Risk Area Mapping for Rickettsioses and Borreliosis in Eastern of Thailand Using Molecular Techniques and Geographic Information System

การสร้างแผนที่เสี่ยงต่อโรคไข้ริคเค็ทเซีย และโรคไข้ข้ออักเสบไลม์ ในบริเวณภาคตะวันออกของประเทศไทย โดยใช้เทคนิคอณูชีวโมเลกุลร่วมกับเทคโนโลยีภูมิสารสนเทศ

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ABSTRACT

Arthropod-borne diseases are difficult to prevent and control due to variety factors involving disease transmission. Gaining insight of specific arthropod vector species harboring specific strains of pathogens in particular area, will allow public health stakeholders to adopt effective measures for disease prevention and control. This study aims to survey for the presence of *Rickettsia* and *Borrelia* pathogens in blood sucking arthropods in the Eastern region of Thailand and to subsequently generate risk area maps. Total 421 pools of arthropods were collected from pets, poultry, livestock and rodents in 166 locations of 7 provinces in Eastern Thailand. Using real-time PCR, *Rickettsial* 17 kDa and *Borrelia* 23S rRNA were detected in 187/421 (44.4%) and 1/421 (0.2%), respectively. Risk areas of rickettsioses and borreliosis were mapped using Geographic Information System. Findings of potential rickettsia vectors in pet's ectoparasites raised concern of high risk infection for owners, thus, clearance of pet ectoparasite is recommended.

บทคัดย่อ

การป้องกันควบคุมโรคติดต่อนำโดยแมลงขาปล้องดูดเลือดพาหะทำได้ยาก เนื่องจากมีปัจจัยหลากหลายที่เกี่ยวข้องกับกลไกการติดเชื้อ ข้อมูลจำเพาะของชนิดสายพันธุ์แมลงพาหะที่นำเชื้อก่อโรคแต่ละสายพันธุ์มีความสำคัญและจำเป็นในการวางมาตรการป้องกันควบคุมโรคที่มีประสิทธิภาพจำเพาะในแต่ละพื้นที่ การศึกษานี้มีวัตถุประสงค์เพื่อสำรวจหาเชื้อก่อโรคไข้ริคเค็ทเซีย และไข้ข้ออักเสบไลม์ในแมลงขาปล้องดูดเลือด พื้นที่ภาคตะวันออกของไทย และสร้างแผนที่เสี่ยงต่อโรค ผลการสำรวจพื้นที่ 166 แห่งใน 7 จังหวัดภาคตะวันออก เก็บตัวอย่างเห็บ หมัด เหาได้ 421 กลุ่มตัวอย่าง จากสัตว์เลี้ยง สัตว์ปีก ปศุสัตว์ และสัตว์ฟันแทะ การตรวจด้วยวิธี real-time PCR พบยีน 17 kDa ของเชื้อริคเค็ทเซีย ร้อยละ 44.4 (187/421) และยีน 23S rRNA ของเชื้อโบริเลีย ร้อยละ 0.2 (1/421) ผลการสำรวจนำเสนอในรูปแผนที่พื้นที่เสี่ยงต่อโรคโดยใช้เทคโนโลยีภูมิสารสนเทศ การพบพาหะนำโรคไข้ริคเค็ทเซียบนตัวสัตว์เลี้ยงที่ใกล้ชิดมนุษย์ เป็นความเสี่ยงที่จะติดเชื้อ ควรรณรงค์ให้มีการกำจัดปาราสิตภายนอกของสัตว์เลี้ยงที่อาจเป็นพาหะ เพื่อตัดวงจรของการเกิดโรค

Key Words: Arthropod-borne diseases, *Rickettsia, Borrelia*
คำสำคัญ: โรคติดเชื้อนำโดยแมลงขาข้อ เชื้อริคเค็ทเซีย เชื้อโบริเลีย

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Introduction

Rickettsioses and Borreliosis are caused the public health problem in worldwide (Azad *et al*., 1998). Rickettsioses and Borreliosis are emerging infectious diseases associated with blood sucking arthropods such as ticks, mites, lice and fleas (Raoult *et al*., 1997 and Steere *et al*., 2004). Rickettsial diseases vary in clinical severity according to the causative agents and hosts. Moreover, some species such as *Rickettsia rickettsii* and *R. prowazekii* are more severe diseases together with killing infected patients by multiple-organ dysfunction syndromes leading to a fatal outcome (Raoult, 2013). These diseases are sufficiently treated with the effectively antimicrobial medication, but they are often under reported due to diagnostic difficulties and misdiagnosis to viral infections (Kelly *et al*., 2002). Human rickettsioses are mainly occurred in Thailand including flea-borne murine typhus and mite-borne scrub typhus (Parola *et al*., 2003; Thitivichianlert *et al*., 2009).

