Surveillance of Coronaviruses in fruit bats (*Pteropus lylei*) of Thailand

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บทคัดย่อ

ไวรัสโคโรนาเป็นไวรัสประเภทที่มีสาย RNA แบบเดี่ยวและมีเปลือกหุ้ม สามารถก่อโรคที่ระบบทางเดินอาหารและทางเดินหายใจในสัตว์เลี้ยงลูกด้วยน้ำนม สัตว์ปีกและมนุษย์ ค้างคาวเป็นแหล่งรังโรคของเชื้อไวรัสโคโรนาอีกทั้งยังมีถิ่นอาศัยอยู่ทั่วประเทศไทย การศึกษานี้เก็บตัวอย่างจากฝูงค้างคาวในจังหวัดพระนครศรีอยุธยาและสระบุรี ตั้งแต่เดือนมีนาคม พ.ศ. 2561 จนถึงเดือนกุมภาพันธ์ พ.ศ. 2562 จำนวนตัวอย่างทั้งหมด 1,487 ตัวอย่างถูกเก็บจากค้างคาวทั้งสิ้น 730 การระบุชนิดของค้างคาวโดยการดูจากลักษณะภายนอกและการวัดความยาวของค้างคาวร่วมกับวิธีการตรวจตรวจหายีน cytochrome B พบว่าค้างคาวจากทั้ง 2 ฝูงคือค้างคาวแม่ไก่ภาคกลาง (*Pteropus lylei*) การตรวจหาเชื้อไวรัสโคโรนามีตัวอย่างที่พบเชื้อไวรัสร้อยละ 4.30 จากวิธีการ phylogenetic analysis ผลการวิเคราะห์พบว่าตัวอย่างไวรัสโคโรนาในการศึกษาครั้งนี้ถูกจัดอยู่ในสกุล Betacoronavirus lineage D ซึ่งมีความคล้ายคลึงกับไวรัสโคโรนาที่เคยถูกรายงานในค้างคาวแม่ไก่ภาคกลางในจังหวัดชลบุรีแต่ถูกจัดอยู่ในคนละประเภทกับเชื้อไวรัสโรโคนา MERS ไวรัสโคโรนา SARS และไวรัสโคโรนา SARS-2 หรือ COVID-19 ผลการวิเคราะห์ทางพันธุกรรมของไวรัสโคโรนาพบว่ามีความใกล้เคียงกับไวรัสโคโรนาในค้างคาวจากจังหวัดชลบุรีถึงร้อยละ 98 - 100 ในทางกลับกันเมื่อเปรียบเทียบกับไวรัสโคโรนากลุ่มอื่นมีความคล้ายคลึงกันทางพันธุกรรมน้อยกว่าร้อยละ 70 โดยสรุปว่าเชื้อไวรัสโคโรนาที่พบในฝูงค้างคาวแม่ไก่ภาคกลางในจังหวัดพระนครศรีอยุธยาและสระบุรีนั้นมีความใกล้เคียงกันทางพันธุกรรมกับไวรัสโคโรนาที่พบในค้างคาวสายพันธุ์เดียวกันจากทางภาคตะวันออกของประเทศไทย แม้ว่าจะมีความใกล้เคียงต่ำระหว่างไวรัสโคโรนาในการศึกษานี้กับไวรัสโคโรนาที่ทำให้เกิดการระบาดทั่วโลก เชื้อไวรัสโคโรนาในค้างคาวยังควรมีการทำการสำรวจและศึกษาเป็นประจำอย่าง ต่อเนื่องจากไวรัสโคโรนาในค้างคาวนั้นมีโอกาสที่ทำให้เกิดโรคระบาดจากสัตว์สู่คนในอนาคต

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
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Abstract

Coronavirus (CoV) is an enveloped single positive-stranded RNA virus CoV can cause enteric and respiratory diseases in mammals, avian and human. Bats are the natural reservoir of CoVs and distribute all over Thailand. Sample collection was performed in two bat colonies in Ayutthaya and Saraburi provinces during March 2018 – February 2019. Total 1,487 samples were collected from 730 bats. The bat species identification via morphological measurement and cytochrome B detection showed that bats in both colonies are Lyle's flying fox (*Pteropus lylei*). The results of CoV detection showed that 4.30% of samples were positive to CoV. Phylogenetic analysis showed the bat CoV in this thesis were clustered with Thai bat CoV of BetaCoV lineage D from *Pteropus lylei* in Chonburi province but were in the different groups of MERS-CoV, SARS-CoV and SARS-CoV-2 (COVID-19). For genetic analysis, bat CoV in this thesis had 98-100% nucleotide identities to Thai Bat-CoV but had <70% identity to others reference sequences. Our results confirmed that CoVs are circulating in two bat colonies in Ayutthaya and Saraburi and the viruses are closely genetic related to bat CoV in bat in the same species from Eastern part of Thailand. Although the bat CoVs in this thesis were far related with pandemic CoVs, but routinely surveillance of CoVs in bats should be performed since bat CoV could be a potential zoonotic virus.

