis* and *B.bigemina*	Cattle	Mozambique	PCR	90% and 82%	Martins *et al*
2008	*B.bovis* and *B.bigemina*	Cattle	North America	PCR	81.5% and 7.5%	Jonsson *et al*
2009	*B.bovis* and *B.bigemina*	Cattle	Central and Southern regions of Portugal	ELISA and PCR	34% - 79%	Silva *et al*
2010	*B.bovis* and *B.bigemina*	Cattle	Semi – arid area of South Africa	IFAT and CI - ELISA	45% and 46%	Marufu *et al*
2011	*B.bovis* and *B.bigemina*	Cattle	Eastern province of Zambia	PCR	Depend on animal age and location	Simuunza *et al*
2011	*B.bovis* and *B.bigemina*	Cattle	Northern Sudan	PCR	1.9% - 4.0%	Awad *et al*
2011	*B.bovis* and *B.bigemina*	Cattle and tick	Taiwan	PCR	1.9% and 0.6%	Tsai *et al*
2011	*B.bovis* and *B.bigemina*	Cattle	8 province in South Africa	ELISA and IFAT	30.0% - 39.7%	Terkawi *et al* (b)
2011	*B.bovis* and *B.bigemina*	White – tailed deer	Tom Green Country in Central Texas	PCR	12%	Holman *et al*
2012	*B.bovis* and *B.bigemina*	Cattle	Espiritu Santu, Costa rica	ELISA and Realtime PCR	Depend on diagnosis method	Shebish *et al*
2012	*B.bovis, B.bigemina* and *B. divergens*	Cattle	Italy	ME, biomolecular and Serological test	Depend on diagnosis method	Cassini *et a*l
2012	*B.bovis*	Cattle	Mongolia	PCR	9.6%	Altangeral *et al*
2012	*Babesia* spp.	Sheep	China	ELISA	31.66%	Guan *et al*

8. Phylogenetic analysis of *Babesia* spp.

Currently, phylogenetic analysis contributed molecular evidences has guided many biologists to question the conventional taxonomy system and focused on remarkable change to the classification (Morrison, 2009). The extrapolated relationship among parasite speciation has extremely increased by the advance of molecular studies, particularly in genetic sequencing information (Luo *et al*., 2005). Although, the phylogenetic analysis presented the relationship which against the taxonomic system which constructed by other method. These genetic information have been used to integrate the speciation previously classified based on morphological and life history criteria (Barta, 1989; 2001).

In piroplasmids, 18S small subunit ribosomal RNA (18S rRNA) genes were used for the evolution studies and preceded to a more accurate classification of some unknown species (Bai *et al*., 2002; Yin *et al*., 2003). In 2003, the partial and complete genome of 18S rRNA has been classified (Babesiidae and Theileriidae) species into 5 groups. This result was identical with high bootstrap value when compare with the previous studies neither use different method (Kimura - Neighbor Joining, Substitution rate and Parsimony as seen in figure 4, 5 and 6, respectively) (Criado – Fernelio *et al*., 2003). Hence, the using of universal gene such as 18S rRNA can use to identify and recognize the unknown *Babesia* isolates and helpful for the taxonomy or epidemiology studies (Altay *et al*., 2008).

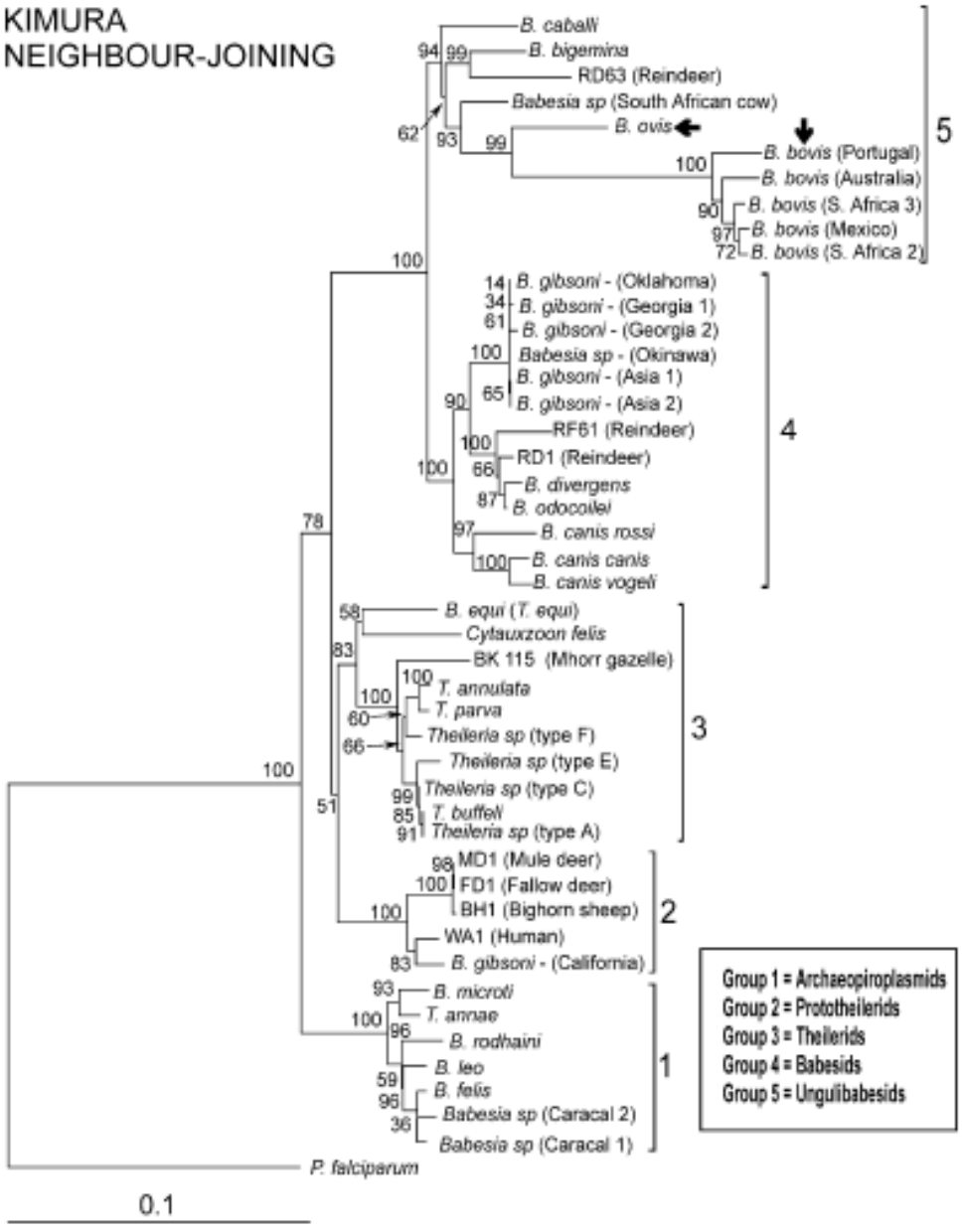


Figure 4 Phylogenetic tree obtained for 45 *Babesia* and *Theileria* species sequenced.

Source: Criado – Fernelio *et al*. (2003)

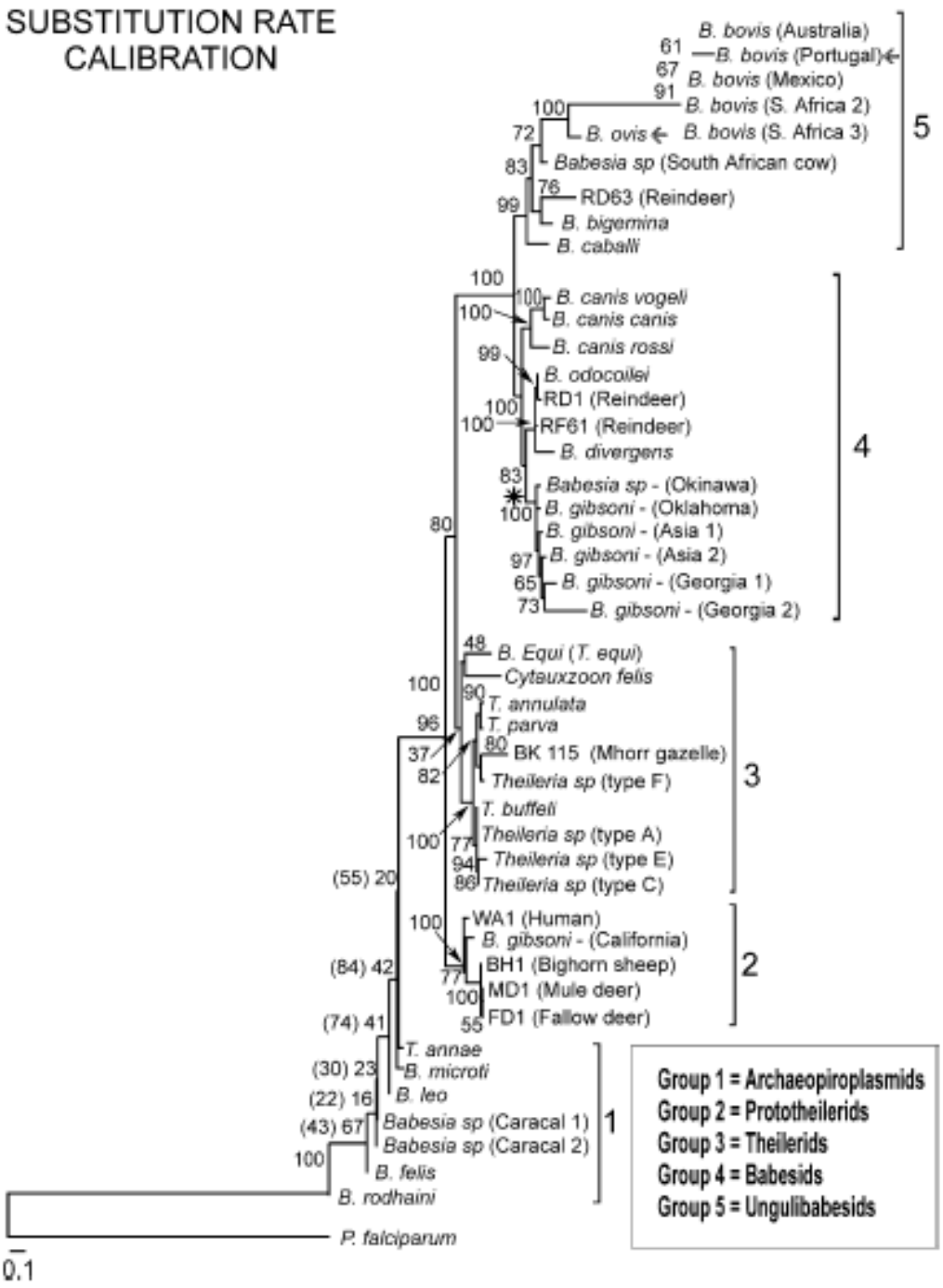


Figure 5 Phylogenetic tree obtained for 45 *Babesia* and *Theileria* species sequenced.

Source: Criado – Fernelio *et al*. (2003)

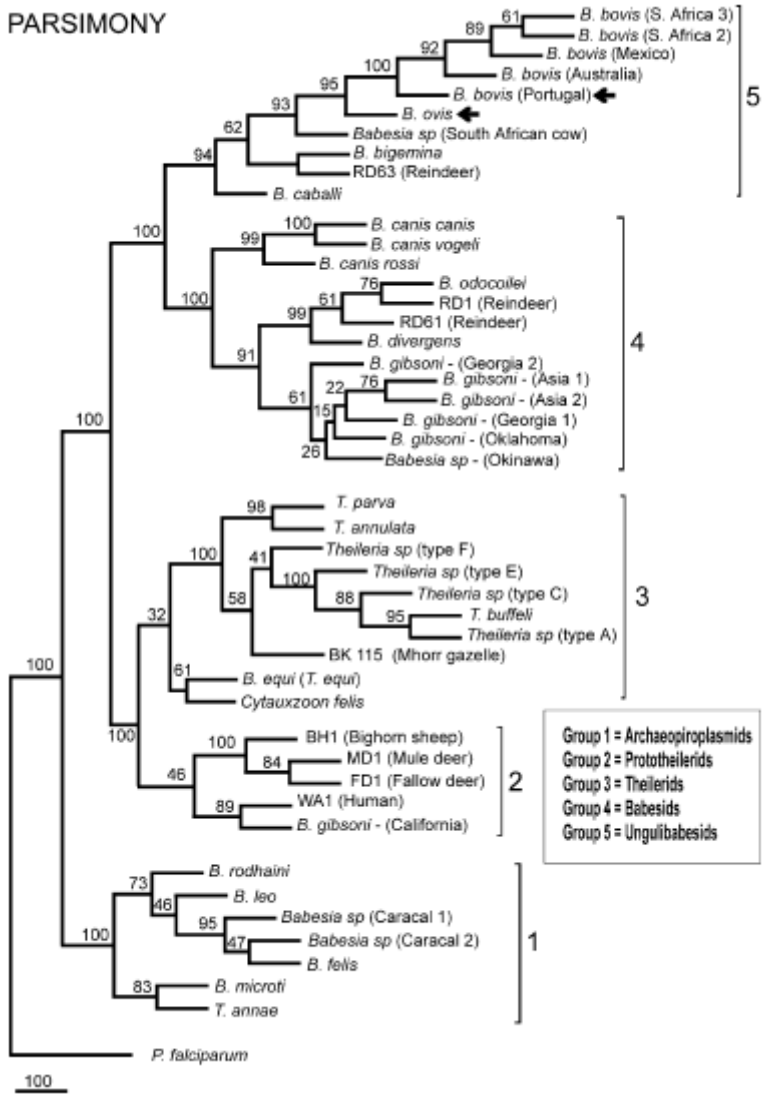


Figure 6 Phylogenetic tree obtained for 45 *Babesia* and *Theileria* species sequenced.

Source: Criado – Fernelio *et al*. (2003)

Recently, beside of the using of 18s rRNA gene, the hsp70 (heat shock protein 70) gene has been used in phylogenetic relationship between species of protozoan parasite because of the highly conserved and important function of hsp 70 (Ruef *et al*., 2000; Yamasaki *et al*., 2002; Fukuda and Endoh, 2008). It was revealed that the hsp70 of protozoan parasite might have important roles in their survival within their hosts (Van der Ploeg *et al*., 1985; Lindquist, 1986; Shapira *et al*., 1988). Hsp 70 enhance the parasites in adapting to the new host environment and have a chaperone function,

forming complexes with a diverse array of cellular proteins and peptides. For example, a temperature increasing from 25 to 37°c induces a significant heat – shock responses in certain *Leishmania* promastigotes *in vitro*, resulting in stage differentiation (Shapira *et al*., 1988). In piroplasm, hsp 70 played an important function and the structure of this protein are varies scarcely among closely related species. The alignment of hsp 70 amino acid demonstrated that hsp 70 are very conservative between each species. Additionally, hsp 70 gene is well conserved among inter – erythrocytic protozoa, and can combine with the phylogenetic classification of *Babesia* and *Theileria spp*. (He *at al*., 2009) as seen in figure 7.

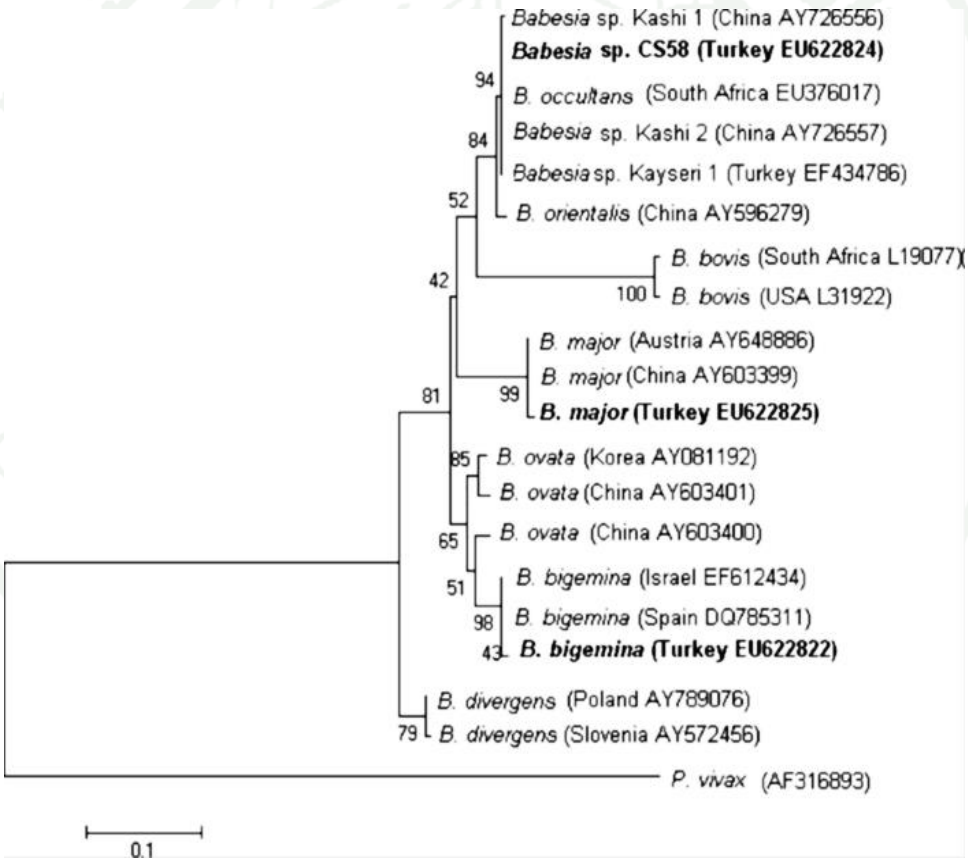


Figure 7 Neighbour-joining analysis of the 18S rRNA gene of the bovine *Theileria* and *Babesia* identified in the study and those present in the GenBank database.

Source: Altey *et al*. (2008)

9. Proteomic analysis of *Babesia* spp.

The study of host cell invasion's mechanism by apicomplexan parasites and the function of apical organelles might be beneficial for the development of the protective vaccine in host by obstructed the cycle of parasite (Baravelle *et al*., 2010). The mechanism of erythrocytic invasion by *Babesia* spp. is resemblance to other apicomplexan parasites such as *Plasmodium* and *Toxoplasma*. Various surface protein and apical secretory organelles mediating the complex multi – steps invasion process (as presented in table 3 and figure 8) were shown as the candidate protein in the vaccine development.