Borreliosis is the most common arthropod-borne disease in the United States and Europe. Lyme disease and relapsing fever are caused by *Borrelia burgdorferi* sensu lato (s.l.) which is transmitted by the *Ixodes* ticks and Ornithodoros ticks, respectively (Steere *et al*., 2004; Babour, 2011). Borreliosis has not been reported in Thailand although; both tick genera are represented in this region (Ahantarig *et al*., 2008). Especially, the eastern region of Thailand is a unique area which covers the wide ranges of geographical terrains from lively cities to urbanize and variety of agricultural fields from mountains to ocean. Interestingly, it is the closest border area to the capital city Bangkok.

Importantly, arthropod-borne diseases are difficult to prevent and control due to variety factors involving disease transmission, animal reservoirs, arthropod vector competence, and host-pathogen interaction. Gaining insight of specific arthropod vector species as well as specific strains of pathogens they harbor in particular area, will allow public health stakeholders to adopt effective measures for disease prevention and control. However, there is only a handful of this information available to date in Thailand. Therefore, an effective survey to gain information of specific vectors together with their harboring pathogens in particular area would be essential.

In recent year, the advent of molecular techniques has resulted in accurate identification of arthropod-borne pathogens and diagnosis of the diseases they cause (Raoult *et al*., 1997). Real-time polymerase chain reaction has widely used because it is rapid, accurate, highly sensitive and can be successfully used with both clinical as well as environmental samples. The technique can greatly enhance our understanding of the epidemiology for these arthropod-borne diseases (Richards, A.L. 2012).

Objectives of the study

1. To survey for *Rickettsia* and *Borrelia* species in blood sucking arthropods; ticks, and fleas collected from vertebrate hosts living in the eastern region of Thailand.
2. To generate the geographic risk area map using Geographic Information System.

Materials and Methods

Study sites

Study site includes seven provinces in the Eastern region of Thailand, Prachinburi, Sa Kaeo, Chachoengsao, Chonburi, Rayong, Chanthaburi, and

Trat. At least 25 points of different terrains such as rural area, urban area, rice field, garden, forest, mountain, river in each province were randomly surveyed for blood sucking arthropods as ectoparasites of vertebrate hosts.

Arthropod specimen collecting

In each survey location, tentative vertebrate hosts such as dogs, cats, cattle, rodents were examined for ectoparasites, ticks, lice and fleas and manually collected. Arthropod specimens were stored in 70% Ethanol at room temperature until processing. GPS latitude and longitude data of specimen collecting site were recorded using GarmineTrex® 30 GPS handheld Device (Garmin, USA).

Arthropod specimen processing

Arthropods was surface decontaminated by immersion in 10% clorox with Tween80, 0.5% benzalkonium chloride, 70%ethanol,final rinsed in sterile distilled water and stored in 70% ethanol. Species identificationwas performed according to classification keys under stereomicroscope (Nikon: SMZ745T, USA). Identified specimens were grouped 1-5/pool, and assigned coding based upon study site, host and species. Coded arthropod pools were preserved into 70% ethanol and kept at 20ºC until used.

DNA extraction

Arthropod genomic DNA was purified using PureLink™ Genomic DNA Kits (Invitrogen, Carlsbad, CA, USA). In brief, arthropods were lysed by Genomic Digestion Buffer, homogenized with clean sterile pestleandincubated at 55°C for 1-4 hours with 20 µL Proteinase K. DNA was precipitated from cell lysate with absoluteethanol and purified by a PureLink™ column. Purified DNA was then eluted from column using 60 µL eluting buffer and stored at -20°C.

Real-time PCR assay

Arthropod DNA was tested for arthropod-borne pathogens, *Rickettsia* and *Borrelia* using real-time PCR according to previous publications (Wright *et al.*, 2011; Courtney *et al.*, 2004). Oligonucleotide probes and primers used are specifically designed for *Rickettsia* 17 kDa genus specific gene and *B. burgdorferi* 23S rRNA. Sequences of primers and probes used as well as product size are listed in Table 1. Real time PCR reaction was performed in 25 µL reaction volume containing 1x PCR buffer (Thermo scientific, EU Lithuania), 0.2 mM of each primer, 0.3 µM probe, 5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.75U/µL of Taq DNA Polymerase (Thermo scientific, EU Lithuania) and 2 µL of arthropod DNA template. Thermocycler conditions was carried out in CFX96™ real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) as followed; 50ºC for 3 minutes, 95ºC for 2 minutes, and 45 cycles of 95ºC for 15 seconds and 60 ºC for 30 seconds. Control panel included *R. honei* TT-118 gDNA as *Rickettsia* positive, JuJp6 plasmid DNA (provided by The Navy Medical Research Center, Silver Spring, MD, USA) as *B. burgdorferi* control, and gDNA of ISE6 tick cell as a negative control.