Field of Study: Veterinary Public Health Student's Signature .....
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Pichamon Moolthep

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Introduction

1.1 Importance and Rationale

Coronavirus is an enveloped, positive-sense single stranded RNA virus of the family *Coronaviridae*, subfamily *Coronavirinae*. The virus can cause wide variety of diseases in mammal and avian species. The symptoms of coronavirus infection are present in both enteric and/or respiratory forms. For example, porcine epidemic diarrhea (PED) and transmissible gastro-enteritis (TGE) causing severe diarrhea in pigs, canine coronavirus (CCoV) causing enteritis in dogs. feline infectious peritonitis (FIP) causing generalized peritonitis in cats. While infectious bronchitis (IB) causing tracheobronchitis in avian species. In human coronavirus (HCoV) causing flu-like symptoms. While Severe Acute Respiratory Syndrome (SARS). Middle East Respiratory Syndrome (MERS) and SARS-CoV-2 causing severe respiratory diseases in human. The transmission routes of coronaviruses are aerosol and fecal-oral routes (Masters, 2006). The viruses could transmit across host species (Baric et al., 1997),(Fehr and Perlman, 2015).

In human, coronaviruses infection (HCoV) have been reported since 1960s (Kahn and McIntosh, 2005). HCoV infection causes mild respiratory signs including sore throat, running nose and sneezing which similar to “Influenza-like symptom”. In 2002, the first case of severe acute respiratory syndrome (SARS) caused by SARS Coronavirus (SAR-CoV) was reported in Guangdong province, southern China. The disease was spread worldwide with high morbidity and mortality, as of 2018 at least 774 deaths in 37 countries. Previous studies showed that the patients had been close contact with wild animals (wild civets) in local market in Southern China (Shi and Hu, 2008). Later, it was confirmed that SARS-like Coronavirus could be isolated from bats in China suggesting that SARS-like CoVs might have been circulating in bats, and the wild civets may acquire the virus from bats (Wang and Eaton, 2007) (Li et al., 2005).

In 2012, the emergence of coronavirus infection occurred in the Middle East. The novel coronavirus was named Middle East respiratory syndrome virus (MERS-CoV). The first case of MERS-CoV infection was identified in Saudi Arabia. Then, the viruses had spread in the middle eastern countries as well as some European and African countries (Reusken et al., 2014; Nassar et al., 2018). Previous studies showed that genome sequences of camel MERS-CoV from camel was 99.9% similar to those of human MERS-CoV suggesting camel is the reservoir of the virus (Hemida et al., 2014). Similar in SARS-CoV, MERS-CoV can be isolated from bats. The genome sequences of MERS-CoV from bats had high similarities to the human MERS-CoV suggesting MERS-CoV transmission from bats to camels and then to human (Memish et al., 2013) (Milne Price et al., 2014).

Recently, in December 2019 a group of unidentified-etiologic pneumonia patients were reported in Wuhan, Hubei province and spread across the mainland of China (Li et al., 2020). Laboratory confirmed the cause of sporadic pneumonia is a novel coronavirus known as 2019-nCoV (Zhu et al., 2020). On February 11, 2020 World Health Organization (WHO) declared this viral infection as Coronavirus Disease-2019 (COVID-19) (WHO; 2020 February, 11). Subsequently the International Committee on Taxonomy of Viruses (ICTV) named the novel coronavirus as SARS-CoV-2 (Gorbalenya et al., 2020). As of November 2020, 50 million confirmed COVID-19 case were reported with over 1.2 million deaths in 253 countries (WHO; 2020 November, 9) and the pandemic is continuing to spread. The mortality rate from COVID-19 is estimated approximately 5.7% (Baud et al., 2020). COVID-19 epidemiological information revealed that the first human cases have visited the local seafood market in Wuhan where not only sell seafood but also trades live wild animals (Jin et al., 2020). Genetic analysis of SARS-CoV-2sequences showed that the viruses were closely related to the bat CoV from bats in China (Zhou et al., 2020).

In Thailand, bats can be found in almost every parts of the country. Bats live and settle down nearby and/or in human habitats. Human can get benefits from the bats living nearby, for example bat's guano mining and bat's meat consuming (Suwannarong and Schuler, 2016) (Suwannarong et al., 2020). Thus, bat-human interface is unavoidable. Direct and indirect contact of bats and bat contaminated environment should not be ignored. In Thailand there were few reports on identification of bat coronaviruses among bat population which poses risk of zoonotic viruses from bats to humans (Wacharapluesadee et al., 2015). In this thesis, the occurrence, genetic characteristics and genetic diversities of coronaviruses isolated from bats were investigated. The results from this thesis will promote the knowledge about coronaviruses in bats of Thailand. The information obtained can be used to support the prevention and control strategies of zoonotic coronaviruses.

1.2 Research Question

What are the genetic characteristics of coronaviruses circulating in bats of Thailand?

1.3 Objectives of study

To answer the research question there were 2 objectives of this study including

1. To detect coronaviruses circulating in bat habitats in two bat colonies of Thailand
2. To determine genetic diversities of coronaviruses circulating in bats of Thailand

1.4 Hypothesis

Bats in the selected bat colonies of Thailand may harbor coronaviruses. Bats may contain Alphacoronavirus and/or Betacoronavirus, which can be potential zoonotic viruses.