Table 3 *B. bovis* proteins involved in Rbc invasion or modification

Protein	kDa	Predicted/known cellular localization	Predicted/ know function	Comments
Invasion				
Merozoite Surface Antigen 1 (MSA-1)	42	Surface of merozoites and sporozoites	Mediates initial attachment of the merozoite to host RBCs	GPI anchored protein Member of VMSA family. Encode by a single copy gene. No known homologues in Plasmodium
Merozoite Surface Antigen 2 (MSA-2) protein	44	Surface of merozoite and sporozoite	Facilitates invasion of host RBCs	GPI anchored protein. Member of VMSA family. Four isoforms. (MSA -2a1, MSA – 2a2, MSA -2b, MSA – 2c) encoded by tandemly arranged gens. Not all isoforms arepresent in all parasite lines. No know homologues in Plasmodium.
Rhoptry Associated Protein 1 (RAP – 1)	60	Apical region of merozoites and sporozoites and in rhoptries	Attachment and invasion of host RBCs	Encoded by two identical tandemly arranged rap -1 genes. Highly conserved amongst other *Babesia*, Theileria and Plasmodium
Apical Membrane Antigen 1 (BbAMA – 1)	82	Apical region of merozoite and in micronemes	Probably involved in RBCs invasion	Homologue in Plasmodium and Toxoplasma species
Thrombo spodin – related anonymous protein (BbTRAP)	75	Apical region of merozoite and in micronemes	Recognition possible attachment and invasion of host RBCs	Conserved throughout apicomplexan parasites. Contain a VWFA and TSP -1 domain
RBC modification				
Spherical Body Protein 1 (SBP1/Bb-1/Bv80)	80	Cytoplasmic face of infected RBC membrane (membrane skeleton) and spherical body organelles	Protein deployed post – invasion and associated with the RBC membrane	Encoded by a single copy gene. No homologue in *B. bigemina* or *Plasmodium*
Spherical Body Protein 2 (SBP2/BvVa1)	225	Cytoplasmic face of infected RBC membrane (membrane skeleton) and spherical body organelles	Protein deployed post – invasion and associated with the RBC membrane	One full length gene and 12 truncated copies of the 5' end. No homologue in *B. bigemina* and *Plasmodium*.
Spherical Body Protein 3 (SBP3)	136	Cytoplasmic face of infected RBC membrane (membrane skeleton) and spherical body organelles	Protein deployed post – invasion and associated with the RBC membrane	Encoded by single copy gene. No homologue in *B. bigemina* or *Plasmodium*.
Variant Erythrocyte Surface Antigen 1 (VESA 1)	~ 100 – 150 per subunit	Surface of ridge – like structure on B. bovis – infected RBCs	Cytoadhesion and antigenic variation	Encoded by the largest multigene family (ves) in the *B. bovis* genome. Two isoforms, VESA1a and 1b form a heterodimer. Possible functional homologue of P. falciparum PfEMP1

Source: Gohil *et al*. (2010)

At the molecular level, initiated of function of erythrocyte are provoked by proteins that expressed from the parasite's plasma membrane to various location within the infected erythrocytes. Recently, the proteins exposed on Rbc surface have been identified by the host cell interaction in *B. bovis* (Hines *et al*., 1995; Dowling *et al*., 1996; Allred *et al*., 2000; Ruef *et al*., 2000). However, the certain function of these proteins and their cellular targets are still unknown (Gohil *et al*., 2010).

When compared with *Plasmodium falciparum*, the details of *Babesia* proteins remain limited. Only a few proteins associated with parasite modification within the infected erythrocyte have been described and characterized (Gohil *et al*., 2010).

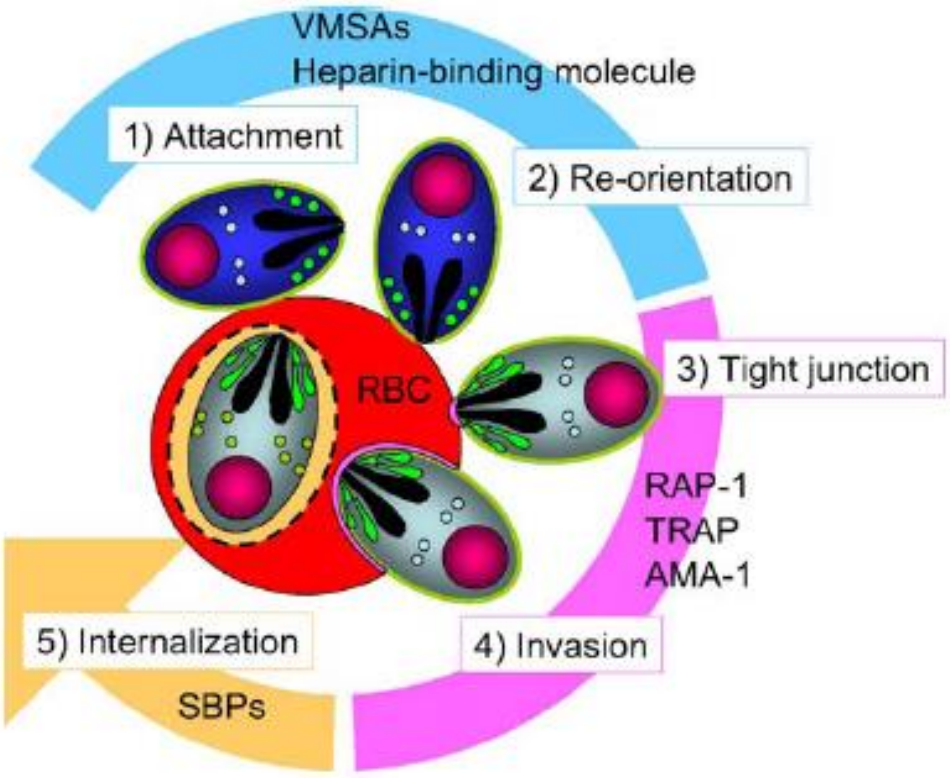


Figure 8 A schematic representation of erythrocyte invasion process of *Babesia bovis* and the gene expression proteins associated in host cells attachment and invasion.

Source: Yokoyama *et al*. (2006)

9.1. Variant Erythrocyte Surface Antigen 1 (VESA1)

Antigenic variation and cytoadhesion of *B. bovis* are mediated through variant erythrocyte surface antigen – 1 (VESA – 1) (Allred *et al*., 1994; O´ Connor *et al*., 1997; Allred *et al*., 2000; O´ Connor and Allred, 2000; Al – Khedery and Allred, 2006). This protein is detected by directed immunofluorescence on live, intact *B. bovis* – infected erythrocyte, consistent with surface expression (O´ Connor *et al*., 1997). The VESA – 1 is highly variable in antigenicity and molecular weight (approximately 100 – 150 kDa per subunit) between different parasites isolates and constructed cluster over the ridge – like protrusions on the infected erythrocyte surface (O´ Connor *et al*., 1999). Similar erythrocyte membrane protein 1 (PfEMP1) of *Plasmodium falciparum*, VESA – 1 is classified in the same multigene family (*ves*) (Allred *et al*., 2000). This group is the largest gene family in the *B. bovis* genome, with approximately 150 genetic copies equally dispensed resemblance between all four chromosomes of the parasite (Brayton *et al*., 2007). Despite of sequence alteration, the length and gene construction, and two distinct types of gene, contain the *ves* family, *ves* 1 α and *ves* 1 β, encoding for the VESA 1α and VESA 1β subunit (Allred *et al*., 2000; Xiao *et al*., 2010). Immunoprecipitation experiments demonstrated two subunits with a large heterodimeric protein on the infected erythrocyte surface (O´ Connor et al., 1997).

For the protein level, VESA – 1 was similar to PfEMP1 and comprised a single, large extracellular domain, followed by a single transmembrane domain and a short cytoplasmic tail (Allred *et al*., 2000; Xiao *et al*., 2010). The resemblance between of these protein groups was such as the construction of a cysteine – and lysine – rich (CKRD) domain in the N – terminal region of VESA – 1a and VESA – 1b and the construction of cysteine – rich interdomain region in PfEMP1 (Allred *et al*., 2000).

9.2. Small Open Reading Frames (SmORFs)

The small open reading frames (SmORFs) family protein is the second massive family in the *B. bovis* genome and were discovered by the analysis of the first completely sequenced and annotated genome of *B. bovis* (Brayton *et al*., 2007). The information of the gene family diagrams is still limited excluding 44 identified SmORFs. All varying sequence lengths were no similar to other genes or proteins in other available species database. All of 44 (except 1 protein) proteins contain a signal peptide that might be important for parasite activities (Brayton *et al*., 2007). Analysis of the genome indicated that the SmORFs genes are closely to the VESA gene existed in chromosomes, suggesting potential involvement in VESA – 1 biology (Brayton *et al*., 2007).

9.3. Spherical Body Protein (SBPs)

The spherical bodies are an organelle analogous to the dense granules of other apicomplaxan parasites. One to four spherical bodies are found in a single *B. bovis* merozoit located subjacent to the rhoptries and micronemes (figure 9) (Hines *et al*., 1995; Dowling *et al*., 1996). Over a decade, three proteins which localized to these organelles have been recognized and known as spherical body protein 1, 2 and 3 (SBP1, SBP2 and SBP3). Immunoelectron microscopy studied have demonstrated that these proteins are come from the spherical bodies following invasion and afterward interacted at the cytoplasmic face of the infected erythrocyte (Jasmer *et al*., 1992; Hines *et al*., 1995; Dowling *et al*., 1996; Ruef *et al*., 2000). The genome sequence of SBP4 has also been submitted to GenBank. As same as to the other SBPs, these sequence were conserved among geographically diverse locations (Hines *et al*., 1995; Ruef *et al*., 2000; De Vries *et al*., 2006; Aboulaila *et al*., 2010).

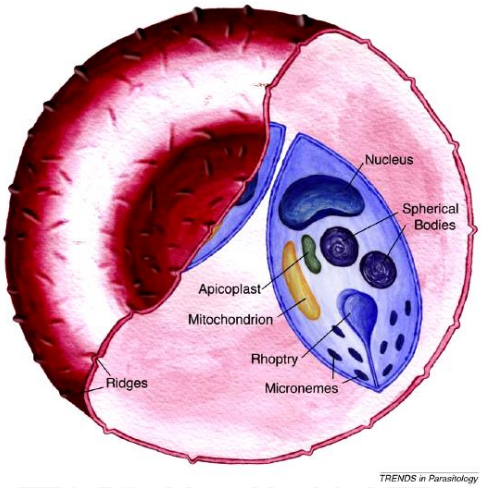


Figure 9 A mechnism of a bovine red blood cell invaded by a mature from of *Babesia bovis*.

Source: Gohil *et al*. (2010)

At the amino acid level, SBP1, SBP2 and SBP3 do not share any conserved domain or homologous sequences. With the exception of the extreme N – terminal ends of SBP1 and SBP2, this could imitate the sorting signal for their pathway to the spherical bodies or export form the parasite into the erythrocyte (Hines *et al*., 1995; Ruef *et al*., 2000). Moreover, none of the SBPs show any significant homology to other know proteins and not found in *B. bigemina*, suggesting an important function related with parasite virulence (Hines *et al*., 1995; Ruef *et al*., 2000).

10. Bovine Babesiosis in Thailand.

The distribution of bovine babesiosis in Thailand has long been studies. Livestock development in Thailand, particularly for dairy cows, has been obstructed by persistently low production of milk and meat due to major blood parasites, including *B. bovis* and *B. bigemina* (Tan – Ariya *et al*., 1992) and the prevalence in some areas as high as 15% since 1988 (Jittapalapong and Leowijak, 1988). In 1990, 428 cattle sera form 12 provinces in the country were tested by serological assay (IFAT) to identify antibody of bovine babesiosis and the seroprevalence of *Babesia*

infection was 74% (Nishikawa *et al*., 1990). The distribution of the bovine *Babesia* spp. in Thailand have been summarized in table 4.

Table 4. Bovine babesiosis in cattle and buffaloes in Thailand.

Year	Organism	Host	Study information	Diagnosis Techniques	Reference
1990	*B.bigemina* and *B. bovis*	Cattle and Buffaloes	Epidemiological study in16 province in the country (74%)	IFAT	Nishikawa *et al*.
1991	*B.bovis*	In vitro	Drug responsiveness study		Tan – ariya and Sarathaphan
1991	*B. bigemina*	Beef cattle	Epidemiological study in Samutprakarn Province (23%)	ME	Pemayothin *et al*
1992	*B. bovis*	In vitro	Drug responsiveness study		Tan – ariya *et al*
1992	Bovine *Babesia spp*	In vitro	In vitro technique development		Tan – ariya
1995	*B.bigemina* and *B. bovis*	Cattle	Isolation and preparation of *Babesia spp* antigen		Sarataphan *et al*
2000	*B.bigemina* and *B. bovis*	Cattle	Epidemiological study in some province (29.5%)	IFAT	Phrikanahok *et al*
2003	*B.bovis*	Cattle	Develop the diagnosis technique	PCR and ELISA	Thammasirirak *et al*
2004	*B.bigemina*	Cattle	Anti – tick vaccine study		Jittapalapong *et al*
2006	*Theileria spp*.	Cattle	Epidemiological study in endemic area in the country (50%)	ME	Kaewthamasorn and Wongsamee .
2008	*B.bigemina* and *B. bovis*	Cattle	Epidemiological study in Lopburi province (1%)	PCR	Sriwarothai *et al.*
2010	*B.bigemina* and *B. bovis*	Dairy cows	Epidemiological study in Chiang Rai, Chiang Mai, Lumpan, and Mae Hong Sorn province (52.9% - 63.8%)	IFAT, ELISA and PCR	Iseki *et al.*
2011	*B.bigemina* and *B. bovis*	Buffaloes	Epidemiological study in Ubon Ratchatani, Roi Ed, Burirum, Surin and Srisaket province (11.2% - 16.8%)	IFAT, ELISA and PCR	Terkawi *et al* (a)
2012	*B.bigemina* and *B. bovis*	Cattle	Epidemiological study in Northern Thailand (3.5% - 39.2%)	PCR	Cao *et al*

Additionally, the prevalence using IFAT and ELISA assay to identification of *B. bovis* and *B. bigemina* infection in dairy cows from northern provinces were 73.8.6% and 69.1%, respectively. These results demonstrated that *Babesia* infections are normally distributed in the northern part of Thailand (Iseki *et al*., 2010). The overall prevalence of *B. bovis* and *B. bigemina* infections by nPCR and ELISA in water buffaloes was 17.5% and 6.9%, respectively. Nevertheless, the widespread of *Rhipicephalus microplus*, a tick vector of *B. bovis* and *B. bigemina* (Bock et *al*., 2004) in Thailand, might be the reason for the high rate of bovine babesiosis in cattle and buffaloes, since 45.6% of the ticks collected from cattle were positive to these

parasites in an enzootic area of Thailand (Jittapalapong *et al*., 2004; Terkawi *et al*,. 2011a).

MATERIALS AND METHODS

1. Blood sample collection

1.1 Dairy cattle

Blood samples from 1,824 dairy cattle in 12 provinces represent the North (641), Northeast (321), Central (637), and South (185) of Thailand were randomly collected from July to September 2010. The animals were reared in small-scale dairy farms under semi-intensive management systems. The animals were categorized under 3 age groups: <1 year, 1–5 years, and >5 years; the sample numbers in each age group were 72, 1091, and 661, respectively. All the animals were apparently healthy when they were sampled. From each animal, approximately 10 ml of blood was drawn from the jugular vein into a vacutainer tube that contained sodium citrate tubes and then stored at -20°C until DNA extraction.

1.2 Beef

A total of 244 cattle blood samples were collected from Salakpra Wildlife Sanctuary in Kanchanaburi including Mueang (94), Si Sawat (25), and Bo Phloi (125). The sample size was calculated from Winepiscope program using randomization model (Multistage sampling) which based on total population at 12,000 individual, the expected prevalence of 30%, absolute precision of ±5% and 95% confidence interval. Ten ml of blood was drawn from jugular vein, transferred to sodium citrate tubes and stored at –20ºc until the laboratory analysis.

Cattle were thoroughly examined for health profiles. The information regarding age, sex, health condition, and location are recorded. In addition, the questionnaire was designed to record these data including herd sized (small: 1 – 40 cattle/farm; medium: 40–80 cattle/farm; large: >80 cattle/farm), seasonal management (Summer/Raining: roaming/in house), insect abundant in barn (low density: 0–

10/cattle; moderate density:11-20/cattle; high density:>20/cattle), Forage density pattern (low density:1 – 10 trees/5 m^2; moderate density:10 – 20 trees/ 5 m^2; high density: over 20 trees/ 5 m^2) deworming, domestic pet in household(found or not found), the tick infestation on the animal, the tick existence in barn, and the using of grazing areas (selected or public areas). All factors were analyzed to identify the significant effect on the distribution pattern of *Babesia* infection among cattle.

1.3 Buffaloes

A total of 617 buffaloes blood samples were included in the study from 6 province in Northeast region as Ubon Ratchathani (138), Roi Ed (81), Surin (73), Buriram (70), Sakon Nakhon (196) and Sri Saked (59), 527 females and 90 males. The individual information include age groups (i) less than 1 year 4 animals (ii) between 1-5 years 362 animals and (iii) more than over 5 years 251 animals, from different areas of Northeast Thailand, were recorded. Ten ml of blood were collected from jugular vein, transferred to sodium citrate tube and stored in at –20ºc until the laboratory analysis.

Table 5 Areas, provinces, type of animals and the number of animals were investigated in this study.

Location	Province	Dairy Cattle	Beef	Buffaloes
North				
	Chiang Rai	392	-	-
	Chiang Mai	150	-	-
	Lumpang	99	-	-
Northeast				
	Udorn Thani	85	-	-
	Khon Khen	185	-	-
	Sakon Nakhon	91	-	196
	Ubon Ratchatani	-	-	138
	Roi Ed	-	-	81
	Surin	-	-	73
	Burirum	-	-	70

Table 5 (Continued)

Location	Province	Dairy Cattle	Beef	Buffaloes
	Sri Saked	-	-	59
Central				
	Ratchaburi	109	-	-
	Kanjanaburi	61	244	-
	Saraburi	268	-	-
	Lopburi	110	-	-
	Nakhon Pathom	89	-	-
South				
	Phathalung	185	-	-
Total	**17 provinces**	**1,824**	**244**	**617**

All samples (dairy, beef and water buffaloes; 2,685 samples) were kept on ice during transportation to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, and stored at -20ºC until use. The total samples in each endemic area were concluded in table 5 and figure 10.