Results and Discussion

From August 2013 to April 2014, 166 surveyed locations of 7 provinces in eastern region of Thailand including variety terrains were successfully obtained blood sucking arthropod specimens as ectoparasites of dogs, cats, wild boars, cows, chickens, buffalo, and rodents (Figure 1). A total 421 pools of arthropod specimens composed of 217 tick, 158 flea and 46 louse pools were collected. In Trat province, 69 arthropod pools comprised of 25 tick, 37 flea and 7 louse pools were collected from 23 survey

locations.Total of 66 pools including 33 tick, 30 flea and 3 louse pools were obtained from 16 locations of Chanthaburi province. In Prachinburi province, 33 tick, 15 flea and 10 louse pools were found from 25 survey locations.Total 57 arthropod composed of 32 tick, 21 flea and 4 louse pools were collected from 26 survey locations of Sa Kaeo. Of 22 survey locations in Chachoengsao province, 52 arthropod pools (31 tick, 16 flea and 5 louse pools) were obtained. Total 55 arthropods pools (32 tick, 19 flea, 4 louse) were received from 31 survey locations in Chonburin province. Of 23 surveyed locations in Rayong, 31 tick, 20 flea and 13 louse pools were obtained. Information of arthropod specimens collected in this study was summarized in Figure 2.

Arthropods were morphological identification under stereomicroscope according to taxonomic keys. However, there were some difficulties hampering species identification such as specimen damage in important parts. Tick samples belonging to five different genera; *Rhipicephalus, Boophilus, Dermacentor, Haemaphysalis* and *Ixodes* were collected from dogs, cats, cows, wild boars and rodents.

Collected tick species included *Rhipicephalus sanguineus* (Brown dog tick), *R. haemaphysaloides* and *Boophilus microplus* (Cow tick). The highest tick population collected in this study was *Rhipicephalus sanguineus*, 89.9% (195/217 pools) of total tick specimens collected (Table 2 and Figure 2). Collected flea specimens composed of *Ctenocephalides felis orientis* (Cat flea), *C. felis felis* (Cat flea) and *Echidnophaga gallinacean* (Chicken flea) were collected from dogs, cats and chicken. The cat flea, *C. felis orientis* was the majority species (93%) found as dog ectoparasite (Table 2 and Figure 2). Six species of lice were collected, *Heterodoxus spinigerum* (Dog louse), *Trichodectes canis* (Canine chewing louse), *Haematopinus eurysternus* (Shortnosed cattle louse), *Chelopistes meleagridis* (Large turkey louse), *Lipeurus caponis* (Poultry wing louse) and *Menopon gallinae* (Bird shaft louse). The dog louse, *Heterodoxus spinigerum* was the highest species found on dogs (87%) (Table 2 and Figure 2). The spatial distribution of arthropods species collected in this study was demonstrated in Figure 3. Dogs were the majority of vertebrate hosts of collected blood sucking arthropods, ticks, lice and fleas (Table 2).

Table 1 Primers and probes used for real-time PCR testing of arthropod-borne pathogens, *Rickettsia* and *Borrelia*.

Name	Gene	Oligonucleotide Sequence (5'-3')	Fragment	Reference
R17K127F2	17-kDa	GGGCGGTATGAAYAAACAAG	111	Wright et al., 2011
R17K238R		CCTACACCTACTCCVACAAG		
R17K202P		FAM-CCGAATTGAGAACCAAGTAATGC-TAMRA		
Bb23Sf	23s rRNA	CGAGTCTTAAAAGGGCGATTTAGT	75	Courtney et al., 2004
Bb23Sr		GCTTCAGCCTGGCCATAAATAG		
Bb23Sp-FAM		6FAM-AGATGTGGTAGACCCGAAGCCGAGTG-TAMRA		