Chapter 2

Literature Review

2.1 Coronaviruses

Coronavirus is an enveloped positive-sense, non-segmented, single-stranded RNA virus (Masters, 2006). Coronavirus belongs to the family *Coronaviridae*, subfamily *Coronavirinae*. The virus has a large genome with the size of approximately 27.3-31.3 kb (Masters, 2006). Coronavirus genome contains multiple Open Reading Frames (ORFs). The major ORFs are ORFs 1a and ORFs 1b which cover two-third of the viral genome. The virus composes of several structural and non-structural genes encoding structural and accessory proteins. Structural proteins of the coronaviruses are spike (S) protein, membrane (M) protein, envelope (E) protein and nucleocapsid (N) protein. Non-structural or accessory proteins are encoded by 2a, HE, 4, 5a and I ORFs at various positions (Masters, 2006).

In the total size of coronavirus approximately 30 kb, only 10 kb contains structural and necessary genes (Fehr and Perlman, 2015). Structural genes encode the structural proteins which are S, M, E and N proteins. The S protein forming in clubbed shape. All over the surface of CoVs makes them look like having spines. S protein is responsible for host receptor (Gallagher and Buchmeier, 2001). E protein, as known as envelope protein, is an integral membrane protein of CoV. E protein has several functions in the viral life cycle such as assembly, budding, envelope formation and pathogenesis (Schoeman and Fielding, 2019). M protein is the most numerous structural protein (Fehr and Perlman, 2015). It helps in virus assembly, and makes viral membrane curvature suitable to bind to neocleocapsid (Neuman et al., 2011). The last structural protein is N protein which forms complexes with viral RNA. It works with membrane during virion assembly and plays important role to promote viral transcription (McBride et al., 2014).

Coronaviruses contain four genera, *Alphacoronavirus, Betacoronavirus, Deltacoronavirus* and *Gammacoronavirus*(Woo et al., 2012). The genus *Alphacoronavirus* such as Porcine Epidemic Diarrhea Virus (PEDV), Transmissible Gastroenteritis Virus (TGEV) and Feline Peritonitis Virus (FIPV) mostly cause enteric diseases and infects mammals. *Betacoronavirus* can be divided into four groups: A, B, C and D usually causes respiratory diseases. *Betacoronavirus* group B can cause lethal diseases in human such as SAR-CoV, MERS-CoV and COVID-19 (de Groot et al., 2013) (Hu et al., 2020). Another genus is *Deltacoronavirus* that has been reported in pigs (Wang et al., 2014) The last one is *Gammacoronavirus* that has been reported in birds and marine mammals (Jackwood et al., 2012),(Woo et al., 2014) Coronavirus can infect in wide host range. The variety of sickness can cause enteric, respiratory, hepatic and neurological diseases (Woo et al., 2012). From farm animals to companion pets, wildlife to marine mammals, CoV can infect several species of avian and mammals. In pigs CoV can cause severe enteritis known as porcine epidemic diarrhea (PED) and transmissible gastro-enteritis (TGE). And recently pigs were found that could carry a novel bat-origin CoV and could be potential intermediate host for CoV from bats to human (Wang et al., 2018a). Another farm animal like cattle can be infected by CoV, too. Bovine coronavirus (BCoV) causes enteric disease not only in cattle but in small ruminant such as goats and sheep presenting sero-prevalence for BCoV in Ghana (Burimuah et al., 2020). Murine hepatitis virus (MHV) is the CoV that cause hepatitis and encephalitis is rodents (Tyrrell et al., 1978). In addition, MHV were reported causing CNS demyelination in primates (Murray et al., 1992). For companion animals, dogs can be infected from a highly pathogenic canine coronavirus (CCoV) with enteritis and diarrhea (Buonavoglia et al., 2006). Feline coronavirus (FCoV) was related in evolutionary trait with CCoV (Pratelli et al., 2003) and causes two different pathotypes. Feline enteric coronavirus (FECV) causes mild and self-limited enteritis in cats. Whereas feline infectious peritonitis virus (FIPV), the virulent mutant of FECV, can cause lethal systemic disease in cats (Chang et al., 2010). For other mammals, CoV was reported in some wildlife for example the founding of *Deltacoronavirus* in

Asian leopard cat (Woo et al., 2012) and discovery of *Gammacoronavirus* in bottlenose dolphin (Woo et al., 2014). In avian, the most relevant CoV is infectious bronchitis virus (IBV) that cause highly contagious in poultry. IBV effects to upper respiratory and reproductive system of chickens also some strain can cause nephritis (Jackwood, 2012). Furthermore, there are detection of high prevalence of CoV in wild aquatic birds (Chu et al., 2011) and some evidences in small wild birds such as Eurasian tree sparrow, gray-baked thrush and oriental magpie robin (Woo et al., 2012).

2.2 Bats and emerging zoonotic diseases

Bat is known as natural reservoir of many zoonotic diseases (Calisher et al., 2006). For example Lyssavirus is a viral zoonotic pathogen in bats, which is related to rabies virus (Hanna et al., 2000). Henipaviruses causing encephalitis in human can be isolated from the fruit bats (Halpin et al., 2000). Ebola virus causing Ebola outbreaks in Africa can be isolated from bats (Leroy et al., 2005). SARS-like coronavirus, MERS coronavirus-like virus and SARS-CoV-2 can be detected from bats (Li et al., 2005),(Woo et al., 2018),(Zhou et al., 2020). The other viruses such as Meangle and Tioman viruses (Calisher et al., 2006), Hepaciviruses and Pegiviruses are also carried by bats (Quan et al., 2013). Furthermore, bats can also harbor pathogenic bacteria, protozoa, fungi and helminths that can transmit to human (Brook and Dobson, 2015).