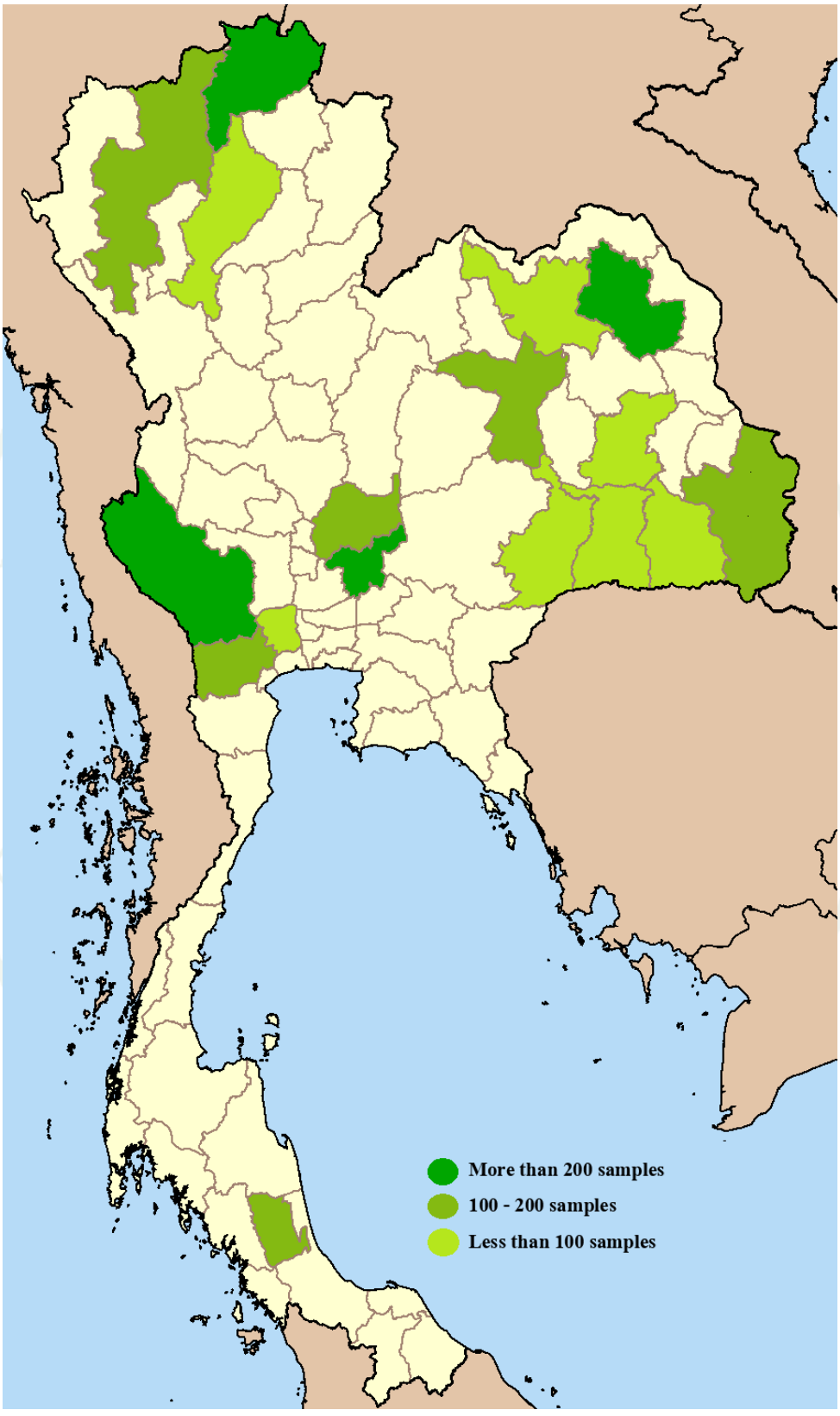


Figure 10 The studied areas and samples for blood collection

2. DNA extraction

Blood samples (100 µl each) were incubated with 500 µl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol, and 0.5% N-lauroylsarcosine). DNA samples were extracted by the phenol-chloroform method, precipitated using ethanol (Sambrook and Russell, 2001), dissolved in a TE buffer (50 mM Tris, pH 8.0, 1 mM EDTA), and then stored at -20°C until use.

3. PCR detection of *B. bovis* and *B.bigemina*

All samples were screened for *B. bovis* using a nested PCR assay based on the spherical body 2 (SBP-2) gene described by Aboulaila *et al* (2010), with modifications. Briefly, 20 µl reaction mixture were prepared to include 1× buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM MgCl2, 1.0 pmol of forward (5'-CCGAATTCCTGGAAGTGGATCTCATGCAACC-3') and reverse (5'-ATCTCGAGTCACGAGCACTCTACGGCTTTGCAG-3') primers, 0.2 mM of each dNTP, 0.75 units of Taq DNA polymerase (DyNAzyme, FINNZYMES), and 1 µg of DNA template. The PCR cycling conditions were adopted as previously described (Aboulaila *et al*., 2010) to amplify a 1236 bp fragment of SBP-2. After the first round of PCR assay, 0.5 µl of each PCR product was transferred into new PCR tubes that contained PCR mixtures similar to that of the first PCR, except that the outer primers were replaced with inner forward (5'-CGAATCTAGGCATATAAGG CAT-3') and reverse (5'-ATCCCCTCCTAAGGTTGGCT AC-3') primers, and then subjected to the PCR condition. The PCR products were visualized under UV light after gel electrophoresis and GelStar® Nucleic Acid Gel Stain. Detection of a 580 bp band was considered as *B. bovis* positive.

For a detection of *B. bigemina*, PCR assay was modified from Terkawi *et al* (2011a). The 20 µl reactions contained 1× buffer (10mM Tris–HCl pH 8.8, 50mM KCl and0.1% Triton X-100), 1.5mM MgCl2, 2 pmol of each primer, 0.2mM of each dNTP, 1.0 Units of Taq DNA polymerase (DyNAzyme, FINNZYMES) and 1 µg of

DNA template. The primer BbigRAP – 1α F1 (5’ GAGTCTGCCAAATCCTTAC 3’) and BbigRAP – 1α R1 (5’ TCCTCTACAGCTGCTTCG 3’) are used to amplified approximately 879 bp of the *B. bigemina* rap 1α gene. The PCR condition was used: pre - denaturation at 94ºC for 5 min, then 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min and followed by final extension at 72°C for 10 min. The nested primers were BbigRAP – 1α F2 (5’–AGCT TGCTTTCACAACTGGCC–3’) and BbigRAP – 1α R2 (5’–TTGGTGCTTTGACCG ACGACAT–3’) and the nested PCR protocol was repeated using the same protocol. The nested PCR product was sized at 419 bp.

4. Statistical analysis

Chi-square and Number Cruncher Statistical System (NCSS) ver. 2000 (Kaysville, UT, USA) programs were used to assess the differences in the prevalence. Analysis was also undertaken to investigate environmental variables associated with the infection patterns, as determined by the probability that individual cattle were infected. P values under 0.05 was considered significant.

5. *B.bovis* and *B. bigemina* alignment and sequence editing

The homologous sequence (99% - 90% similarity) of related SBP2 gene and *rap1* – α gene (table 6), including recently sequences (table 7) were aligned by using ClustalOmega (McWilliam *et al*., 2013) and manually editing the align sequence by Genedoc version 2.7.

Table 6 The *B.bigemina rap1* – α and *B.bovis* SBP2 sequence used in phylogenetic analysis and their accession numbers

Organism	Accession No	Gene	Source	host	Node code
B.bigemina	AB617643	*rap1 – α*	Syrain merozoite DNA	cattle	Syrian C1:AB617643
	M60878	*p58*		clone	p58Bg 1: M60878

Table 6 (Continued)

Organism	Accession No	Gene	Source	host	Node code
	AB617644	*rap1 – α*	Syrain	cattle	Syrian C2:AB617644
	M85187	*p58*	merozoite DNA	clone	p58Bg 2: M85187
	M85187	*p58*	merozoite DNA	clone	p58Bg 3: M85186
	AB586126	*rap1 – α*	Thai	buffalo	Thai Buf1:AB586126
	AB594816	*rap1 – α*	Thai	buffalo	Thai Buf3:AB594816
	AB594817	*rap1 – α*	Thai	buffalo	Thai Buf2:AB594817
	KF192811	*rap1 – α*	Egypt	cattle	EgyptC1:KF192811
outgroup	**KF059876**	***B.caballi rap1***	**Israel**	**horse**	***Bcaballirap1*:KF059876**
B.bovis	AB742545	*SBP2*	Veitnam	cattle	VeitnamC1:AB742545
	AB742547	*SBP2*	Veitnam	cattle	VeitnamC2:AB742547
	AB772318	*SBP2*	Thai	cattle	ThaiC1:AB772318
	JX648555	*SBP2*	Philippines	cattle	PhilippinesC1:JX648555
	JN974305	*SBP2*	Thai	cattle	ThaiC2:JN974305
	AB742546	*SBP2*	Veitnam	cattle	VeitnamC4:AB742546
	AB742547	*SBP2*	Veitnam	cattle	VeitnamC3:AB742547
	AB772320	*SBP2*	Brazil	cattle	BrazilC1:AB772320
	AB772321	*SBP2*	Brazil	cattle	BrazilC2:AB772321
	AB742548	*SBP2*	Veitnam	buffalo	VeitnamB1:AB742548
outgroup	**FJ537057**	***B.bovis VESA1***	**Mexico**	**clone**	***Bbovis*VESA1:FJ537057**

Table 7. The information of *B.bigemina rap – 1* α gene and *B.bovis* SBP2 gene Thailand sequence

Gene	No.	Host	Source	Code	Node label
B.bigemina rap – 1 α gene	1	Water buffalo	Ubon Ratchathani	UB.01	*B.bigemina*:Buffalo:Ubon Ratchathani (UB.01)
	2	Water buffalo	Sakon Nakhon	SN.142	*B.bigemina*:Buffalo: Sakon Nakhon (SN.142)
	3	Beef	Kachanaburi: Bo Phloi	AA.117	*B.bigemina*:Beef: Kanchanaburi BP (AA.117)
	4	Beef	Kachanaburi: Sri Sawat	I.037	*B.bigemina*:Beef: Kanchanaburi SW (I.037)
	5	Beef	Kachanaburi: Mueang	FF.162	*B.bigemina*:Beef: Kanchanaburi M (FF.162)
	6	Dairy Cattle	Lumpang	LP.86	*B.bigemina*:Dairy Cattle: Lumpang (LP.86)

Table 7 (Continued)

Gene	No.	Host	Source	Code	Node label
B.bigemina rap – 1 α gene	7	Dairy Cattle	Saraburi	CC.214	*B.bigemina*:Dairy Cattle: Saraburi (CC.214)
B.bovis SBP2 gene	1	Water buffalo	Ubon Ratchathani	UB.107	*B.bovis*: Buffalo: Ubon Ratchatani (UB.101)
	2	Water buffalo	Sakon Nakhon	SS.23	*B.bovis*: Buffalo: Sakon Nakhon (SN.23)
	3	Beef	Kachanaburi: Bo Phloi	UU.111	*B.bovis*: Beef: Kanchanaburi BP (UU.111)
	4	Beef	Kachanaburi: Sri Sawat	N.50	*B.bovis*: Beef: Kanchanaburi SW (N.50)
	5	Dairy Cattle	Saraburi	CC.212	*B.bovis*: Dairy Cattle: Saraburi (CC.212)
	6	Dairy Cattle	Chiang Rai	CR.37	*B.bovis*: Dairy Cattle: Chiang Rai (CR.37)

6. The construction of phylogenetic tree

A phylogenetic tree was constructed as described by Altangarel *et al*. (2012) using the gene sequences determined in the present study and those available from other countries. Briefly, after the initial analyses using GENETYX 7.0 software (GENETYX, Tokyo, Japan), construction of a phylogenetic tree and estimation of bootstrap values were performed using a MAFFT program available online (Katoh *et al*., 2002).

7. Amplification, sequencing, and phylogenetic analyses of the MSA-2b gene

Full-length MSA-2b genes were amplified from 23 *B. bovis*-positive DNA samples representing all the surveyed regions. PCR assay was performed using 30 µl of PCR mixture that contained 1 µg of DNA template, 1× buffer (Applied Biosystems, New Jersey, USA), 0.2 mM of dNTPs (Applied Biosystems), 1 pmol each of forward (5'-ATGATCGGGAAAATCTTCTTGTTAA-3') and reverse (5'-TTAAAATGCAGAGAGAACGAAGTAGC-3') primers, and 1.0 unit of Taq polymerase. After an initial enzyme activation step at 95°C for 5 min, the PCR mixtures were subjected to 45 cycles, each of which included a denaturing step at

95°C for 1 min, an annealing step at 52°C for 1 min, and an extension step at 72°C for 2 min. After the final elongation at 72°C for 10 min, PCR products were visualized. Subsequently, appropriately sized PCR amplicons were gel-extracted, ligated to plasmid vectors, and then sequenced as described previously (Sivakumar *et al*., 2012). The MSA-2b sequences generated in the present study were registered in GenBank to obtain the accession numbers (AB745695–AB745717).

8. B-cell epitope prediction

Nucleotide sequences of MSA-2b genes were converted to amino acid sequences, and the percentage of similarity between Thai sequences was calculated using EMBOSS Needle Pairwise Sequence Alignment software (http://www.ebi .ac.uk/Tools/psa/emboss_needle/). In addition, the B-cell epitopes in MSA-2b sequences were predicted by the method of Kolaskar and Tongaonkar (1990) (http://tools.immuneepitope.org/tools/bcell/iedb_input).

9. The Satellite mapping

The mapping of parasitic distribution was created by the GIS program, ArcGIS ver. 9 (USA) to find the location of the infected monasteries. The satellite image were constructed for beef cattle to display the location and cluster of beef habitats in Salakpra Wildlife Sanctuary that related to the forest boundary araeas.

RESULTS

1. The prevalence and factors associated with *Babesia* spp. infection in dairy cattle

Using SPB2 gene specific to *B. bovis*, 162 positive samples from 1,824 samples (8.8%) were shown. The highest prevalence were found in Lumpang province (28.5%, 28/99). However, the highest endemic areas was settled on the central region of Thailand where demonstrated 96 positive sample from 637 local field sample (15%) or 59.2% from all positive sample (96/162). Additionally, the statistical calculation by using NCSS software show the significant value of the region factor ($p<0.01$, $\chi 2=64.2$, df=3) and odd ration reveal that the central region was the risk area of infection more than the other region of Thailand (Odds Ratio = 4.7, 95%= 3.4 – 6.5). However, there is no significant evaluation between age group since the most infection rate was located between 1 – 5 years (105/1090; 9.6%) as data was shown in Table 8.

The result of *B.bigemina* infection showed 186 positive samples (10.2%). The highest prevalence was found in Lumpang province as 34 positive samples from 99 samples (34.3%). However, the most infected location was the central region where the highest prevalence was 89 positive from 637 samples (14%) or 47.8% from all positive samples (89/186) with significant effect ($p<0.01$, Odds Ratio = 1.82, 95%CI = 1.345 – 2.477). The most infected age was found in younger animal (age <1 year) (12/72; 16.7%) but no significant correlation as showed in Table 9.

Table 8 PCR results of *B. bovis* infection of dairy cows associated with region and age group in Thailand

Region	Province	Total no. of samples	Positive samples (%)	Positive among age groups			Odds Ratio	95% CI
				<1 yr	1 – 5 yrs	5>yrs		
North		**641**	**56 (8.7)**	**1/18 (5.6%)**	**31/313 (8.1%)**	**24/240 (10%)**	**0.9**	**0.9 – 1.7**
	Chiang Rai	392	27 (6.8)	0	7/212 (3.3%)	20/180 (11.1%)		
	Chiang Mai	150	1 (0.6)	0	1/96 (1.0%)	0		
	Lumpang	99	28 (28.5)	1/7 (14.3%)	23/75 (30.7%)	4/17 (23.5%)		
Northeast		**361**	**9 (2.4)**	**0**	**3/185 (1.6%)**	**6/148 (4.1%)**	**0.2**	**0.1 – 0.4**
	Udorn Thani	85	2 (2.3)	0	0	2/28 (7.1%)		
	Khon Khen	185	1 (0.5)	0	0	1/91 (1.1%)		
	Sakon Nakhon	91	6 (6.5)	0	3/55 (5.5%)	3/29 (10.3%)		
Central		**637**	**96 (15.0)**	**2/18 (11.1%)**	**71/408 (17.4%)**	**23/211 (10.9%)**	**4.7**	**3.4 – 6.5**
	Ratchaburi	109	1 (0.9)	0	1/50 (2%)	0		
	Kanjanaburi	61	1 (1.6)	0	1/48 (2.1%)	0		
	Saraburi	268	65 (24.2)	2/18 (11.1%)	54/179 (30.2%)	9/71 (12.7%)		
	Lopburi	110	27 (24.5)	0	14/68 (20.6%)	13/42 (31.0%)		
	Nakhon Pathom	89	2 (2.2)	0	1/63 (1.6%)	1/26 (3.8%)		
South		**185**	**1 (0.5)**	**0**	**0**	**1/63 (1.6%)**	**0.04**	**0.0 – 0.3**
	Phathalung	185	1 (0.5)	0	0	1/63 (1.6%)		
Total (%)		**1824**	**162 (8.8)**	**3/72 (4.2)**	**105/1090 (9.6)**	**54/662 (8.2)**		

Table 9 PCR results of *B. bigemina* infection of dairy cows associated with region and age group in Thailand

Region	Province	Total no. of samples	Positive samples (%)	Positive among age groups			Odds Ratio	95% CI
				<1 yr	1 – 5 yrs	5>yrs		
North		**641**	**78 (12.2)**	**3/18 (16.7%)**	**44/383 (11.5%)**	**31/240 (12.9%)**	**1.38**	**1.01 – 1.80**
	Chiang Rai	392	43 (11.0)	0	20/212 (9.4%)	23/180 (53.5%)		
	Chiang Mai	150	1 (0.7)	1/11 (9.1%)	0	0		
	Lumpang	99	34 (34.3)	2/7 (28.6%)	24/75 (32%)	8/17 (47.1%)		
Northeast		**361**	**18 (5.0)**	**1/28 (3.6%)**	**10/185 (5.4%)**	**7/184 (4.7%)**	**0.41**	**0.25 – 0.67**
	Udorn Thani	85	4 (4.7)	0	1/42 (2.4%)	3/28 (10.7%)		
	Khon Khen	185	1 (0.5)	0	1/88 (1.1%)	0		
	Sakon Nakhon	91	3 (14.3)	1/7 (14.3%)	8/55 (14.5%)	4/29 (13.8%)		
Central		**637**	**89 (14)**	**8/18 (44.4%)**	**46/408 (11.3%)**	**35/211 (16.6%)**	**1.82**	**1.34 – 2.48**
	Ratchaburi	109	17 (5.6)	0	1/50 (2.0%)	16/59 (27.1%)		
	Kanjanaburi	61	5 (8.2)	0	2/48 (4.2%)	3/13 (23.1%)		
	Saraburi	268	52 (19.4)	8/18 (44.4%)	31/179 (17.3%)	13/71 (18.3%)		
	Lopburi	110	11 (10)	0	9/68 (13.2%)	2/42 (4.8%)		
	Nakhon Pathom	89	4 (4.5)	0	3/63 (4.8%)	1/26 (3.8%)		
South		**185**	**1 (0.5)**	**0**	**0**	**1/63 (1.6%)**	**0.04**	**0.01 – 0.31**
	Phathalung	185	1 (0.5)	0	0	1/63 (1.6%)		
Total (%)		**1824**	**186 (10.2)**	**12 (16.7)**	**100 (9.2)**	**74 (11.2)**		

The result showed the prevalence of *B.bovis* and *B.bigemina* in all endemic areas at 8.8% and 10.2%, respectively.

2 The prevalence of *Babesia* spp. infection in beef cattle

2.1 The prevalence of *Babesia* spp. infection of beef cattle in Salakpra Wildlife Sanctuary areas

A total of 244 *Babesia*-positive cattle among 36 farms was distributed in the Salakpra Wildlife Sanctuary areas as shown in Fig.11. The highest infection was found at Mueang district (47.9%, 45/94) while Sri Sawat had the lowest prevalence (44%, 11/25). The overall *Babesia* infection of cattle in Salakpra Wildlife Sanctuary was 46.7% (114/244) for individual prevalence and 88.9% (32/36) for herd prevalence. The prevalence of *B. bovis* infection is 5.3% (13/244) and was mostly found in Sri Sawat district (16%, 4/25). The prevalence of *B. bigemina* infection was 38.9% (95/244) and was frequently distributed in Bo Phloi district (45.6%, 57/125). In addition, the mixed infection between *B. bigemina* and *B. bovis* was also found at 2.5% (6/244).