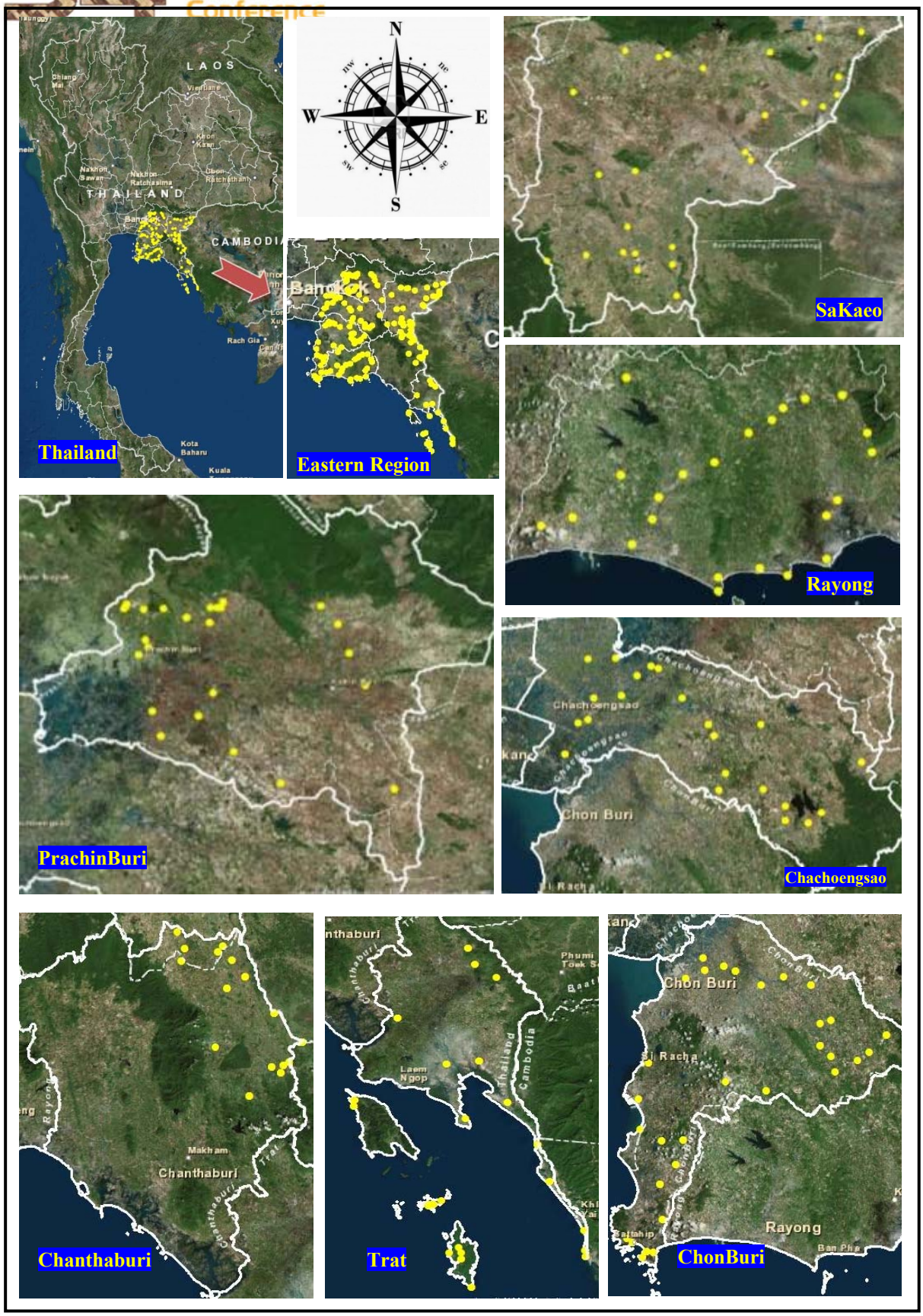

Figure 1 Spatial distribution of arthropods' collecting sites (yellow dots) in the Eastern Thailand during August 2013 to April 2014.

Table 2 Summary information of arthropod specimens collected in the Eastern Thailand during August 2008 to April 2014 and laboratory results to evaluate for rickettsia 17 kDa and borrelia 23S rRNA genes using real-time PCR.

Province	Arthropod species	Host	No. of Pools	Number of positive (%)	
				17kDa	23S rRNA
Chonburi	*Ctenocephalides felis felis*	Dog	1	0	0
	Ctenocephalides felis felis	Cat	1	0	0
	Ctenocephalides felis orientis	Dog	17	14	0
	Heterodoxus spinigerum	Dog	4	1	0
	Rhipicephalus sanguineus	Dog	32	3	0
	Total		**55**	**18 (32.7)**	**0**
Prachinburi	*Boophilus microplus*	Cow	1	0	0
	Ctenocephalides felis orientis	Dog	15	13	0
	Heterodoxus spinigerum	Dog	10	7	0
	Rhipicephalus sanguineus	Dog	31	13	0
	Rhipicephalus sanguineus	Cat	1	0	0
	Total		**58**	**33 (56.9)**	**0**
Rayong	*Boophilus microplus*	Dog	1	0	0
	Ctenocephalides felis felis	Cat	2	0	0
	Ctenocephalides felis orientis	Cat	3	1	0
	Ctenocephalides felis orientis	Dog	15	5	0
	Heterodoxus spinigerum	Dog	13	6	0
	Rhipicephalus sanguineus	Dog	29	11	0
	Rhipicephalus sanguineus	Cat	1	1	0
	Total		**64**	**24 (37.5)**	**0**
Sa kaeo	*Boophilus microplus*	Cow	3	0	0
	Boophilus microplus	Dog	1	0	0
	Ctenocephalides felis orientis	Dog	20	19	0
	Echidnophaga gallinacea	Dog	1	1	0
	Haematopinus eurysternus	Buffalo	1	0	0
	Heterodoxus spinigerum	Dog	3	1	0
	Rhipicephalus haemaphysaloides	Dog	1	0	0
	Rhipicephalus sanguineus	Dog	27	7	0
	Total		**57**	**28 (49.1)**	**0**