For coronavirus, many studies reporting that bats harbor coronaviruses. In China 2005, SARS-like virus has been identified in Chinese horseshoe bats (Lau et al., 2005). In China 2013, the novel SARS-like coronavirus in bats of China was discovered (Yang et al., 2013) (Li et al., 2005) (Hu et al., 2017). In Europe, bat coronavirus was reported in northern Germany (Gloza-Rausch et al., 2008). In north America also has reported about the discovery of coronavirus in insectivore bats (Dominguez et al., 2007). In South Africa, the close relative of human MERS-CoV was isolated from bats (Ithete et al., 2013). There are some studies showed that bat SARS-like CoV can be

potential to infect human (Menachery et al., 2015; Menachery et al., 2016; Wang et al., 2018b).

About recently outbreak of COVID-19 since late of 2019, there are some evidences showing that the genome of SARS-CoV-2 has percentage identity to SARS-CoV approximately 79% and 50% identical to MERS-CoV (Lu et al., 2020). Furthermore, SARS-CoV-2 shares 96% genomic similarity to SARS-like CoV discovered in Hoseshoe bats at whole genome level (Zhou et al., 2020) (Zhu et al., 2020). From the study showed that SARS-CoV-2 probably use the same receptor which is angiotensin-converting enzyme 2 (ACE2) as well as SARS-CoV (Zhou et al., 2020). The virus can transmit from human to human easily via aerosol and direct contact. The COVID-19 patients have common symptom similar to seasonal flu such as fever, cough, fatigue, sputum production, shortness of breath and headache. Some patients have combination symptom with gastrointestinal signs like diarrhea and vomiting (Guan et al., 2020).

2.3 Human-bat interface and coronaviruses

There is a high number of bats in Thailand and their distribution is all over the country. Bats live and forage nearby and/or in human's habitats such as temples, abandoned constructions or human's orchards. Besides the distribution of bats, human's behaviors to hunt and consume bat's flesh encourage human-bat interface. Moreover, the expansion of human habitats and urbanization in the country promote the opportunity of human-bat interface and subsequently zoonotic diseases transmission from wildlife to human. Therefore, human has high risk to be directly and/or indirectly infected with zoonotic pathogens from bats. In Thailand, there were some reports of prevalence of coronaviruses from bats. The evidences of *Alphacoronavirus* and *Betacoronavirus* in bats in Eastern part of Thailand were documented (Wacharapluesadee et al., 2015). *Betacoronavirus* lineage C had been found in bat guano fertilizer from Ratchaburi province (Wacharapluesadee et al.,

2013). Recently *Betacoronavirus* lineage D isolated from flying fox (fruit bat) was reported (Wacharapluesadee et al., 2018). Therefore, the surveillance and monitoring of coronaviruses in bats in Thailand should be routinely conducted.

Chapter 3

Materials and Methods

This thesis consists of 3 phases including: Phase 1. Sample collection from bats; Phase 2. Bat species identification and coronavirus identification; Phase 3. Genetic characterization of coronaviruses in bats. The conceptual framework of this study is shown in Figure 1