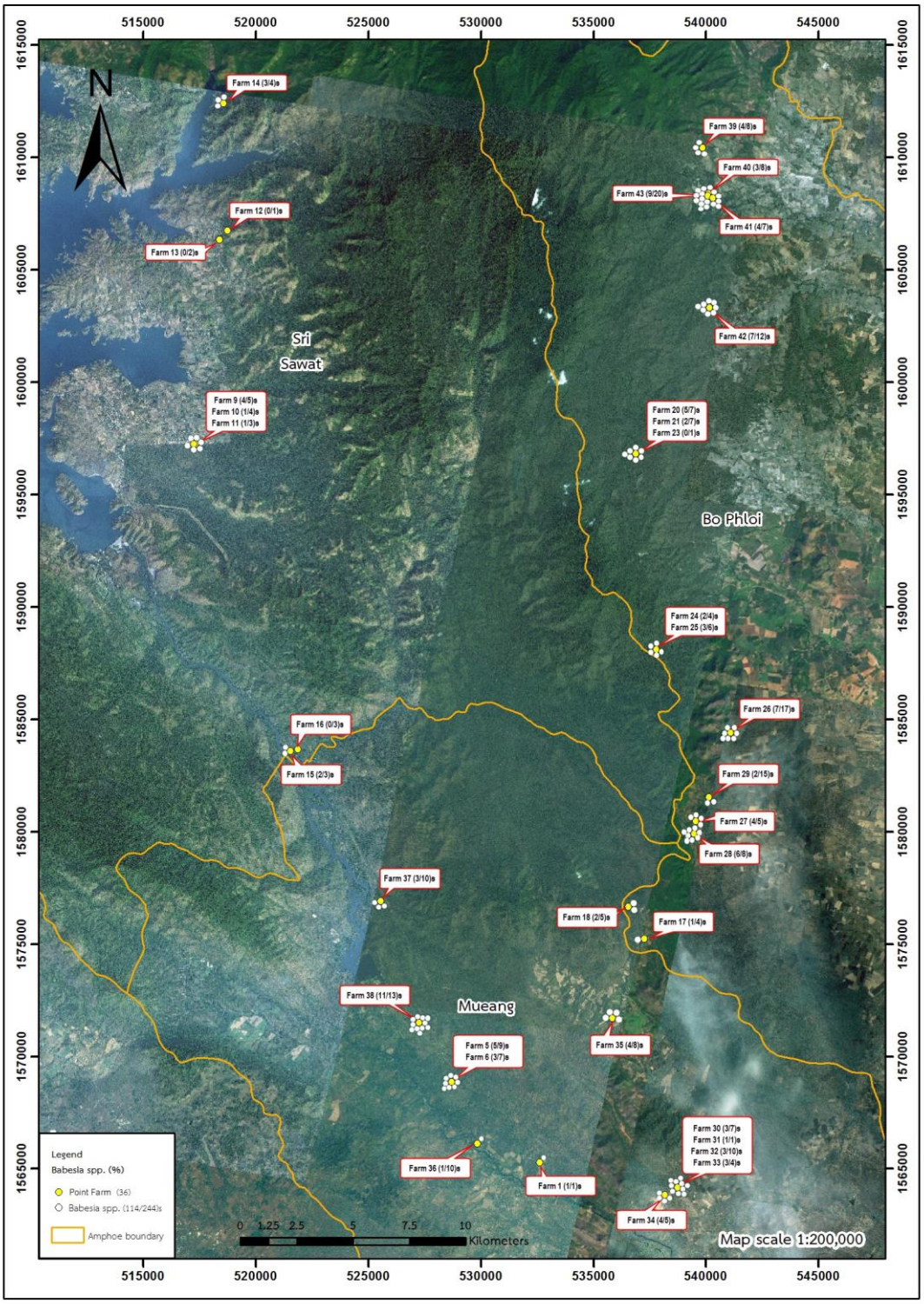


Figure 11 The satellite map demonstrated the distribution of *Babesia* infection of beef cattle in the Salakpra Wildlife Sanctuary areas

2.2 Factors associated with *Babesia* spp. infection in beef cattle

The univariation analysis of the associated factors such as location, age, sex, herd size, seasonal management, insect density, forage density, deworming, pet in farm, tick infestation of cattle, the tick existence in house, and the using of grazing areas were shown in table 10. Some potential factors associated with *Babesia* infections including herd sizes, age, and forage density division were statistical significance ($p<0.05$). However, the multivariable analysis using logistic regression models significantly showed the affect on the age of animal (between 1 – 5 years) and the medium herd size (40 – 80 cattle/farm) as shown in table 11.

Table 10. Univariate analysis for the risk factors associated with *Babesia* spp. infection

Parameters	No.	Positive No. (%)	X^2 values	df	*p-value*	*B.bovis*	*B.bigemina*	Mixed infection
Areas			0.13	2	0.93			
1. Mueang	94	45/94 (47.9%)				8 (8.5%)	33 (35.1%)	4 (4.2%)
2. Sri Sawat	25	11/25 (44%)				4 (16%)	5 (20%)	2 (8.0%)
3. Bo Phloi	125	58/125 (46.4%)				1 (0.8%)	57 (45.6%)	0
Herd size			16.37	2	0.0002*			
1. Small (1 – 40 cattle)	60	25/60 (41.7%)				6 (10%)	18 (30%)	1 (1.7%)
2. Medium (40 – 80 cattle)	105	64/105 (61.0%)				5 (4.8%)	54 (51.4%)	5 (4.8%)
3. Large (over 80 cattle)	79	25/79 (31.6%)				2 (2.5%)	23 (29.1%)	0
Age group			6.01	2	0.049*			
1. 0 – 1 year	37	23/37 (62.2%)				5 (13.5%)	14 (37.8%)	4 (10.8%)
2. 1 – 5 years	129	52/129 (40.3%)				7 (5.4%)	43 (33.3%)	2 (1.6%)
3. > 5 years	78	39/78 (50.0%)				1 (1.3%)	38 (48.7%)	0
Sex			1.63	1	0.20			
1. Male	40	15/40 (37.5%)				1 (2.5%)	14 (35.0%)	0
2. Female	204	99/204 (48.5%)				12 (5.9%)	81 (39.7%)	6 (2.9%)
Dry season management			1.21	1	0.27			
1. Free roaming	231	106/231 (45.9%)				13 (5.6%)	87 (37.7%)	6 (2.6%)
2. in Barn	13	8/13 (61.5%)				0	8 (61.5%)	0
Insect Density			2.70	2	0.25			
1. Low (0 – 10/cattle)	99	40/99 (40.4%)				6 (6.1%)	34 (34.3%)	0
2. Moderate (11 – 20/cattle)	50	25/50 (50%)				2 (4%)	22 (44%)	1 (2%)
3. High(>20/cattle)	95	49/95 (51.6%)				5 (5.3%)	39 (41.1%)	5 (5.3%)
Forage Density			7.94	2	0.018*			
1. Low	84	38/84 (45.2%)				8 (9.5%)	27 (32.1%)	3 (3.6%)
2. Moderate	147	65/147 (44.2%)				3 (2.0%)	60 (40.8%)	2 (1.4%)

Table 10 (Continued)

Variables	No.	Positive No. (%)	X^2 values	df	*p-value*	*B.bovis*	*B.bigemina*	Mixed infection
3. High	13	11/13 (84.6%)				2 (15.4%)	8 (61.5%)	1 (7.7%)
Deworming			0.85	1	0.35			
1. Yes	195	94/195 (48.2%)				12 (6.2%)	76 (40.0%)	6 (3.1%)
2. No	49	20/49 (40.8%)				1 (2.0%)	19 (38.8%)	0
Pet in the farm			2.28	1	0.13			
1. Yes	208	93/208 (44.7%)				11 (5.3%)	79 (37.9%)	3 (1.4%)
2. No	36	21/36 (58.3%)				2 (5.6%)	16 (44.4%)	3 (8.3%)
Tick infestation on the animal			0.08	1	0.77			
1. Yes	219	103/219 (47.0%)				13 (5.9%)	84 (38.4%)	6 (2.73%)
2. No	25	11/25 (44.0%)				0	11 (44.0%)	0
Tick existence in barn			0.14	1	0.70			
1. Yes	80	36/80 (45.0%)				6 (7.5%)	28 (35%)	2 (2.5%)
2. No	164	78/164 (47.6%)				7 (4.3%)	67 (40.9%)	4 (2.4%)
Grazing areas			1.55	1	0.21			
1. Selected area	157	78/157 (49.7%)				10 (6.4%)	66 (42.0%)	2 (1.3%)
2. Public area	87	36/87 (41.4%)				3 (3.4%)	29 (33.3%)	4 (4.6%)
Total	**244**	**114 (46.7%)**				**13 (5.3%)**	**95 (38.9%)**	**6 (2.4%)**

Table 11. Results of the multivariate analysis of risk factors associated with *Babesia* infection in SWS areas.

Risk factors	β	SE β	Adjusted OR[a] (95CI)[b]	*p-value*[c]
Age group				
1. 0 – 1 year	-	-	1	
2. 1 – 5 years	-1.138	0.049	0.32 (0.14 – 0.72)	0.005
3. > 5 years	-5.01	0.425	0.60 (0.26 – 1.39)	0.23
Herd size				
1. Small (1 – 40 cattle)	-	-	1	
2. Medium (40 – 80 cattle)	0.856	0.402	2.35 (1.07 – 5.18)	0.033
3. Large (over 80 cattle)	-0.360	0.409	0.69 (0.31 – 1.55)	0.37

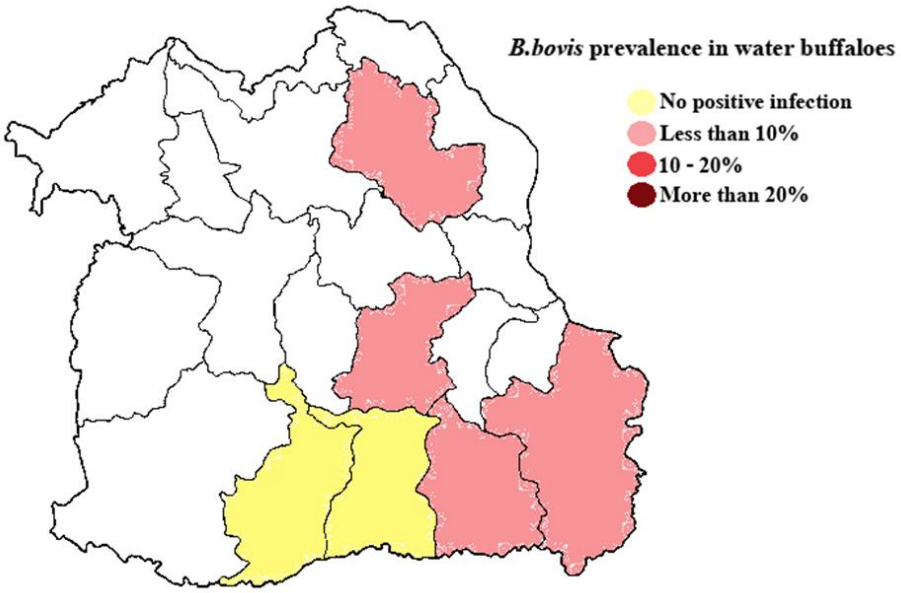
[a]OR, odds ratio

[b]CI, confidence interval

[c]Wald test

3 The prevalence and factor associated of *Babesia* spp. infection of water buffaloes

The molecular results showed 3.7% (23/617) and 2.1% (13/617) positive samples for *B.bovis* and *B. bigemina*, respectively. The highest infection for both agents was found in Sakon Nakorn province (6.1% for *B.bovis* and 5.6% for *B.bigemina*). The highest herd prevalence was 10.6% (9/85) and 7.05% (6/85) for *B.bovis* and *B.bigemina* which found in Sakon Nakon province (table 12). However, Surin and Burirum province showed negative results for *Babesia* spp. infection (Figure 12). Moreover, the highest infective age in water buffaloes was showed at the older group (more than 5 years) as 4.4% (11/251) and 4.0% (10/251) for *B.bovis* and *B.bigemina*, consequently. The highest infection of *B. bovis* was found in male group at 4.4% (4/90) while *B.bigemina* were found in female group at 2.2% (12/527). However, there was no significant associated factor from sex and age in buffaloes's babesiosis prevalence.



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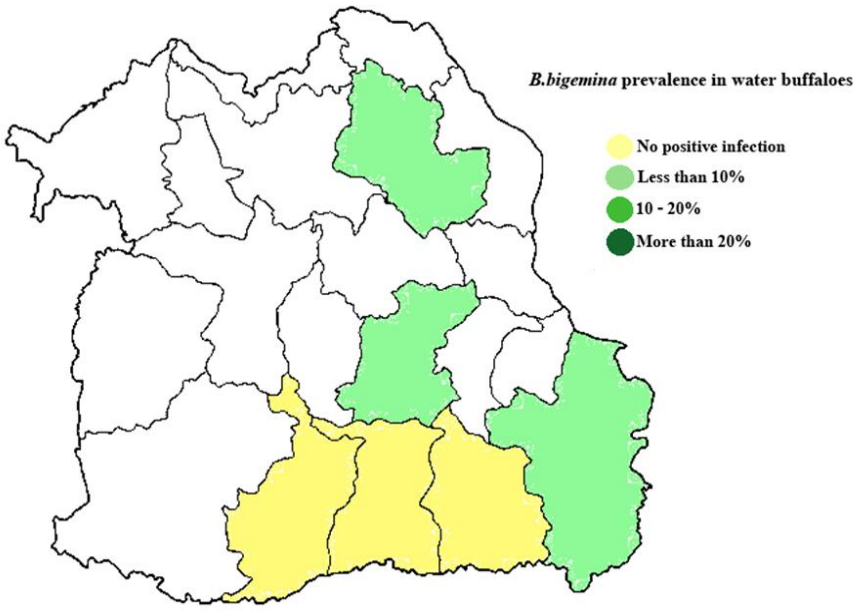

B

Figure 12 The positive areas of buffaloes's babesiosis in Northeast region (A) *B.bovis* positive areas, (B) *B. bigemina* positive areas

4. The comparative prevalence among host species.

The different prevalence between host animals (dairy cattle, beef and buffaloes) were calculated and shown in table 13 and the prevalence mapping showed in figure 13 - 19. The highest prevalence of *B.bovis* and *B.bigemina* was found in beef cattle while the least affective animal among these species were water buffaloes. Moreover, in water buffaloes, the age associated – factor was differed to the other host species. Most *Babesia* – positive water buffaloes were found in the older animals (more than 5 years group) while most positive cattle were found in younger animals.

The molecular analysis by using SBP2 gene for *B.bovis* and *rap 1α* gene for *B.bigemina* revealed 16.4% (440/2,685) for *Babesia* spp. infection. The highest infection species was *B.bigemina* as 11.2% (300/2,685) while *B.bovis* found 7.6% (204/2685) from total samples as presented in table 13. The highest infection age group was the younger animal as 31% (35/311) positive to the infection for *Babesia* spp. and the lowest presented in the youth (1 – 5 years) cluster.

Table 12 PCR results of *B.bovis* and *B. bigemina* infection of water buffaloes associated with sex and age group in Northeast, Thailand

Causative agent	Province	Total no. of samples	Positive samples (%)	Total no. of Farm	Positive Farm (%)	Sex		Positive among age groups		
						Male	Female	<1 yr	1 – 5 yrs	5>yrs
B. bovis										
	Ubon Ratchatani	138	6 (4.3)	59	6 (10.1)	2/29 (6.9)	4/109 (3.7)	0/2	3/74 (4.1)	3/62 (4.8)
	Roi Ed	81	4 (4.9)	58	4 (6.8)	0/7	4/74 (5.4)	0	4/74 (5.4)	0/7
	Surin	73	0	28	0	0/12	0/61	0	0/42	0/31
	Burirum	70	0	21	0	0/8	0/62	0/1	0/45	0/24
	Sakon Nakhon	196	12 (6.1)	85	9 (10.6)	2/23 (8.7)	10/173(5.8)	0/1	5/87 (5.7)	7/108(6.5)
	Sri Saked	59	1(1.7)	22	1 (4.5)	0/11	1/48 (2.1)	0	0/40	1/19 (5.3)
	Total	**617**	**23(3.7)**	**273**	**20 (7.32)**	**4/90 (4.4)**	**19/527 (3.6)**	**0/4**	**12/362 (3.3)**	**11/251 (4.4)**
B. bigemina										
	Ubon Ratchatani	138	1 (0.7)	59	1 (1.7)	0/29	1/109 (0.9)	0/2	0/74	1/62 (1.6)
	Roi Ed	81	1(1.2)	58	1 (1.7)	0/7	1/74 (1.4)	0	1/74 (1.4)	0/7
	Surin	73	0	28	0	0/12	0/61	0	0/42	0/31
	Burirum	70	0	21	0	0/8	0/62	0/1	0/45	0/24
	Sakon Nakon	196	11(5.6)	85	6 (7.05)	1/23 (4.3)	10/173 (5.8)	0/1	2/87 (2.3)	9/108
	Sri Saked	59	0	22	0	0/11	0/48	0	0/40	0/19
	Total	**617**	**13 (2.1)**	**273**	**8 (2.3)**	**1/90 (1.1)**	**12/527 (2.2)**	**0/4**	**3/362 (0.8)**	**10/251 (4.0)**

Table 13 All prevalence of of *B.bovis* and *B. bigemina* infection of dairy cattle, beef and water buffaloes associated age group in Thailand

Causative agent	Host	Total no. of samples	Positive samples (%)	Positive among age groups			Statistical analysis
				<1 yr	1 – 5 yrs	5>yrs	
B. bovis							**Host:** X^2 = 17.4, df = 2, p < 0.01
	Dairy Cattle	1824	162 (8.8)	3/72 (4.2)	105/1090 (9.6)	54/662 (8.2)	
	Beef Cattle	244	19 (7.8)	9/37 (24.3)	9/129 (7.0)	1/78 (1.3)	**Age:** X^2 = 3.02, df = 2, p = 0.22
	Water Buffaloes	617	23 (3.7)	0/4	12/362 (3.3)	11/251 (4.4)	
	Total	**2685**	**204 (7.6)**	**12/113 (10.6)**	**126/1581 (8.0)**	**66/991 (6.7)**	
B. bigemina							**Host:** X^2 = 277.3, df = 2, p < 0.01
	Dairy Cattle	1824	186 (10.2)	12/72 (16.7)	100/1090 (9.2)	74/662 (11.2)	
	Beef Cattle	244	101 (41.4)	18/37 (48.6)	45/129 (34.9)	38/78 (48.7)	**Age:** X^2 = 33.4, df = 2, p < 0.01
	Water Buffaloes	617	13 (2.1)	0/4	3/362 (0.8)	10/251 (4.0)	
	Total	**2685**	**300 (11.2)**	**30/113 (26.5)**	**148/1581 (9.4)**	**122/991 (12.3)**	
Babesia spp.							**Host:** X^2 = 217.3, df = 2, p < 0.01
	Dairy Cattle	1824	292 (16.0)	12/72 (16.7)	171/1090 (15.7)	109/662(16.5)	
	Beef Cattle	244	114 (46.7)	23/37 (62.2)	52/129 (40.3)	39/78 (50.0)	**Age:** X^2 = 19.7, df = 2, p < 0.01
	Water Buffaloes	617	34 (5.5)	0/4	15/362 (4.1)	19/251 (7.6)	
	Total	**2685**	**440 (16.4)**	**35/113 (31.0)**	**238/1581 (15.1)**	**167/991 (16.9)**	