Table 2 Summary information of arthropod specimens collected in the Eastern Thailand during August 2013 to April 2014 and laboratory results to evaluate for rickettsia 17 kDa and borrelia 23S rRNA genes using real-time PCR. (Cont.)

Province	Arthropod species	Host	No. of Pools	Number of positive (%)	
				17kDa	23S rRNA
Trat	*Ctenocephalides felis orientis*	Dog	37	36	0
	Dermacentor spp.	Dog	2	0	0
	Haemaphysalis spp.	Dog	1	1	0
	Heterodoxus spinigerum	Dog	6	0	0
	Menopon gallinae	Chicken	1	0	0
	Rhipicephalus haemaphysaloides	Dog	1	0	0
	Rhipicephalus sanguineus	Dog	21	1	0
	Total		**69**	**38 (55.1)**	**0**
Chanthaburi	*Chelopistes meleagridis*	Dog	1	0	0
	Ctenocephalides felis felis	Dog	2	0	0
	Ctenocephalides felis orientis	Dog	27	24	0
	Dermacentor spp.	Wild boar	2	1	0
	Dermacentor spp.	Dog	1	0	0
	Echidnophaga gallinacea	Chicken	1	0	0
	Haemaphysalis spp.	Dog	2	0	0
	Heterodoxus spinigerum	Dog	1	0	0
	Ixodes spp.	Rat	1	0	1
	Lipeurus caponis	Dog	1	0	0
	Rhipicephalus haemaphysaloides	Dog	3	0	0
	Rhipicephalus sanguineus	Dog	24	6	0
	Total		**66**	**31 (46.9)**	**1(1.5)**
Chachoengsao	*Ctenocephalides felis orientis*	Dog	16	15	0
	Heterodoxus spinigerum	Dog	3	0	0
	Rhipicephalus sanguineus	Dog	31	0	0
	Trichodectes canis	Dog	2	0	0
	Total		**52**	**15 (28.8)**	**0**
	Grand Total		**421**	**187 (44.4)**	**1 (0.2)**

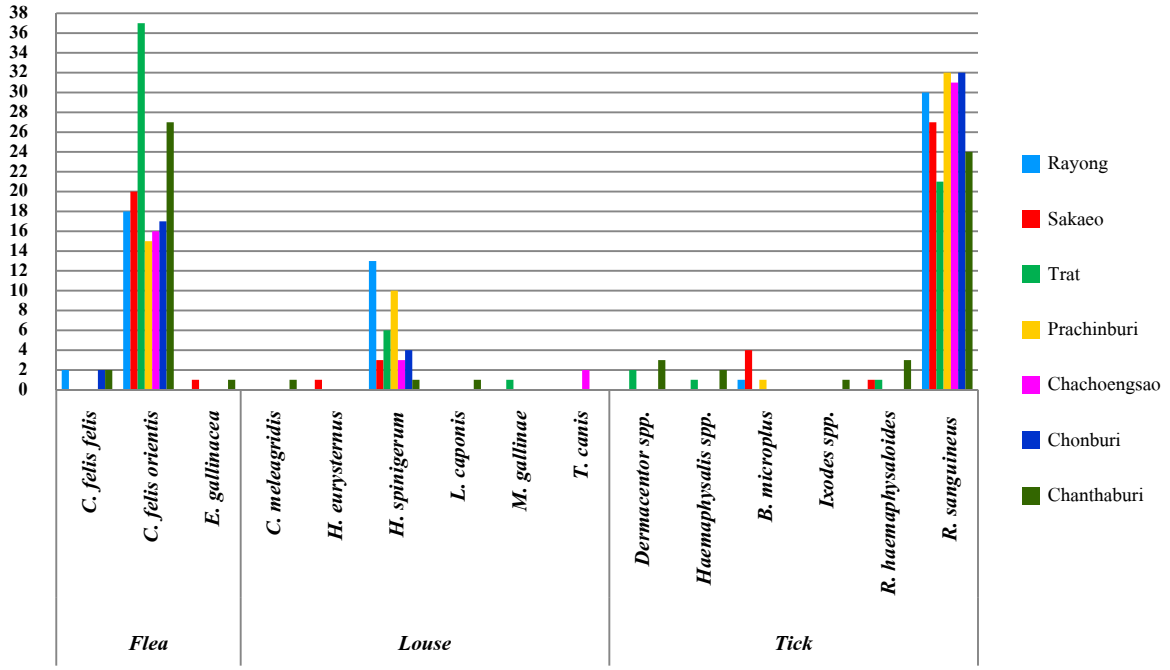

Figure 2 Arthropod species collected from the Eastern Thailand during August 2013 to April 2014.