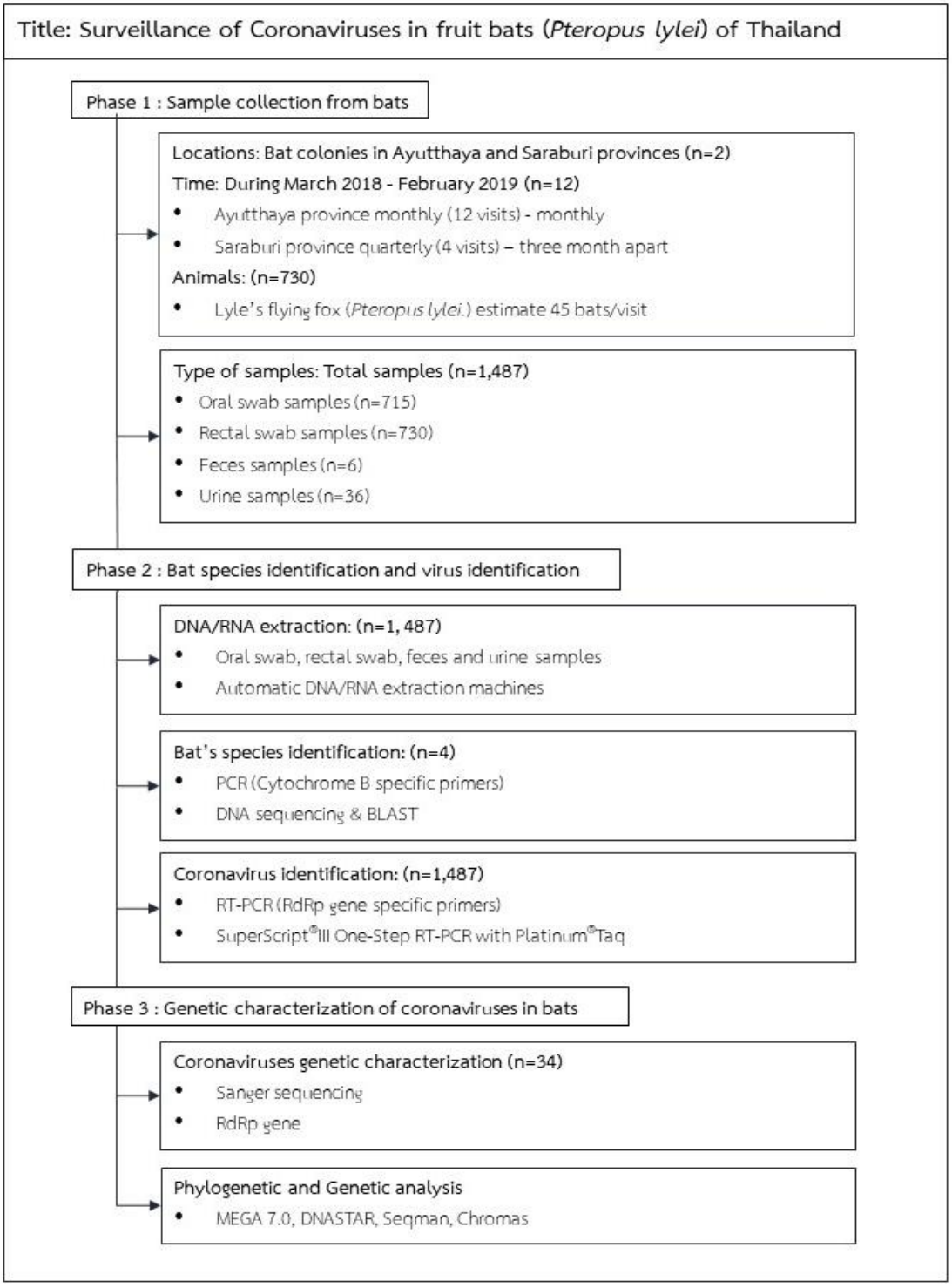

Figure 1 conceptual framework of this study

3.1 Phase 1. Sample collection from bats

3.1.1 Bat colonies for sample collection

In this thesis, sample collection was conducted in bat colonies in Saraburi and Ayutthaya provinces (Figure 2). The bat colonies (n=2) are Lyle's flying fox (*Pteropus lylei*) colonies. Two bat colonies were selected for longitudinal sample collection. The bats colonies were chosen based on the information from preliminary cross-sectional study which coronaviruses had been detected in these two locations. One bat colony is located in Nong Sida temple, Nong sida subdistrict, Nongsang district, Saraburi province (GPS: 14.514243, 100.828617) (Figure 3). Another bat colony is located in Tan-en temple, Tan-en subdistrict, Bang Pahun district, Ayutthaya province (GPS: 14.517727, 100.559685) (Figure 4). These two bat colonies are located in the center of village where provide suitable environment of human-bat interface.