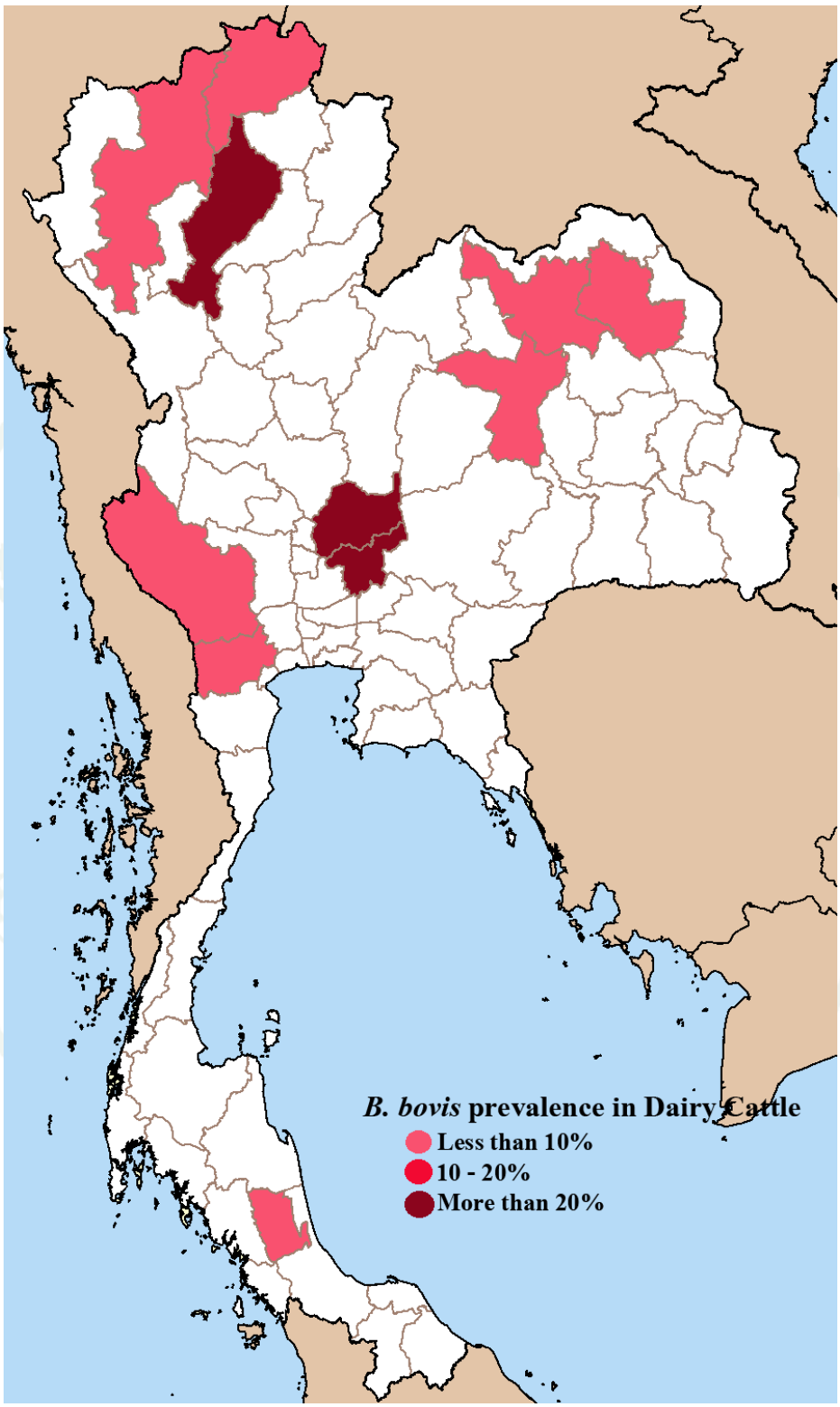

Figure 13 The result of *B.bovis*'s prevalence in dairy cattle in this study

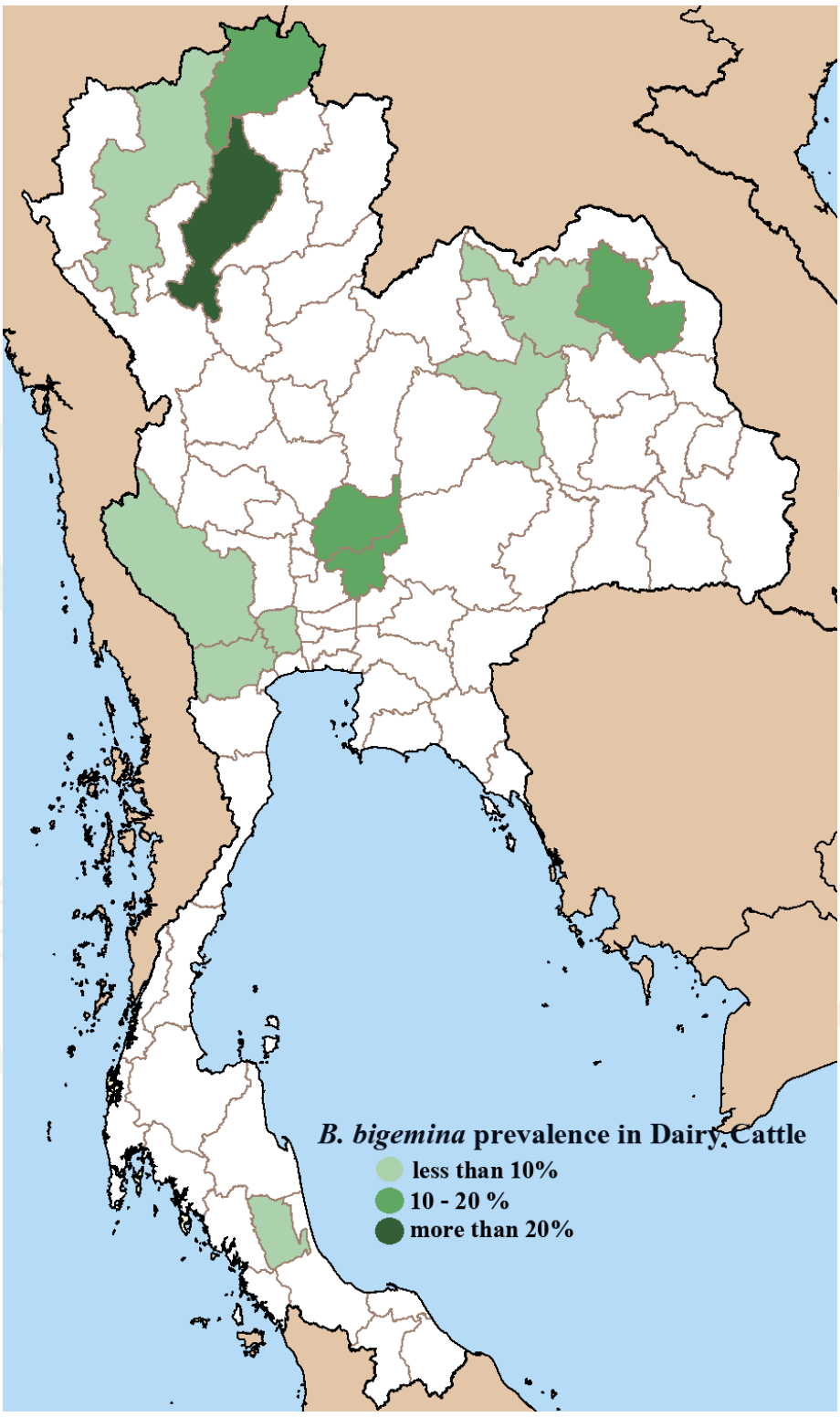

Figure 14 The result of *B.bigemina*'s prevalence in dairy cattle in this study

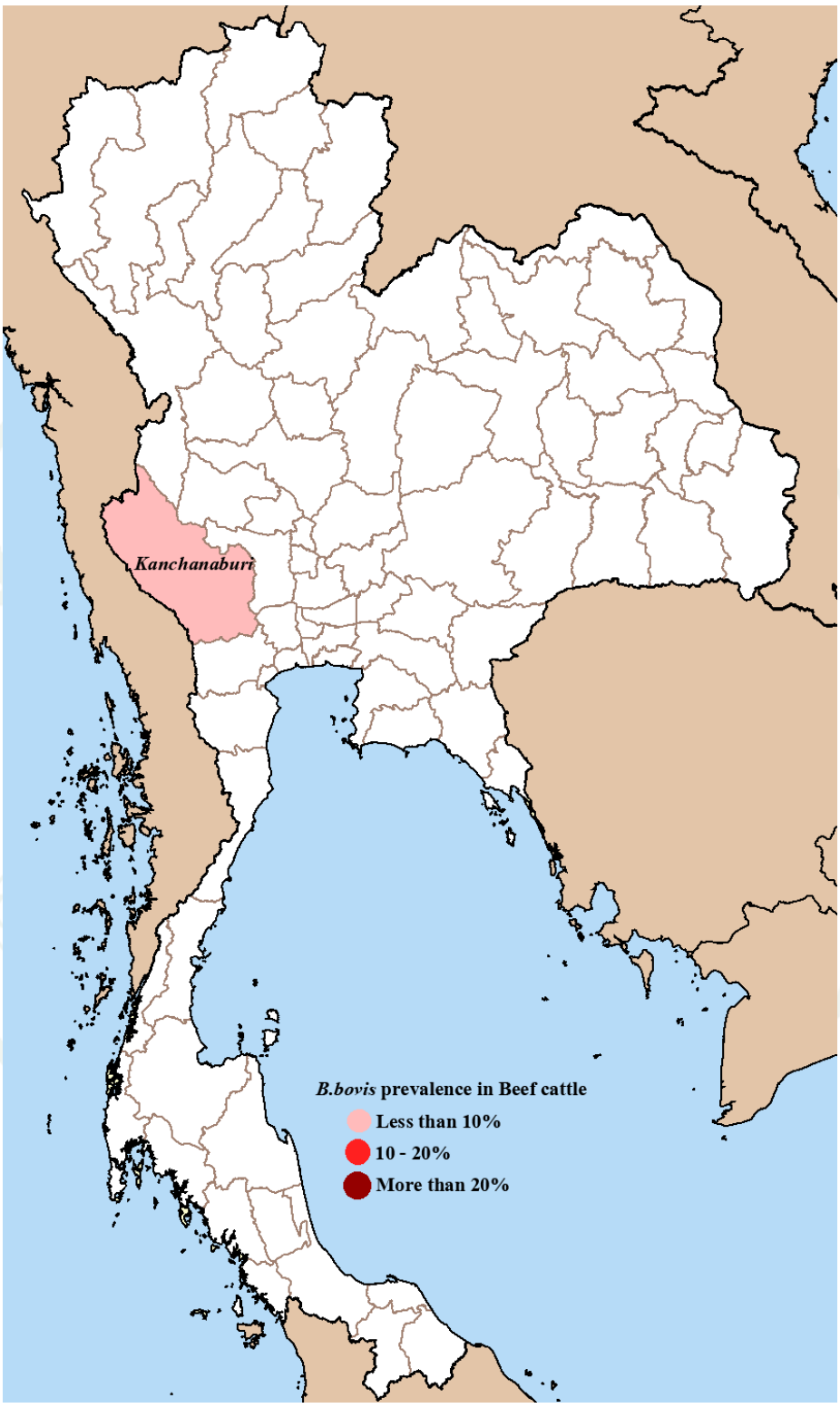

Figure 15 The result of *B.bovis*'s prevalence in beef cattle in this study

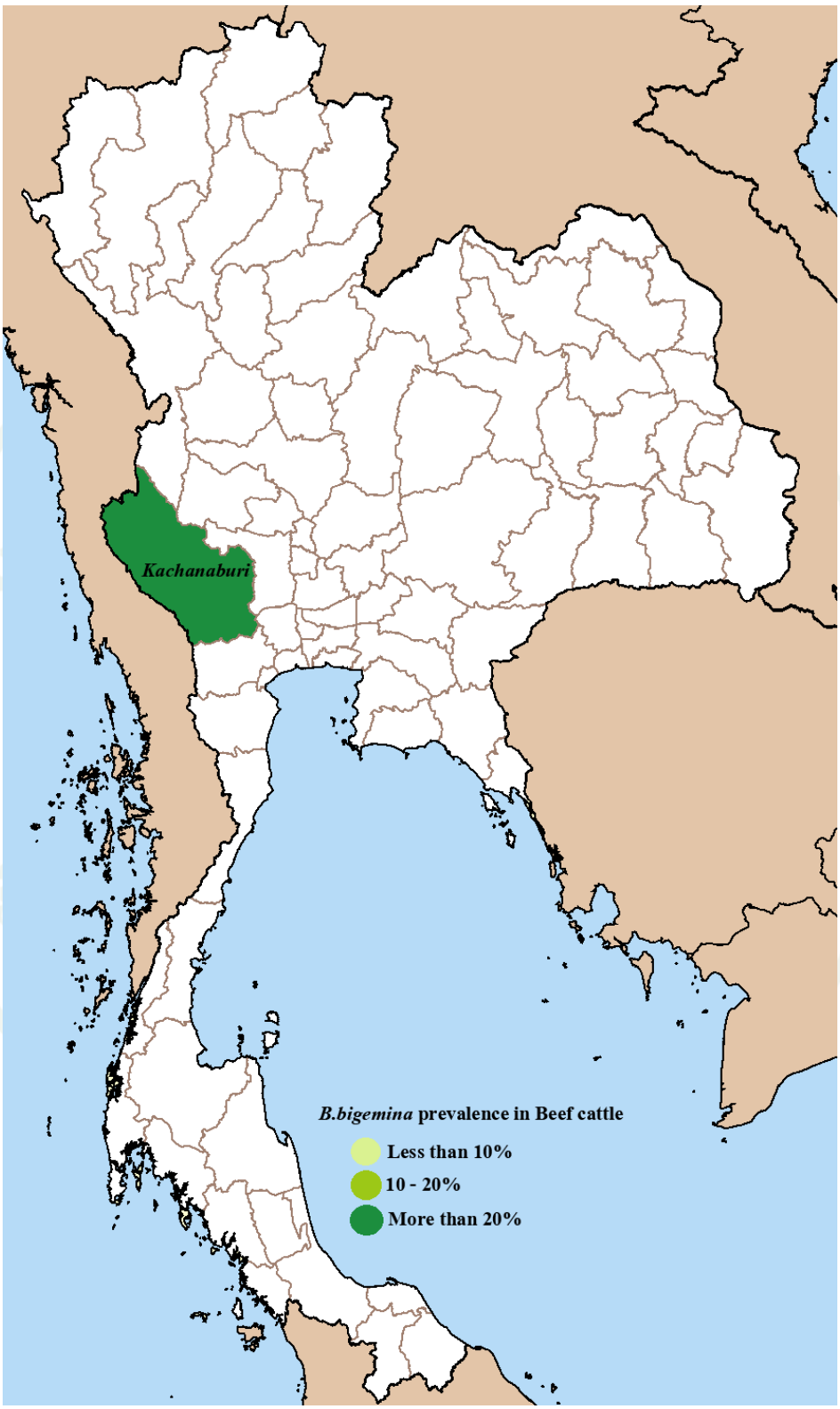

Figure 16 The result of *B.bigemina*'s prevalence in beef cattle in this study

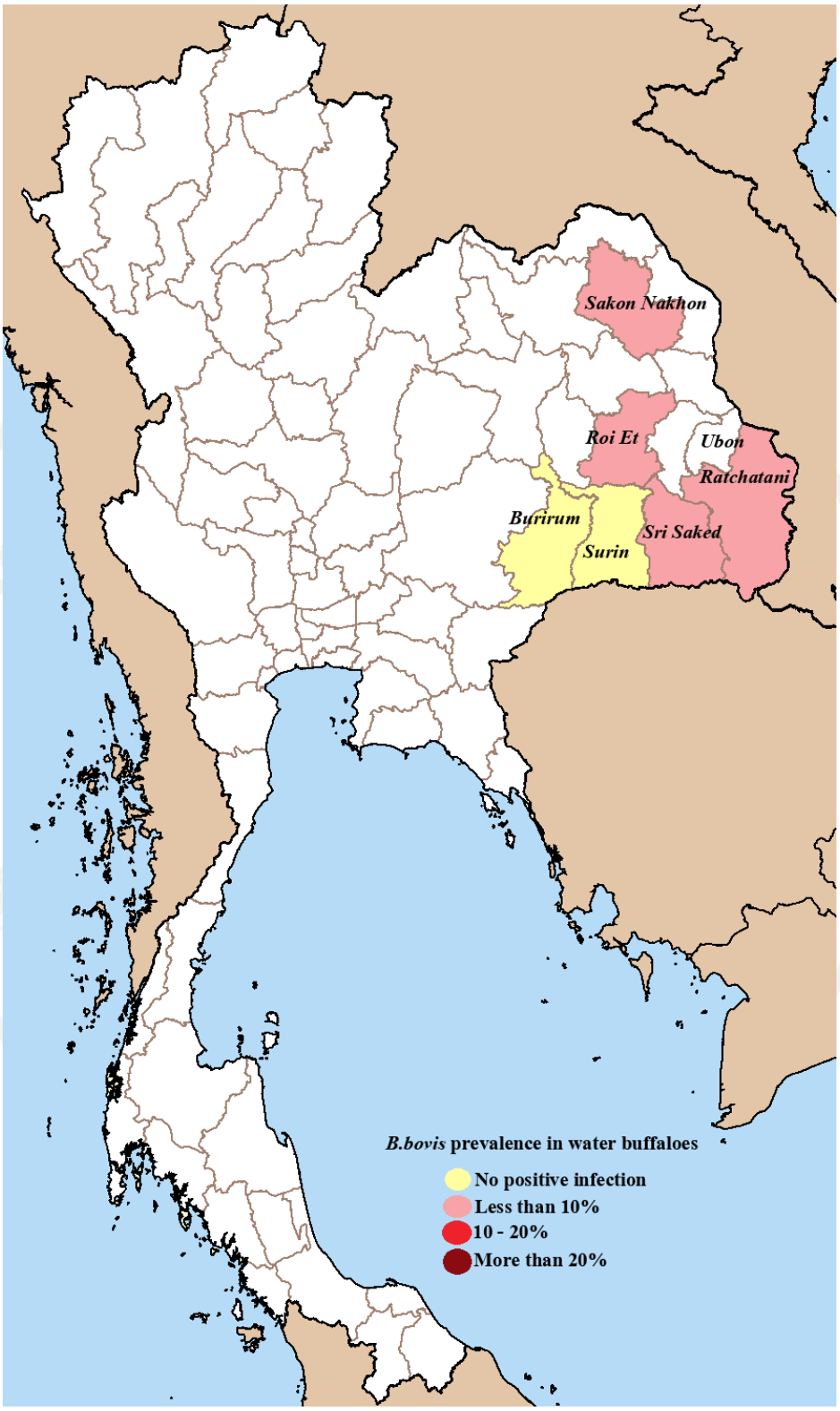

Figure 17 The result of *B.bovis*'s prevalence in water buffaloes in this study

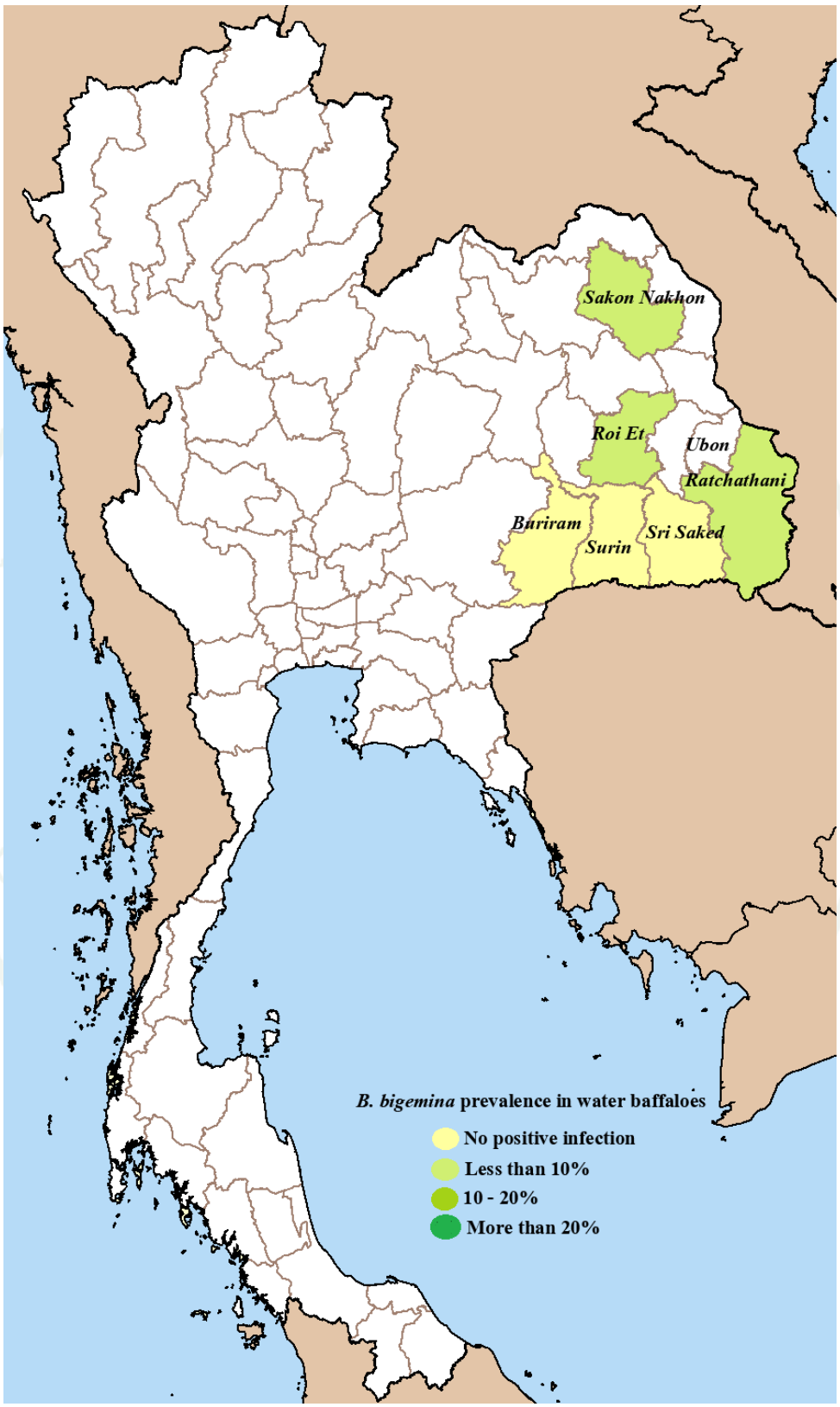

Figure 18 The result of *B.bigemina*'s prevalence in water buffaloes in this study