In Thailand, there were several survey studies of blood sucking arthropods, ticks lice and fleas. Sarataphan and others have surveyed for ticks as ectoparasite of cattle and buffaloes in 25 provinces of Thailand (Sarataphan *et. al*., 1998). The cattle tick, *Rhipicephalus (Boophilus) microplus*, was dominantly found with an extensive distribution. Likewise, a high percentage of cattle were parasitized by this tick species in our survey. Four out of total 5 pools of ticks found on cow were *Boophilus microplus* (Table 2). Sangvaranond and Colleagues have surveyed ectoparasites of domesticated dogs and cats in 19 provinces of Thailand and found that the majority of blood sucking arthropods commonly found as ectoparasites on domestic dogs were *Rhipicephalus sanguineus* (Brown dog tick) and *Heterodoxus spiniger* (Dog louse) (Sangvaranond *et.al.*, 1990a). This finding is similar to our survey information (Table 2 and Figure 2). Other reports from a survey by Beaucournu *et. al.* (2001) in Lao PDR and a survey in Tak province, located along the Thai-Myanmar border by Changbunjong and colleagues also demonstrated the similar results (Beaucournu *et. al.*, 2001; Changbunjong, *et.al*., 2009).

Of the two flea species, *C. felis orientis* was the highest prevalence found only on domestic dogs and *C. felis felis* was confined to cats (Table 2). The survey of flea in 15 Thai provinces by Sangvaranond *et.al.* reported in 1990 that *C. felis orientis*, *C. felis felis* and *C. canis* were found on dogs and most ectoparasitic fleas on cats were *C. felis felis* and *C. felis orientis* (Sangvaranond *et.al.*, 1990b). Beaucournu *et. al.* reported in 2001 that *C. felis felis* and *C. felis orientis* were found on dogs in neighboring LaoPDR

(Beaucournu *et. al*., 2001). Others lice species found were *Echidnophaga gallinacea* (sticktight flea) and *Menopon gallinae*. These fleas are primarily a pest of domestic poultry, but may also parasitize cats, dogs, rabbits and humans (Wall and Shearer, 1997). *Rhipicephalis sangunieus*, like its name (brown dog tick) was found only on dogs.

Using Real Time PCR, Rickettsial 17 kDa and *Borrelia* 23S rRNA was detected in 187/421 (44.4%) and 1/421 (0.2%) of collected arthropod specimens, respectively. Ticks 217 pools of 5 genera were positive for *Rickettsia* 20.3%, and *Borrelia* 0.5%. This is the first report for the existing evidence of *Borrelia* spp. in tick in Thailand. Fleas 158 pools of 2 genera were positive only for *rickettsia* 81.0%. Lice 46 pools of 6 genera were positive for only *rickettsia* 32.6% (Table 2). There were several reports of arthropod borne pathogens survey in Thailand. Rickettsia DNA belong to spotted fever group were detected in 30% (9/30) *Amblyoma testudinarium* ticks collected from Khao Yai National Park, Nakorn nayok Province, adjacent to Prageneburi Province, eastern border province in this survey and 16.8% (16/95) *Haemaphysalis ornithophila* ticks collected from Khao Yai National Park, Nakorn nayok Province and Khao Ang Rue Nai Wildlife Sanctuary, Chachoengsao Province (Hirunkanokpun, *et.al.,* 2003). The pathogen, *Rickettsia felis* like species was detected in 67.4% (66/98 clones) of fleas collected from dogs in Bangkok during 2006-2007. Fleas collected from cats (54 pools) and 304 *Rhipicephalis sangunieus* tick pools collected from dogs were all negative for rickettsia (Foongladda, *et.al.*, 2011). To better coordinating geographical survey location with arthropod species data and laboratory results, Geographic Information System (GIS) technology has been used. Survey data was summarized and illustrated as risk area maps (Figure 4-5). These risk maps are easy to understand and convenient to use as public health application. However, the relevant of geographical location and arthropod vectors harboring pathogens remained to be further analyzed.