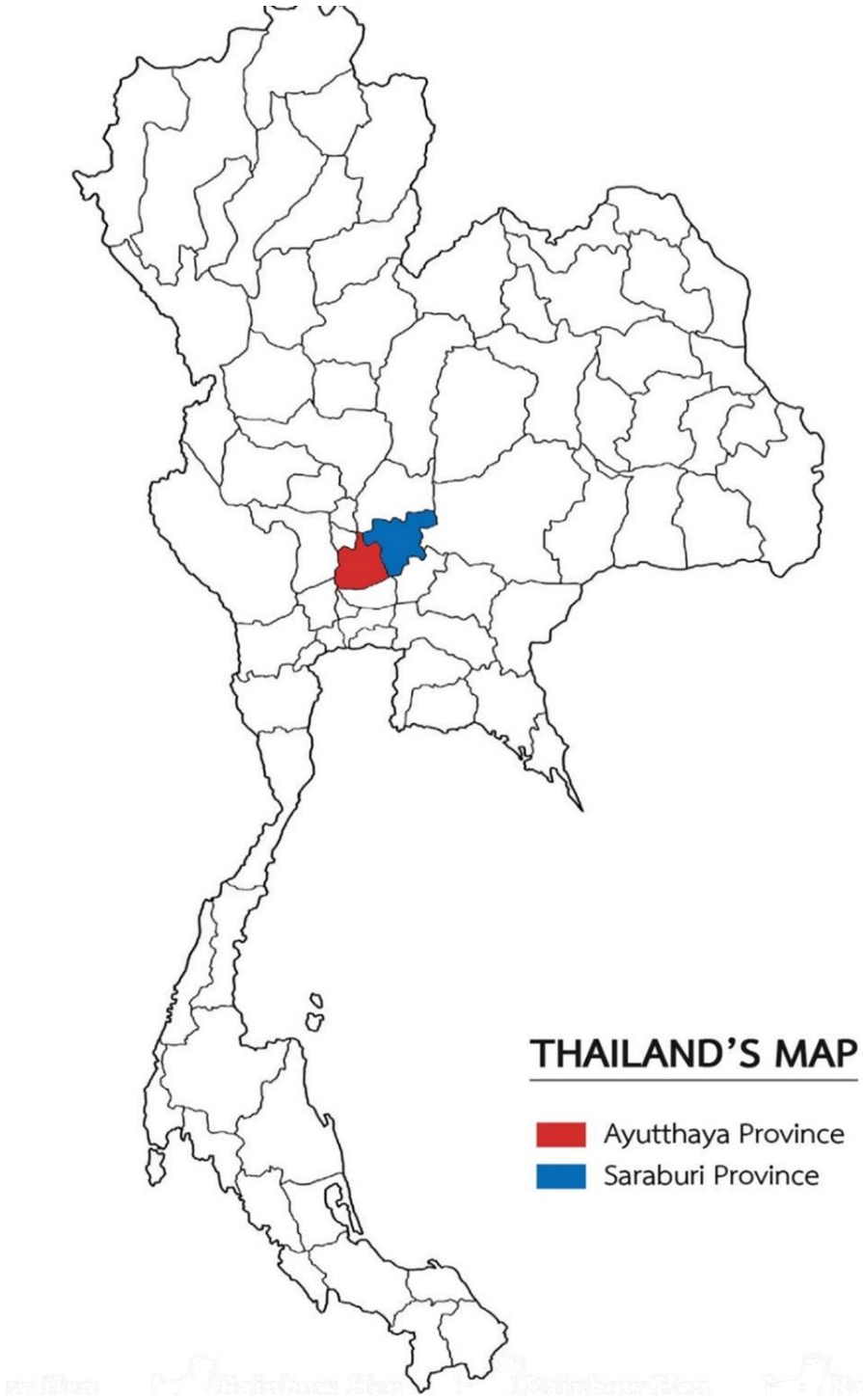

Figure 2 Map of provinces and location of bat colonies for sample collection in this thesis, Thailand's map and locations of Saraburi and Ayutthaya provinces

Figure 3 Satellite photo of location of bat colony at Nong Sida temple; Saraburi province, Thailand (GPS: 14.514243, 100.828617)

Figure 4 Satellite photo of location of bat colony at Tan-en temple; Ayutthaya province, Thailand (GPS: 14.517727, 100.559685)

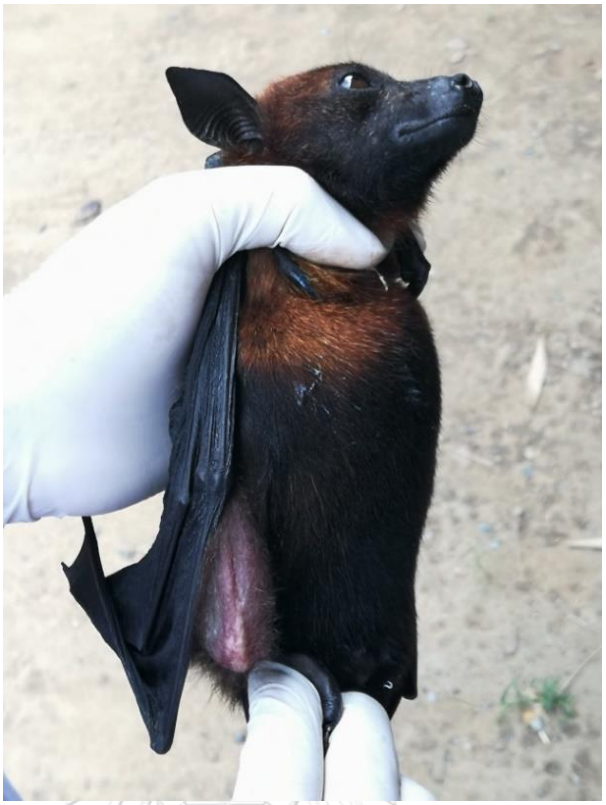

Figure 5 The bat in sample collection in this thesis

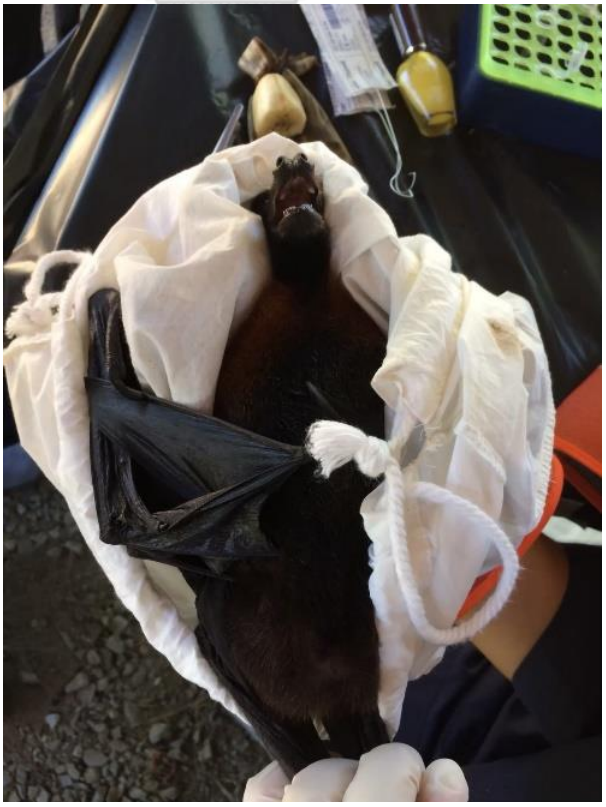

Figure 6 The bat in this thesis was captured and kept in individual bag

3.1.2 Bat sample collection

Bat sample collection was conducted during March 2018 to February 2019 (12 months). In Ayutthaya province, bat samples were collected monthly for 1 years (12 visits), while the sample collection in Saraburi were quarterly conducted for 1 years (4 visits). During sample collection, demographical distribution of bats including sex, age, forearm length, body length and weight were recorded and/or pictured.