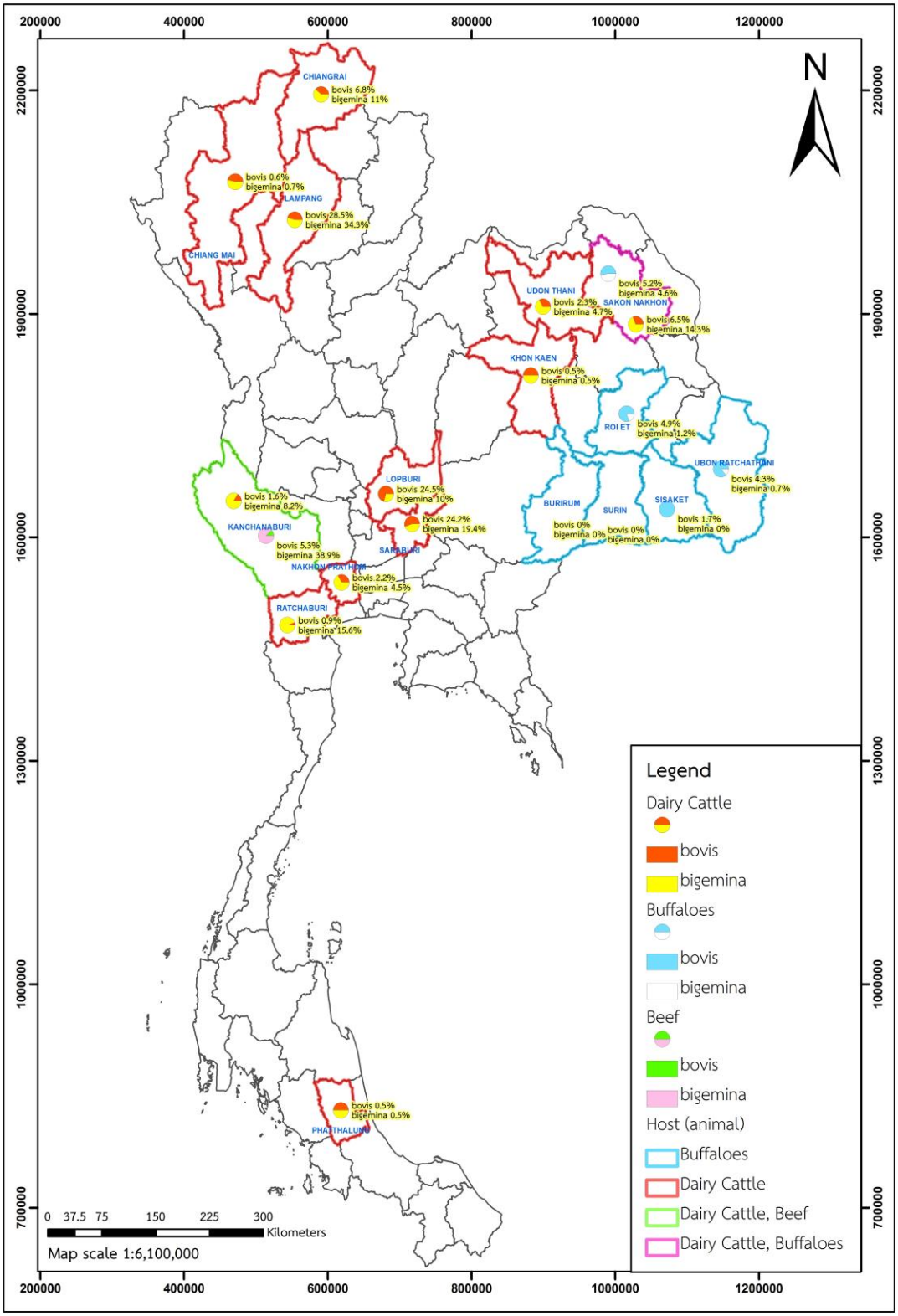

Figure 19 The overall prevalence of *Babesia* infection in Thailand

Table 14 The risk factor analysis of factors associated with *Babesia* spp. infection

Factor		Odds Ratio	95% CI
Host			
	Dairy cattle	1.81	1.24 – 2.66
	Beef	15.04	9.80– 23.06
	Water buffaloes	1	-
Age			
	<1 year	2.53	1.66 – 3.86
	1 – 5 years	1	-
	Over 5 years	1.14	0.92 – 1.42

5. Phylogenetic analysis

5.1 The phylogenic analysis of of *B.bigemina rap - 1α* gene

The phylogenetic analysis of *rap - 1α* gene in *B.bigemina* between 7 sequence from this recent study and 10 sequence from GeneBank database was showed in figure 20. The rap – 1 α gene cluster of recent study still present the specificity of organism to the host. However, the high diversity was presented between the cluster as the bootstrap values were ranging between 71 – 35 in each subtree (Figure 21).

Figure 20 The phylogram of *B.bigemina rap - 1α* demonstrated the high variation of each gene cluster.

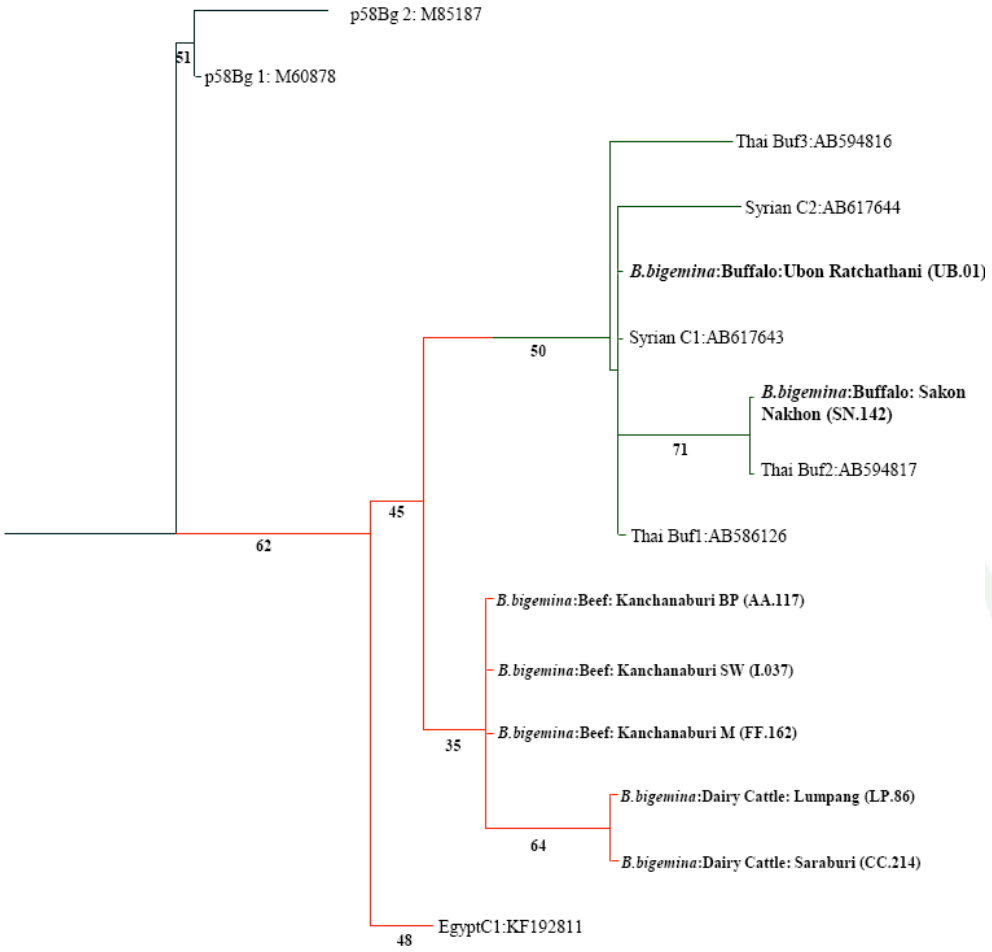

Figure 21 Subtree of *B. bigemina rap 1* – α phylogram showed the bootstrap value and the cluster of host – pathogen specificity.

5.2. Phylogenetic analysis of *B.bovis SBP2* gene

The phylogenetic tree of *B.bovis* SBP2 gene were construct from 6 sequence in this study and 10 homologous gene from genebank database. The phylogram showed the grouping of host specific in figure 22. However, the gene cluster showed the variation with the isolated location as the cluster of Veitnam and Brazil and Thailand cattle with Philippines. The bootstrap value showed the high variation among the gene cluster

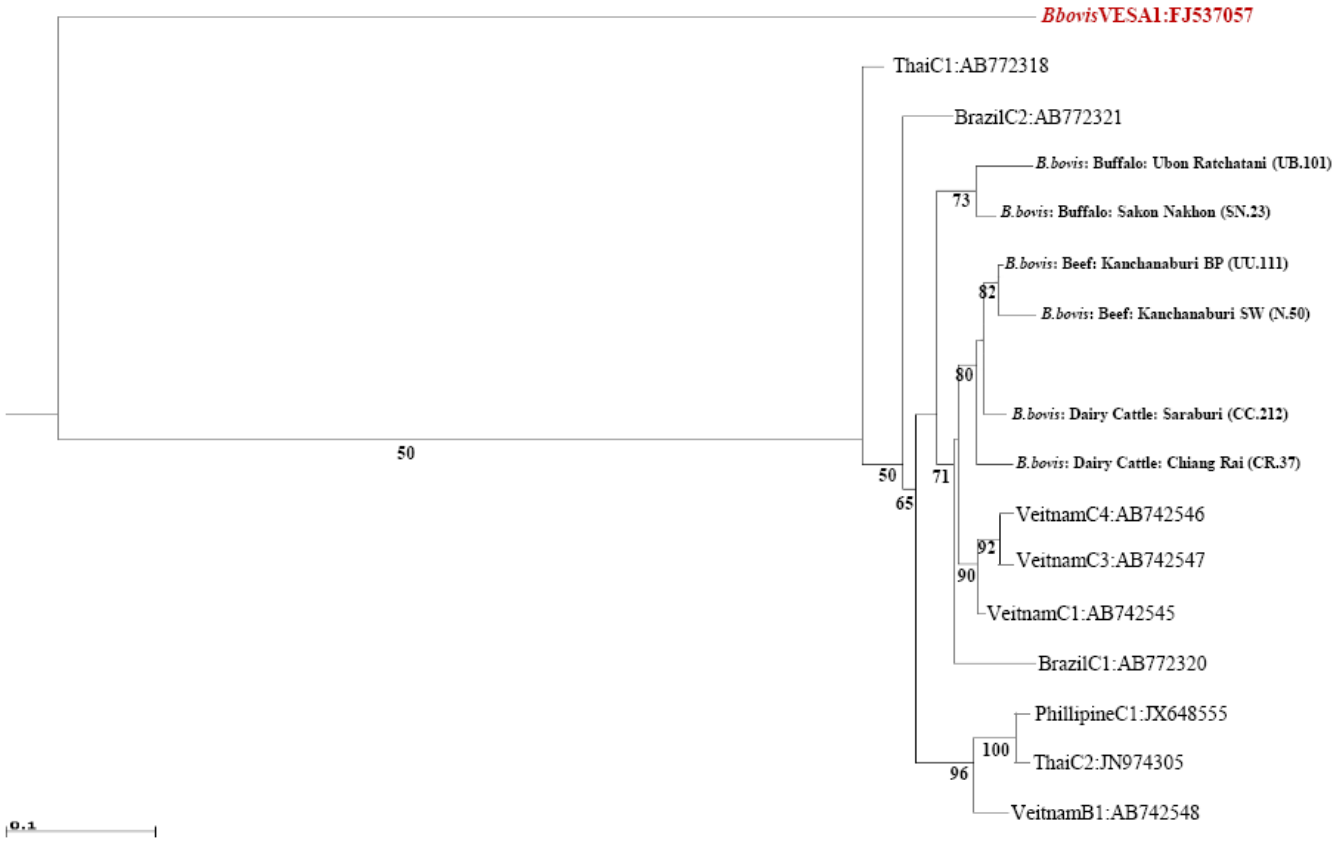

Figure 22 The phylogenetic tree of *B.bovis* SBP2 gene showed the genetic diversity among the *B.bovis* isolation.

6. DNA sequencing and phylogenetic analysis of *B. bovis* MSA2b gene

Form 162 positive samples of *B. bovis* infection, 23 samples were successfully cloned and sequenced by using the full – length amplification of MSA2b gene which yield at approximately 800 bp band. All sequences were processed in BLAST program and show the maximum identity at 99.9% to MSA2b gene from the Genebank database. The sequences were already submitted to the Genebank data (AB745695 – AB745717).

The phylogram divided the *MSA-2b* gene sequences into thirteen clades (designated as 1–13), in which Thai sequences were found in eight different clades (1, 2, 3, 5, 6, 9, 10, and 13) (Figure. 23). In particular, three of these eight clades (clades 2, 6, and 10) were grouped only of Thai sequences. While most of the Thai *MSA-2b* sequences were clustered together with Mexican sequences in clade 13, 3. Thai sequences (AB745697, AB745696, and AB745712) were located in 3 different clades (clades 1, 3, and 5, respectively), which only existed in the Australian *MSA-2a/b* sequences. In addition, a single Thai sequence was also found in clade 9, together with those from Mexico and USA. Furthermore, sequences originated from different regions of Thailand were often found in the same clades. The pairwise comparison of deduced MSA-2b amino acid sequences showed that the similarity among Thai isolates ranged from 68.3 to 100% (Figure 24).

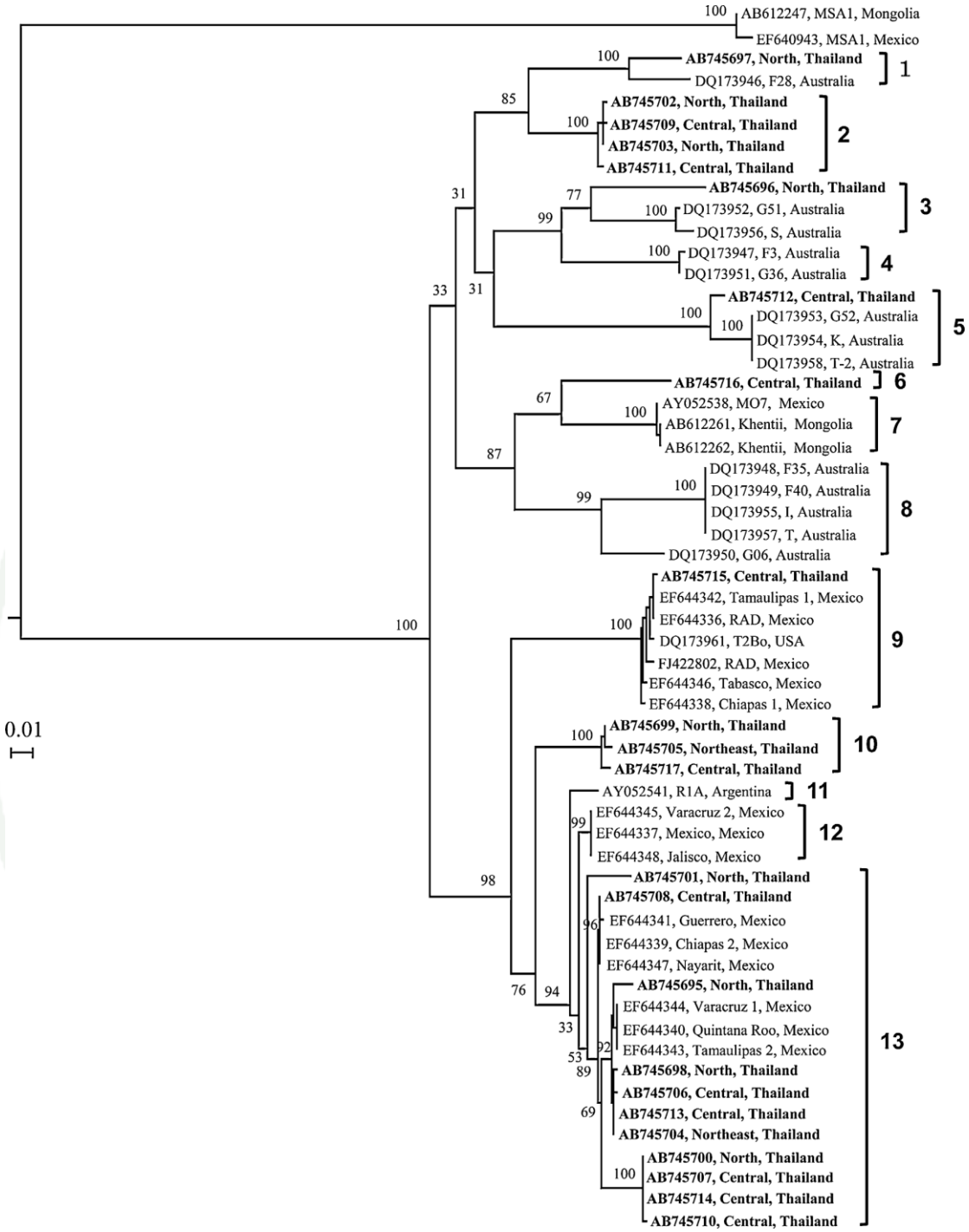

Figure 23 The phylogenic tree of *B. bovis* MSA-2b gene sequences generated in the present study (boldface letters) and sequences reported from other countries were used to construct the phylogram.