In summary, our finding demonstrated that arthropods collected from pet dogs are the potential vector for rickettsia, pathogen of rickettsioses. It raised concern of high risk infection for owners, thus, clearance of pet ectoparasite is recommended. The other interest survey result was the detection of *Borrelia* DNA in rodent tick. This result also raised concern of Lyme disease vector existing along Thai-Cambodia border. Public Health and medical awareness of this emerging disease is encouraged. Findings from this study are crucial to establish an effective disease prevention and control strategy for arthropod-borne diseases, rickettsioses and borreliosis.

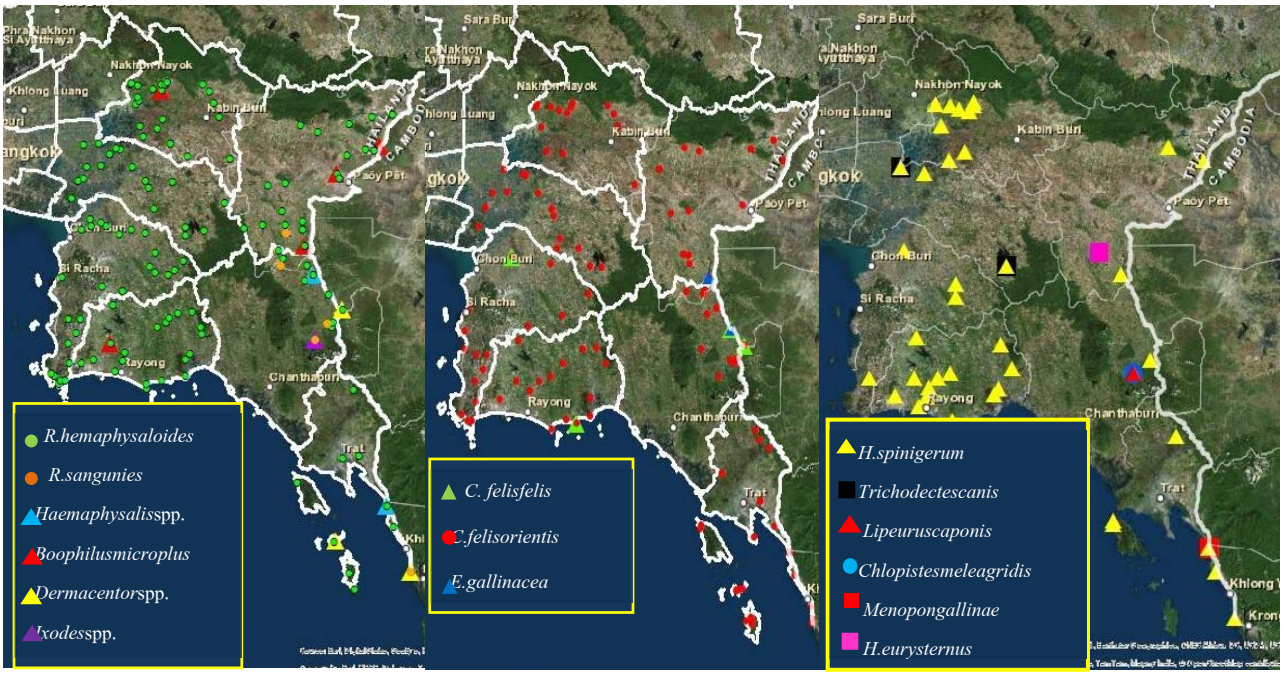

Figure 3 GIS mapping demonstrated the distribution of arthropod species collected from the Eastern Thailand during August 2013 to April 2014.