In case of working with wild animals, the sample collection was conducted under the permission protocols from the Department of National park and Wildlife and Plant Conservation (DNP). All the animals were captured unharmfully from the experienced DNP officers. The DNP officers helped trap and restrain the bats in each individual bag. The well-trained researchers measured and collected samples of the bats carefully. The animals were fed by banana and mineral water before released. This thesis was conducted under the approval of Chulalongkorn University Institutional Animal Care and Use Committee (IACUC), animal use protocol no, 1631006. And this thesis was approved of Institutional Biosafety Committee (IBC), biosafety use protocol no. IBC1831028.

In this thesis, we collaborated and worked with DNP for appropriate and standard protocol of animal capture and sample collection. In total of 1,487 bat samples were collected during the course of the study. In detail, a total 730 bats were sampled including oral swabs (n=715) and rectal swabs (n=730). In addition, feces (n=6) and urine (n=36) were randomly collected from bats. All samples preserved in lysis buffer (RNA carrier in AVL solution) (GeneAll®; Seoul, South Korea). The samples were transported in triple packaging system (WHO/EMC/97.3) to the laboratory. The samples were aliquoted and processed in the BSL2 laboratory.

3.2 Phase 2. Bat species identification and coronavirus identification

3.2.1 Bat species identification

For bat species identification was combined with two methods. First was morphologiacal identification. The other method is molecular technique which is cytochrome B detection via PCR. Morphological identification, we distinguished the bats by their appearances for example sizes, faces, ears, etc. Bat personal informations were recorded which are body weight, body length and forearm length.

For molecular technique, rectal swabs from 2 locations (n=4) were randomly selected and subjected to genomic DNA extraction by using Automatic DNA/RNA extraction machine; GENTi $^{TM\ 32}$ (GeneAll®; Seoul, South Korea). Bat species identification was carried out by characterizing cytochrome B gene (housekeeping gene) (Bradley and Baker, 2001). The genomic DNA was tested with cytochrome B specific PCR by using TopTaq Master Mix Kit (QIAGEN^{TM}; Hilden, Germany). In detail, a 30 µl reaction of mixture consist of 15 µl of 2X TopTaq Master Mix (consist of TopTaq DNA Polymerase, 3 mM of MCl_2 and 400 µM of each dNTP), 1.2 µl of each specific primer (Table 1), 3 µl of coral load, 1 µl of DNA template and RNAase free water. The amplification performed 40 cycles. In each cycle started with denaturation at 94 ℃ for 30 seconds, following with annealing process at 50 ℃ for 30 seconds and extension at 72 ℃ for 30 seconds (Table 2) (Kocher et al., 1989). PCR products was examined by gel electrophoresis in 1.5% agarose gel. The PCR products were purified by QIAquick PCR Purification kit (QIAGEN^{TM}; Hilden, Germany) and were submitted for DNA sequencing. The nucleotide sequences of cytochrome B gene were validated and analyzed by BLAST program to identify bat's species (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1 List of oligonucleotide primers for cytochrome B gene specific PCR detection for bat species identification

Primer	Length (bp)	Sequence (5'-3')
CytB-H15149	34	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA
CytB-L14724	28	CGAAGCTTGATATGAAAAACCATCGTTG

(Kocher et al., 1989)

Table 2 PCR conditions for cytochrome B gene specific PCR assay for bat species identification

PCR process	Temp (°C)	Time
Pre-denaturation	94	3 mins
Denaturation	94	30 sec
Annealing	50	30 sec
Extension	72	30 sec
Final extension	72	7 mins

*Modified from (Kocher et al., 1989)

3.2.2 Coronavirus identification

To identify coronavirus all samples (oral swabs, rectal swabs, feces, urine and environmental samples; (n=1,487) were subjected to viral RNA extraction by using Automatic DNA/RNA extraction machine; GENTi $^{TM\ 32}$ (GeneAll®; Lisbon, Portugal). The RNA samples were then tested for coronaviruses by using one step RT-PCR with specific primers to RdRp gene (Table 3) (Woo et al., 2005). One step RT-PCR was performed by using SuperScript®III one step RT-PCR with Platinum®Taq (Invitrogen™; CA, USA). In detail 10 µl of mixture consist of 5 µl of 2X Reaction mix (Invitrogen™; CA, USA) (containing 0.4 mM of each dNTP and 3.2 mM $MgSO_4$), 0.2 µl of each specific primer, 0.4 µl of SuperScript™ III RT/Platinum Taq Mix (Invitrogen™; CA, USA), 1 µl of RNA sample and RNAase free water (Woo et al., 2012). The amplification condition is 55°C 30 minutes for reverse transcription process, following by 35 cycles of 94°C for denaturation for 30 seconds, next 59°C 45 seconds for annealing and 68°C for 1 minute for extension process (Table 4). The PCR products were illuminated by gel electrophoresis in 1.5% agarose gel.