Clade		1	2				3	5	6	9	10			13									
Acc. Num.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
AB745697	1	100	87.1	87.5	87.5	87.5	82.0	75.7	73.8	70.4	74.6	75.0	75.5	77.2	79.1	78.7	79.3	78.7	79.1	79.1	77.9	77.9	77.9	77.9
AB745702	2		100	99.6	99.6	99.6	84.2	76.4	71.7	71.7	77.1	77.5	78.2	82.5	86.3	85.9	85.1	85.9	86.3	86.3	85.5	85.5	85.5	85.5
AB745709	3			100	100	100	84.6	76.4	71.7	72.0	77.5	77.8	78.5	82.9	86.6	86.3	85.5	86.3	86.6	86.6	85.9	85.9	85.9	85.9
AB745703	4				100	100	84.6	76.8	72.0	72.0	77.5	77.8	78.5	82.9	86.6	86.3	85.5	86.3	86.6	86.6	85.9	85.9	85.9	85.9
AB745711	5					100	84.6	77.2	72.0	72.0	77.5	77.8	78.5	82.9	86.6	86.3	85.5	86.3	86.6	86.6	85.9	85.9	85.9	85.9
AB745696	6						100	77.9	72.5	70.3	70.5	70.9	71.2	77.1	78.2	77.8	78.6	77.8	78.2	78.2	79.3	79.3	79.3	79.3
AB745712	7							100	70.4	72.7	69.0	69.0	68.3	71.0	72.5	72.1	72.1	72.1	72.5	72.5	73.2	73.2	73.2	73.2
AB745716	8								100	71.7	74.8	75.2	74.5	71.3	70.0	69.7	70.1	69.7	70.0	70.0	69.0	69.0	69.0	69.0
AB745715	9									100	84.4	84.0	84.7	78.5	79.9	79.9	80.4	79.9	80.2	80.2	80.5	80.5	80.5	80.5
AB745699	10										100	99.6	97.4	87.6	89.1	88.4	89.0	88.4	88.7	88.7	87.6	87.6	87.6	87.6
AB745705	11											100	97.8	87.3	88.7	88.0	88.6	88.0	88.4	88.4	87.3	87.3	87.3	87.3
AB745717	12												100	86.4	88.7	88.0	88.6	88.0	88.4	88.4	87.3	87.3	87.3	87.3
AB745701	13													100	95.4	94.7	93.9	94.7	95.1	95.1	93.9	93.9	93.9	93.9
AB745708	14														100	99.2	98.5	99.2	99.6	99.6	98.5	98.5	98.5	98.5
AB745695	15															100	98.5	99.2	99.6	99.6	97.7	97.7	97.7	97.7
AB745698	16																100	98.5	98.9	98.9	96.9	96.9	96.9	96.9
AB745706	17																	100	99.6	99.6	97.7	97.7	97.7	97.7
AB745713	18																		100	100	98.1	98.1	98.1	98.1
AB745704	19																			100	98.1	98.1	98.1	98.1
AB745700	20																				100	100	100	100
AB745707	21																					100	100	100
AB745714	22																						100	100
AB745710	23																							100

Figure 24 The pairwise comparison analysis of amino acid sequence translate of MSA2b gene from 23 Thailand sample. The result of pairwise correlation show 8 cluster of *B. bovis* speciation in the country. The comparison was constructed by using EMBOSS needle program to calculated the percentage homology.

7. B cell epitope prediction and Protein 3dimetion prediction

The B cell epitope prediction shown 15 predicted epitopes that specific for *B.bovis* to invade to host's erythrocyte (Table 15). The PDB (Protein Data Base) entry of template structures for homology modeling of C122, C152, and N49 were 2OPK, 1PEW, and 2NR4 respectively (Figure 25). All of models were assessed the quality of structure by using Ramachandran plot. The Ramachandran plot of each structure revealed the appropriate result for proven the accuracy of the prediction structure. By the intra variation of each epitope, the prediction structures demonstrated the different variable domain that might play the role of B – cell attachment and cell invasion.

Table 15 Analysis of B-cell epitopes predicted from MSA-2b sequences determined in the present study

Start position	Stop position	Predicted B cell epitope amino acid sequence	Length (aa)	Conserved amino acid/Total
4	22	KIFLLTACCCASLLSVSAS[a]	19	19/19
27	38	DTSTTLHYEMK	12	Only one sample
40 - 41	47 - 48	VANLITYL	8	4/8
68	87	DCSRDALKALKDILVVLKEE	20	11/20
85	93	KEKVPFKTS	9	Only one sample
95	103	FDDYVLGNL	9	4/9
108	126	TDQVFKSLLERVLLIKK[b]	17	6/17
152	158	YKKHISA	7	2/7
163	176	VKDYTFLVKFCNDF[b]	14	3/14
184	190	MKIYKAF	7	4/7
194	200	EELVKKK	7	4/7
206	211	SSPPSS	6	2/6
221	229	PQAPAAQSQ	9	5/9
243	247	AAPQD	5	2/5
260	266	PSTIPEQ	7	Only one sample

After analyzing B-cell epitopes among all MSA-2b sequences, the lengthiest epitopes were selected and used for comparisons.

[a] The conserved epitope located in the signal peptide

[b] These epitopes were detected in all MSA-2b sequences

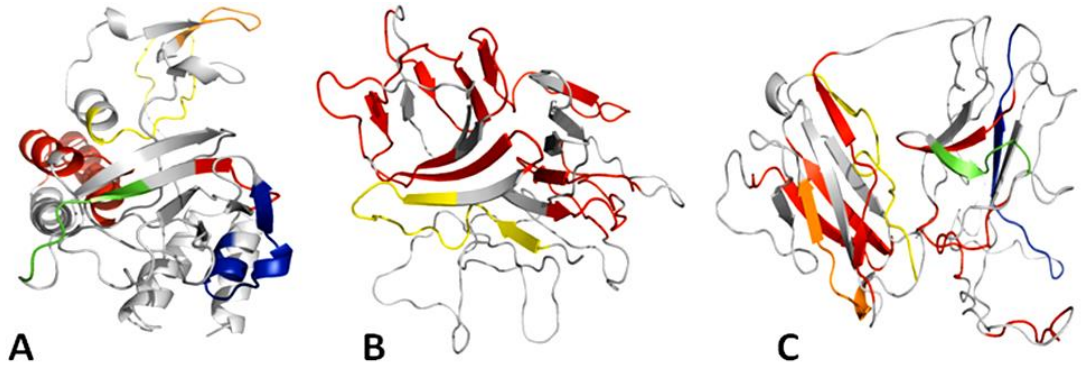

Figure 25 Three dimensional structure of positive sample, all of structures were modeled by using homology modeling technique. The yellow ribbon is represented the signal peptide which found in every sequence of MSA2b gene. The orange, blue and green ribbons refer as the conservative peptide that almost found in the MSA2 gene but have intra – variation in amino acid sequence. The red ribbon is represented the individual B cell epitope which is predicted and found in each sequence. A: sample N49, B: sample C122, C: sample C152

DISCUSSIONS

1. The prevalence of *Babesia* spp. infection

1.1 The prevalence of *Babesia* spp infection in dairy cattle

In the dairy cattle study, although Lumpang Province showed the higher positive rate for *B. bovis* and *B.bigemina*, the most infected area per region was Central Thailand. The high prevalence of *Babesia* spp. infection in Central Thailand might correlated with the abundance of tick vectors (*R. microplus*) in this region (Ahantarig *et al*., 2008). In addition, the current findings showed that the prevalence of *B. bovis* and *B.bigemina* in dairy cattle were higher among the animals in 1 – 5 years and less than 1 year age – grouped, respectively. This result in dairy cattle group was supported to the recently study that bovine babesiosis had no relationship among age group (Iseki *et al*., 2010). However, young animals have been reported to be more susceptible to *Babesia* infection than the old animals (James, 1988; Awad *et al*., 2011).

1.2 The prevalence of *Babesia* spp infection in beef cattle

The result of this study demonstrated that 5.3% and 38.9% of cattle in SWF areas were infected with *B. bovis* and *B. bigemina*, respectively. Specific genes such as spherical body protein 2 (SBP-2) and rhoptry-associated protein 1 (*rap-1*) gene are used to improve their specificity and sensitivity to identify *B. bovis* and *B. bigemina*, respectively and beneficial for epidemiological investigation (McElwain *et al*., 1987; Figueroa *et al*., 1990; Machado *et al*., 1993,; Suarez *et al*., 1994; Vidotto *et al*., 1995). The genetic variation of subpopulation within *B. bovis* or *B. bigemina* was also shown distinct biological characteristic (Timms *et al*., 1990).

Contrasted to the study in dairy cattle, the age of animals was significantly potential related with the infection ($p<0.05$). In this study, the young animal (<1 year) had the highest infection (67.6%) compared to the other range of age (Odds

ratio=3.88, CI= 1.19 – 12.6). The prevalence of *B. bovis* and *B. bigemina* in calves (<1 year) were 13.5% and 37.8%, respectively. Compared to the previous publication (Phrikanahok *et al*., 2000), the prevalence of *B. bovis* and *B. bigemina* in calves (6-7.4 months) were 29.5% and 23.5%, respectively. The differences of prevalence were depended on the technique since different genes primers might yield different prevalence (Cao *et al*., 2012). After birth, calves immediately receive passive immunity via the colostrums from premunized mothers, which is considerable to be a challenge during the first month of life (James, 1988). In the endemic areas, mild *Babesia* spp. infections of cattle were normally occurred and induced immunity against babesiosis particularly in native animals. This immunity was also correlated with the herd immunity.

For the other related factors, medium sized herd (40–80 animals/herd) had more infection than the large (>80 animals/herd) and small herd (1–40 cattle) due to the overcrowded cattle in the herd and the poor management. Moreover, the high forage density (over 20 trees/ 5 m2) might be implicated with the humidity of the environment since the temperature and humidity were the essential factors to support the tick survival and population. For seasonally effect, the annual precipitation of Kanchanaburi was over 1000 mm (data form Thai Meteorological Department) while the annual precipitation above 700 mm is suitable for enzootic stability (Barros *et al*., 2005). The high humidity (>50%) has influenced the tick biological cycle led to the stability of the pathogen in the areas. However, there were no significant differences by seasonal factors in this study. In addition, *Babesia* cannot be transmitted by the other vectors such as stable flies or tabanus. Therefore, the insect factor might not have the effect on *Babesia* infection in this area.

The other factors such as sex, farm management, seasonal change, grazing location, and insect vector had no significant related to the *Babesia* infection in this study. Evidently, sharing of the grazing location for rearing the cattle might increase the spreading of the cattle ticks leading to the great risks of transmitted babesiosis (Simuunza *et al*., 2011). The rearing of cattle in SWS areas was by roaming the animals in the public pasture. Frequently, these animals had been intentionally or

unintentionally grazed in the SWS areas. Naturally, this protected forestry area is the habitat of the wildlife such as guar, sambar deer, bantang and elephant (Chaiyarat and Srikosamatara, 2009). This trespassing cattle combined with available ticks might create the high risk environment for the wildlife. The numbers of trespassing cattle in the protected forestry areas is currently increasing. Salakpra wildlife sanctuary is one of the protected forestry areas that encounter to the problem of the cattle trespassing (Chaiyarat and Srikosamatara, 2009). Most cattle were freely wandered in or nearby the forestry areas since no real boundary to limit the trespassing (Barros *et al*., 2005). For the update information, SWS contain 859 square kilometer with 28 villages, 1,277 cattle, and 392 buffaloes live nearby the wild areas (Chaiyarat and Srikosamatara, 2009). Some cattle diseases can be transmitted by the invaded cattle to the wildlife or vice versa. Both infected wildlife and cattle can become a carrier with high potential of disease transmission (Aboulaila *et al*., 2010, Chaiyarat and Srikosamatara, 2009).

1.3 The prevalence of *Babesia* spp infection in water buffaloes samples

Among these significantly factor (host and age), the beef cattle showed the highest significantly risk factor for *Babesia* infection (Odds Ratio=15.04, CI= 9.80 – 23.06) as present in table 14. This risk factor might be correlated the chance to exposure to the tick vector in the environment and the herd management. While the dairy cattle almost household management, the beef cattle mainly roamed in the habitat where the endemic areas of the tick vector. Generally, young animals have been reported to be more susceptible to *Babesia* infection than the old animals (James, 1988; Awad *et al*., 2011).

In this study, the younger animal showed the significant affect to the *Babesia* infection and the highest prevalence also found in the younger animal (less than 1 year) both three host groups. The prevalence of *B.bovis* and *B.bigemina* hase been reported 29.5% and 23.49% in the young animals (age 6.0 – 7.4 months) (Phrikanahok *et al*., 2000).Moreover, previous study has been reported calves can immediately receive antibody via the colostrum after birth from immune mothers, which is considerable to be a challenge during the first month of life (James, 1988).

Bovine babesiosis in Thailand has been reported over the past decades, and the distribution of this disease had the impact on the economic losses in herd production. Recently, the serological studies in water buffaloes in Northeast areas of Thailand demonstrated the prevalence ranged 3.6 – 16.8% in five provinces (Terkawi *et al*., 2011a). The highly prevalent vector, the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, is the important factor of the disease's distribution in the endemic areas and cattle ticks were the main vector of babesiosis in dairy cows (Jittapalapong *et al*., 2004). As one of the fatal diseases prevalent among cattle populations in Thailand, the upward trend of the disease burden is currently observed because of the spreading of the tick species *Rhipicephalus* (*Boophilus*) *microplus*, which is known as a transmission vector of *Babesia* parasites (Pemayodhin *et al*., 1991)

2. The phylogenic analysis of *B.bigemina rap-1* gene and *B. bovis SBP2* gene

B.bigemina rhoptry-associated protein 1 (*rap-1*) gene expressed the immunogenicity of rhoptry protein was formed at the surface of *B. bigemina* 's merozoites and was one of the identified major proteins in *B. bigemina* merozoites.(McElwain *et al*., 1987; Figueroa *et al*., 1990; Machado *et al*., 1993; Suarez *et al*., 1994; Vidotto *et al*., 1995). The nested PCR (nPCR) using specific *rap 1 α* gene of *B. bigemina* that is highly conserved and shown 96 – 97% identity to the referent sequences in Genbank (Terkawi *et al*., 2011a). This gene is conserved and manipulated in two identical gene copies arranged in tandem making it suitable for discriminating against field *B. bigemina* (Petrigh *et al*. 2008). The variation of *rap 1* gene among geographic isolations may be affected the survival of parasite in host cells. From the high variation within this gene, the sequence of *B.bigemina rap – 1α* gene is difficult to analysis genetic diversity when comparing with another universal gene such as the rRNA internal transcribed spacer (ITS) regions (Cao *et al*., 2012). For this reason, the phylogenic analysis which the combination of another universal gene marker might be result more accuracy phylogram for *B.bigemina* isolation

The SBP2 gene was successfully developed as the nested PCR to detect and identify *B. bovis* infection as the screening test for epidemiological investigation

(Aboulaiala *et al*., 2010). This gene was conserved among the geographically diverse isolates of *B. bovis* and is not present in *B. bigemina*. so it is suitable for the detection of *B. bovis* (Aboulaila *et al*. 2010) Furthermore, the genetic variation of subpopulation within *B. bovis* also show distinct biological characteristic (Timms *et al*., 1990). However, the information and the consensus gene in the Genebank database still limited when comparing with the ribosomal small subunit gene. For this reason, the genetic variation analysis of *B.bovis* should process by using ITS region or partial small subunit ribosomal gene combination with the other gene marker to discrimination the diversity of the pathogen within the endemic area.

3. B – cell epitope analysis of *B.bovis* MSA – 2b gene in dairy cattle

The VMSA gene are a group of immunodominant, GPI anchored protein (Reduker *et al*., 1989; Hines *et al*., 1989; Mosqueda *et al*., 2002). Significantly, the VMSA protein were demonstrated to contain neutralization – sensitive B cell epitopes and though to participate in erythrocyte invasion (Hines *et al*., 1992; Sauerez *et al*., 2000; Florin – Christensen *et al*., 2002; Mosqueda *et al*., 2002; Wilkowsky *et al*., 2003). Furthermore, a variable B – cell epitope was identified by the monoclonal antibody 23/70.174 and mapped in a repetitive region MSA2 of *Babesia* paraite in Texas and Mexico strain (Goff *et al*., 1988; Palmer *et al*., 1991; Jasmer *et al*., 1992). The previous study has been shown that the structure of α – helix bundle almost conserved despite extensive amino acid variation, suggesting this predicted structure might be have the important roles for their functionality (Dominquez *et al*., 2010).

For this study, B cell epitope prediction was calculated by using Kalaskar and Tongaonkar method (1990). This algorithm is calculated from the variation of hydrophilicity, aminocidic environment, and comparison with another epitopes in the other species that already proven. All of the positive sequences were result at 15 putative B cell epitope which 3 epitope were found in every sample (Table 15). However, in this study shown that the motif **YYKKHIS** is not present in all isolation opposite to the previous study which shown this peptide sequence coincide with the most hydrophilic peak predicted for MSA2b antigen (Florin – Christensen *et al*.,

2002; Dominquez *et al*., 2010). Additionally, sequence number N49, C122 and C152 were selected to analysis by 3D Protein modeling structure prediction by Discovery studio 2.5 (Accelrys, USA) and displayed the differentiation structure (Figure.25). This structure show the variable of the genetic code that influence on the protein structure which function as the immunogenicity protein on B – cell surface and might play a role for the different pattern of the clinical sign, the successful of veterinary threatening of the infected animal and also useful for the development of recombination vaccine against the bovine babesiosis infection.

The high genetic diversity of MSAs observed among different isolates could be linked to the immune evasion strategies of *B. bovis*. Among the *MSAs*, *MSA-2b* was considered to be a relatively good marker that can separate *B. bovis* isolates into different genotypes (Genis *et al.*, 2009; Altangerel *et al.*, 2012). Therefore, in the present study, *MSA-2b* gene sequences were used to determine the genetic diversity of Thai isolates.

The phylogenic analysis indicated that *B. bovis* isolates in Thailand can be classified into at least eight groups. Obvious geographical relationships among isolates were not established, since several Thai sequences were clustered together with the sequences from Australia, Mexico, and the USA. However, the genotypes which were formed only of Thai *MSA-2b* sequences might be an evidence of epidemic clones. The genetic diversity observed in the phylogram was further confirmed by the low similarity values estimated for Thai MSA-2b sequences. These findings collectively showed that the *B. bovis* isolates in Thailand are genetically diverse.