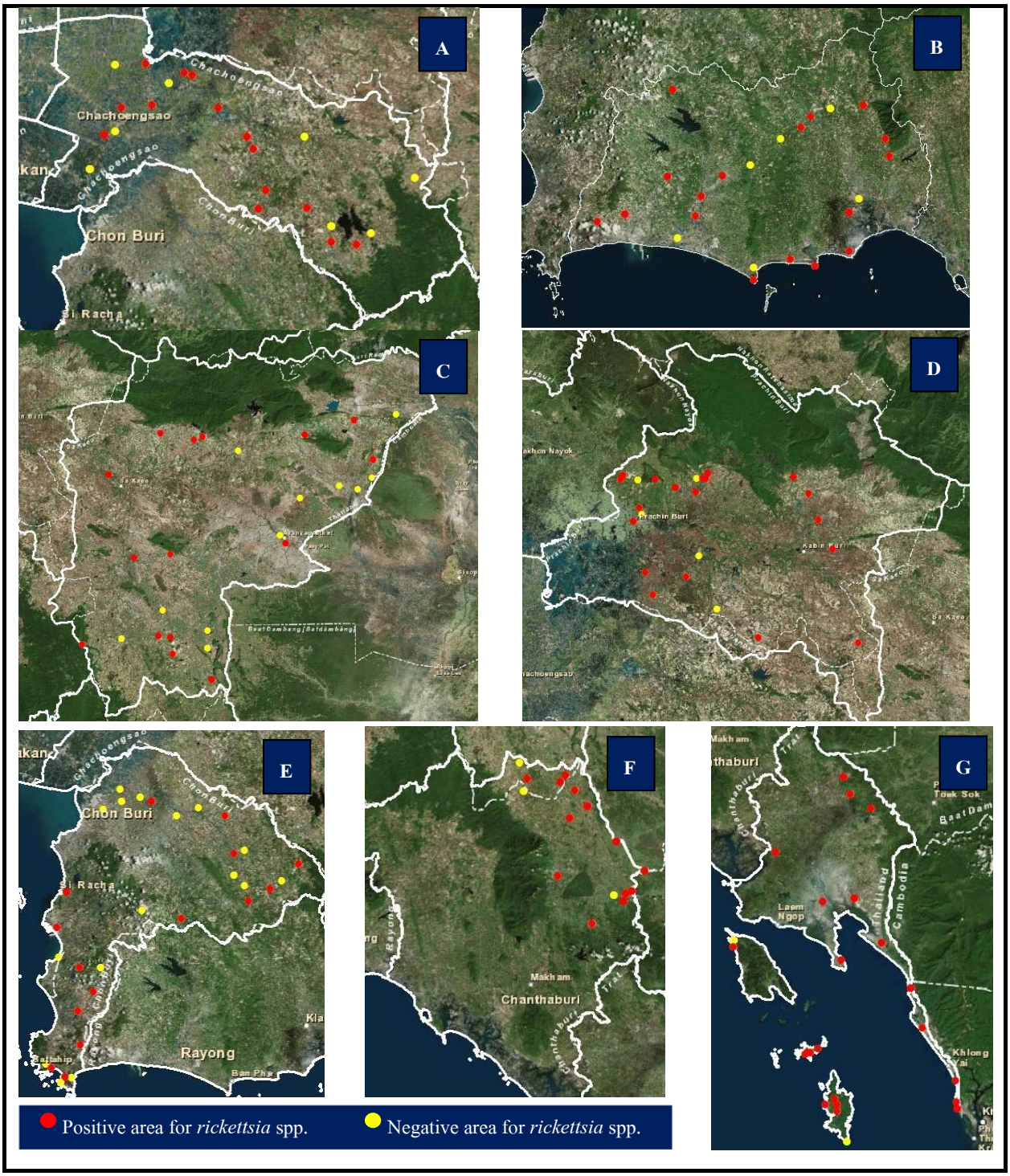

Figure 4 GIS mapping demonstrated risk areas for rickettsioses (red dot) in 7 provinces of the Eastern Thailand. Yellow dots represented surveyed locations. A: Chachoengsao 14 areas (red dot) out of 22 survey locations (yellow dot), B: Rayong 17 areas (red dot) out of 23 survey locations (yellow dot), C: Sa kaeo 15 areas (red dot) out of 26 survey locations (yellow dot), D: Prachinburi 20 areas (red dot) out of 25 survey locations (yellow dot), E: Chonburi 15 areas (red dot) out of 31 survey locations (yellow dot), F:Chantaburi 13 areas (red dot) out of 16 survey locations (yellow dot), G: Trat 21 areas (red dot) out of 23 survey locations (yellow dot).

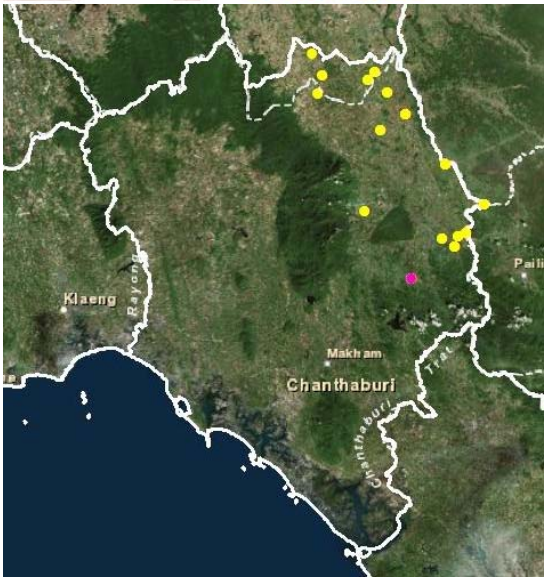

Figure 5 GIS mapping of Chantaburi province demonstrated risk area for Borreliosis (pink dot) from 16 surveyed locations (yellow dot).

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