CONCLUSION AND RECOMMEDATION

Conclusion

As located in tropical climate, the livestock development in Thailand have been hampered by the bovine babesiosis cause economically damaged to the cattle's owner due to the loss of milk, animal illness and dead, cost for treatment and chemical contamination. Our current study has demonstrated the prevalence of bovine babesiosis caused by *B. bovis* and *B.bigemina* in buffaloes, beef and dairy cattle in all endemic areas in Thailand was 7.6 % (204 / 2,685) and 11.2% (300/2,685), respectively. The highest prevalence of *Babesia* spp. infection was found in beef cattle as 46.7% (114/244) and the lowest infection was in the buffaloes group at 5.5% (34/617). However, the water buffaloes are served as another carrier of this disease but shown the lower prevalence because of the decrease opportunity to attach by the tick (Somparn *et al*., 2004). The significant factor for disease distribution was the host specificity and animal age. The phylogenetic analysis of specific gene, *B.bigemina rap 1 – α* and *B.bovis* SBP2 gene, showed the variation within gene cluster but can identification between the host groups. The phylogenic analysis by using MSA2b demonstrated 8 clusters of *B. bovis* that wide spreading in Thailand. Additionally, the B cell epitope prediction and 3D Protein structure modeling prediction present the variation among the antigenicity epitope that specific to B cell surface. The conservation of surface exposed B cell epitope are the one of the risk factor that affected by selection pressure of the host immunological system. This selection pressure suggested the influence of the physiological compression to genetic variation of the parasite to survive in host cell (Dominquez *et al*., 2010). The results of this study provide the important information about the prevalence and the genetic diversity of *B. bovis* and *B.bigemina* infected in dairy cattle that be benefit to the preventing and controlling strategies, and also to develop recombinant candidate vaccine against the bovine babesia infection in the high endemic areas in Thailand.

Recommendation

The B cell epitope analysis presented the conformation variation of MSA2b protein from each region that might influence to the severity of the babesiosis in each endemic area. This information will be useful for the development of vaccine candidate against bovine babesiosis. The most conservative epitope might be selected and analyzed for candidate protein in the pilot vaccine development. Further study is needed to analyze the immunogen protein form *Babesia* spp. in our country that might be use as the candidate vaccine for the livestock in all endemic areas and have the certain protective capacity for against all *Babesia* spp.

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APPENDICES

Appendix A

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. Gel loading buffer (loading dye)

The loading dye buffer composed of 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol and 35 ml of ultrapure distilled water. The loading dye solution was kept at 4°C.

2. Tris acetate buffer (50x TAE)

The stock 50x TAE was prepared by dissolved 242 grams of Tris-base in 500 ml of distilled water. After the ingredient was completely dissolved, 57.1 ml of concentrate glacial acetic acid and 100 ml of 0.5 M EDTA, pH 8.0, were added into the solution. The final volume was adjusted to 1,000 ml by distilled water. The 50x TAE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 50x TAE buffer with distilled water.

3. Working (0.5x TAE)

Twenty milliliter of 50X TAE was added to 980 ml of UDW. This solution can be reused three times.

Appendix B

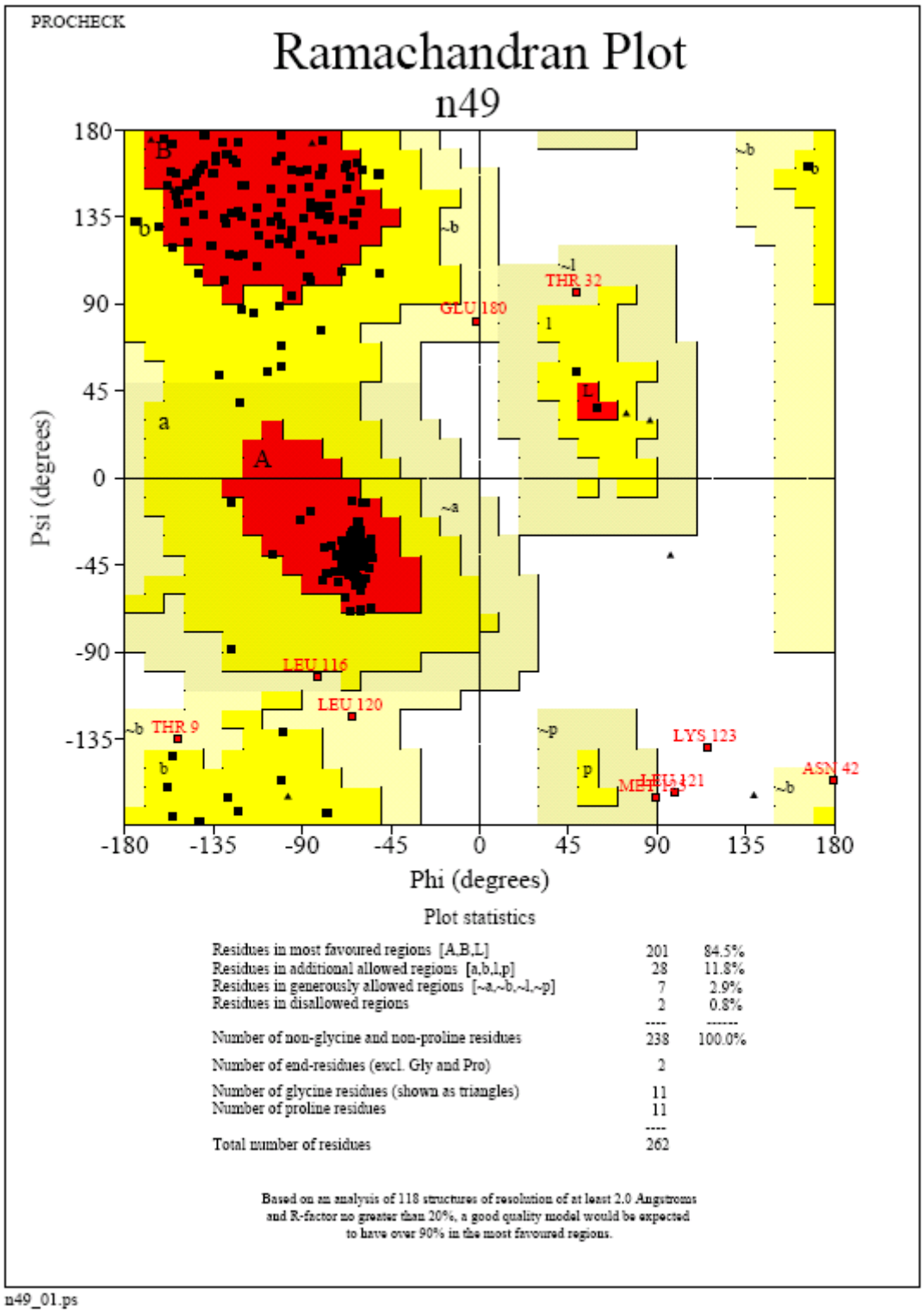
The phenol – chloroform DNA extraction method

The phenol – chloroform DNA extraction method

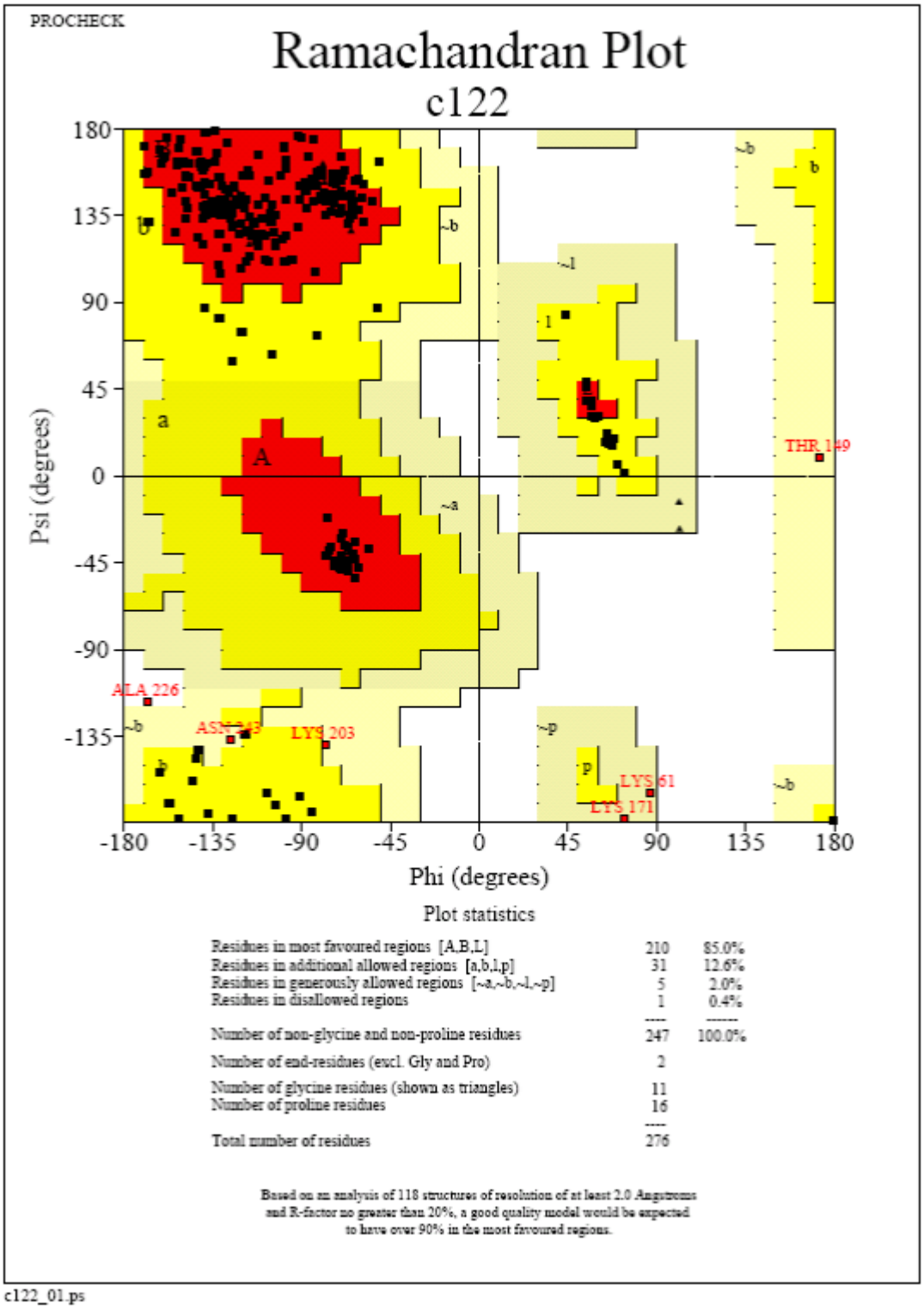
1. DNA was extracted from blood sample 100 µl mixed with denature solution 500 µl by shaken to 5 – 10 minutes.
2. Add chloroform 150 µl and DNA phenol (pH 7.9) 150 µl (chloroform : phenol = 1:1), shaken for 10 minutes.
3. Centrifuge the mixture at 13,000 rpm for 5 minutes to separate the phases.
4. Collected the supernatant for 550 – 600 µl to the clean microtube (1.5 ml), carefully avoiding protein at the aqueous phenol interface at the last collecting.
5. Repeated the same protocol to clean the supernatant (step 2 – 4). In the second time, collected 400 µl of the supernatant and transfer to new microtube (1.5 ml).
6. Precipitated DNA by adding 1,000 µl (1 ml) of absolute ethanol (99.99%), invert gentally upside down and keep in -80°c for 30 minutes or -20°c for overnight.
7. Centrifuge at 13,000 rpm for 10 minutes. Remove the supernatant carefully.
8. To wash the DNA pallet with 75% ethanol. Centrifuge at 13,000 rpm for 5 minutes. Decant the supernatant, and dry the pallet by air.

Appendix C

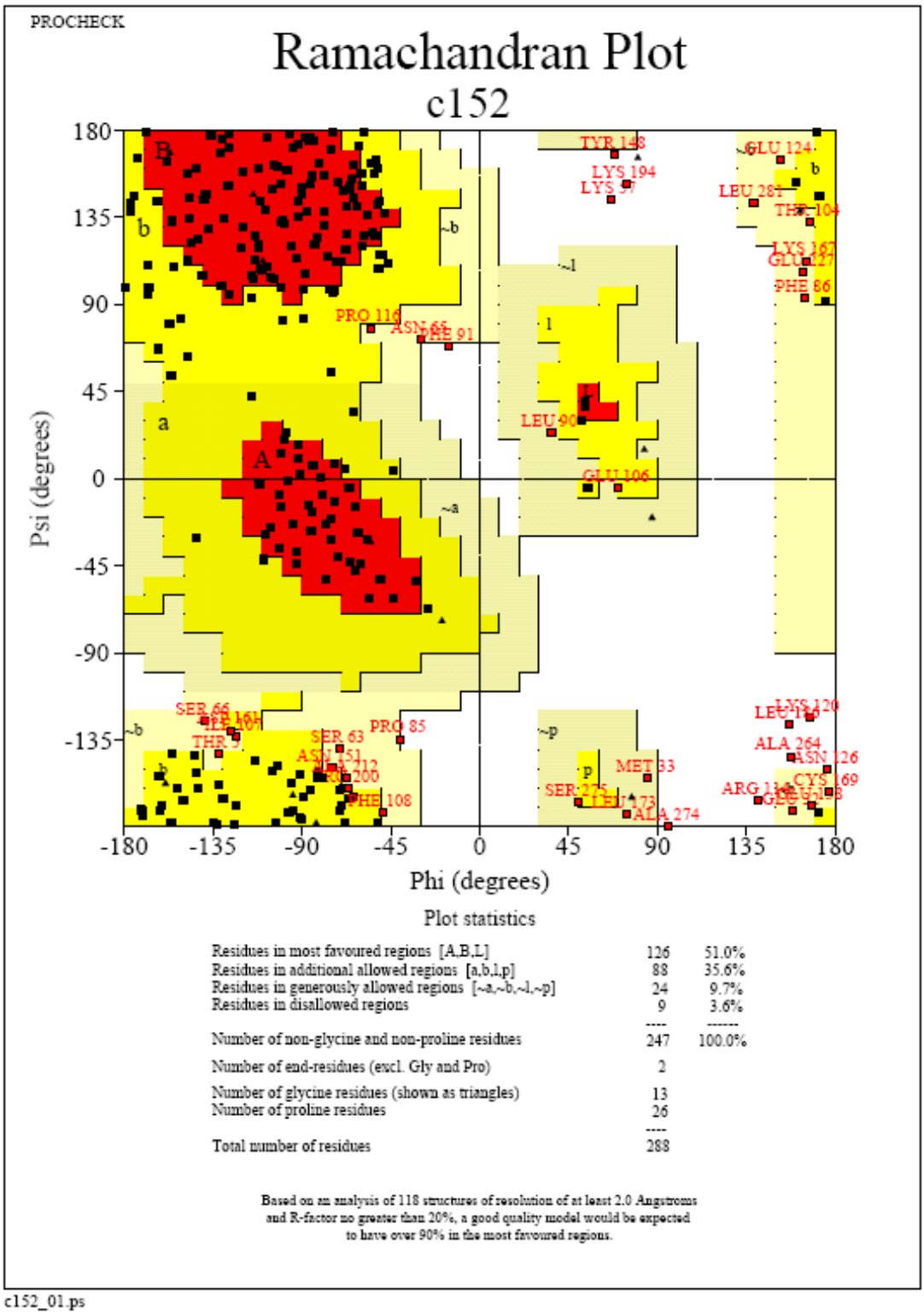
Ramachandran plot of MSA 2b protein from N49, C122 and C159



Appendix Figure C1 Ramachandorn plot of sample N. 49



Appendix Figure C2 Ramachandorn plot of sample C.122



c152_01.ps

Appendix Figure C2 Ramachandorn plot of sample C.152

CIRRICULUM VITAE

NAME : Miss Pacharathon Simking

BIRTH DATE : April 28, 1980

BIRTH PLACE : Bangkok, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE
2004	Faculty of Veterinary Medicine, Kasetsart University.	D.V.M.
2008	the Graduated School, Kasetsart University	Master of Science Veterinary Parasitology
2009 to present	Central of Agricultural Biotechonology, Kasetsart University	a Ph.D. student in Agricultural Biotechnology program

POSITION/TITLE : -

WORK PLACE : Faculty of Veterinary Medicine, Kasetsart University

SCHOLARSHIP/AWARDS : -

Training Experience

2 June 2012 – 14 September 2012: Training in "Molecular and Cell Culture technique of *Babesia spp*." at National Research Center for Protozoan Disease, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (NRU support)

Presentation

2005: **The 43th Kasetsart University Annual Conference**. "Prevalence of Heartworm Infections of Stray Dogs and Cats in Bangkok Metropolitan Areas". Sathaporn Jittapalapong, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Burin Nimsupan, Sinsamut Saengow, Pacharathon Simking and Gun Kaewmongkol.

2009: **The 47th Kasetsart University Annual Conference**. "Humoral Immune Response of Dairy Cows Immunized by Anti – tick Vaccine against *Rhipicephalus microplus* (KU – VAC1)". Sathaporn Jittapalapong, Chanya Kengradomkit, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Paitoon Kaewhom, Sinsamut Saengow, Pacharathon Simking, Amnuay Thamlangka, and Nachai Sarataphan.

2012: **The 50th Kasetsart University Annual Conference**: "Identification of Babesia spp. in dairy cows of Central Thailand by PCR". Patcharathorn Simking, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Pipat Arunvipas and Sathaporn Jittapalapong

2012: **The First Regional STVM 2012 Conference**: A change in Global Environment, Biodiversity, Disease, and Health. "Molecular detection of *Babesia spp*. infections of dairy cows in Thailand" Patcharathorn Simking, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Pipat Arunvipas, Wanut Sricharern, Sirichai Wongnarkpet, and Sathaporn Jittapalapong

2012: **The First Regional STVM 2012 Conference**: A change in Global Environment, Biodiversity, Disease, and Health. "Molecular prevalence of

Anaplasma spp. infection of dairy cows in Thailand". Natiya Saetiew, Pacharathon Simking, Sinsamut Saengow, Pipat Arunvipas, Sirichai Wongnarkpet, Nachai Sarataphan and Sathaporn Jittapalapong

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2012: **The 5th AG-BIO/PERDO Graduate Conference on Agricultural Biotechnology & KU-UT Joint Seminar II**. The molecular prevalence and genetic diversity of *Babesia bovis* infection of Thai dairy cattle using MSA2b gene. Patcharathorn Simking and Sathaporn Jittapalapong

2013: **The 51th Kasetsart University Annual Conference**: "Molecular detection of *Babesia bigemina* in Dairy cattle of Thailand by using rap 1 α gene". Patcharathorn Simkin, Sinsamut Saengow, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Pipat Arunvipas and Sathaporn Jittapalapong

2014: **The 52th Kasetsart University Annual Conference**: "Prevalence and factors associated with *Babesia spp*. infections of cattle raised nearby forest in Salakpra Wildlife Sanctuary, Kanchanaburi province". Patcharathorn Simking, Nantawan Yatbantoong, Nantiya Saetiew, Sinsamut Saengow, Nongnuch Pinyopanuwat, Wissanuwat Chimnoi, Rattanawat Chaiyarat and Sathaporn Jittapalapong

Publication

Jittapalapong, S., Pinyopanuwat, N., Chimnoi, W., Nimsupan, B., Saengow, S., **Simking, P**. and Kaewmongkol, G. 2005. Prevalence of Heartworm Infections of Stray Dogs and Cats in Bangkok Metropolitan Areas. Proceedings of 43th Kasetsart University Annual Conference (Feb 1-4, 2005), 97-105

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