Table 3 List of oligonucleotide primers for the detection of RdRp gene of coronavirus in bats

Primer	Length (bp)	Sequence (5'-3')
CoVWat_out_F1	24	ATGGGMTGGGAYTATCCWAARTGT
CoVWat_out_F2	24	ATGGGKTGGGAYTATCCWAARTGT
CoVWat_out_R	24	CCATCATCAGATAGAATCATCATA

*Modified from (Woo et al., 2005)

Table 4 PCR conditions for one step RT-PCR for the detection of RdRp gene of coronavirus in bats

PCR process	Temp (°C)	Time
cDNA synthesis	55	30 mins
Pre-denaturation	94	2 mins
Denaturation	94	30 sec
Annealing	59	45 sec
Extension	68	1 min
Final extension	68	7 mins

*Modified from (Woo et al., 2012)

3.3 Phase 3. Genetic characterization of coronaviruses from bats

3.3.1 Coronaviruses genetic characterization

For genetic characterization of the virus, RdRp gene of coronaviruses were sequenced. The RdRp gene of positive samples was amplified by using SuperScript®III one step RT-PCR with Platinum®Taq (Invitrogen™; CA, USA). In detail 50 µl of mixture consist of 25 µl of 2X Reaction mix (Invitrogen™; CA, USA) (containing 0.4 mM of each dNTP and 3.2 mM $MgSO_4$), 1 µl of each specific primer, 2 µl of SuperScript™ III RT/Platinum Taq Mix (Invitrogen™; CA, USA), 5 µl of RNA samples and RNAase free water (Woo et al., 2012). The amplification condition is 55°C for 30 minutes for reverse transcription process, then 35 cycles of 94°C for denaturation for 30 seconds, 59°C for annealing for 45 seconds and 68°C for extension process for 1 minute (Table 3.4). The PCR templates were examined by gel electrophoresis in 1.5% agarose gel and were purified by using QIAquick PCR Purification kit (QIAGEN™; Hilden, Germany). The purified products were submitted to sanger sequencing (n=34).

3.3.2 Phylogenetic and genetic analysis and Genetic analysis

To construct the phylogenetic tree, first the nucleotide sequences of reference coronaviruses were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The reference nucleotide sequences of RdRp gene of coronaviruses in this study consisted of Alphacoronovirus for example PED-CoV, Human CoV, FIP-CoV, Gammacoronavirus, Deltacoronavirus and Betacoronavirus for example SAR-CoV, SAR-like-CoV, MERS-CoV, MERS-like-CoV etc (n~50) including the pandemic SARS-CoV-2. Phylogenetic analysis, the nucleotide sequences of the viruses were aligned to reference sequences by using ClustralW algorithm (Rui et al.; Pavlović-Lažetić et al., 2004). Then, the phylogenetic tree were generated by using MEGA7.0 program with Neighbor-Joining method (Tamura et al., 2011). The confidence of the tree topology was assessed by Bootstrapping method with 1,000 replications (Hillis and Bull, 1993).

To conduct genetic analysis and amino acids analysis of coronaviruses, the nucleotide sequences were validated, assembled and edited by using Chromas program (Technelysium Pty Ltd, Helensvale, Australia) (Moës et al., 2005). Then, the RdRp gene sequences and the reference sequences were compared by multiple alignment using ClustalW algorithm via Megalign Program. The percentage of similarities were evaluated and presented in table.

Chapter 4

Results

4.1 Sample collection from bat colonies

In this thesis, the samples (n=1,487) were collected from bats from two bat colonies. One flock is located in Tan-en temple; Tan-en district, Bang Pa Hun Aumphur, Ayutthaya province. Another flock is located in Nong Sida temple; Nong Sida district, Aumphur Nong Saeng, Saraburi province. The bats are living and foraging nearby human villages for generations allowing frequent bat-human interface. The sample collection was conducted during March 2018 to February 2019. The bat colony in Ayutthaya province was visited monthly (n=12 visits). While the bat colony in Saraburi province was visited every 3 months (n=4 visits). Since the bat colony in Nong Sida temple; Saraburi province is a tourist attraction, we conducted sample collection for only every 3 months to avoid interruption to the bats. In conclusion, we collected 1,487 samples from total 730 bats including oral swab samples (n=715), rectal swab samples (n=730), urine samples (n=36) and feces samples (n=6) (Table 5). By locations of sample collection, Ayutthaya province, we collected 1,027 samples from bats including oral swab samples (n=490), rectal swab samples (n=505), urine samples (n=26) and feces samples (n=6) (Table 4.2). For Saraburi province, we collected 460 samples from bats including oral swab samples (n=225), rectal swab sample (n=225), urine samples (n=10) (Table 4.2).

.

Table 5 Detailed description of bat sample collection by time of sample collection (March 2018 – February 2019)

Year	Month	Location (province)	No. of Bats	Oral Sample	Rectal Sample	Urine Sample	Fecal Sample
2018	Mar-2018	Ayutthaya	49	49	49	-	-
	Apr-2018	Saraburi	50	50	50	2	-
	Apr-2018	Ayutthaya	51	51	51	-	-
	May-2018	Ayutthaya	48	35	48	7	3
	Jun-2018	Ayutthaya	50	49	50	3	-
	Jul-2018	Saraburi	50	50	50	-	-
	Jul-2018	Ayutthaya	50	49	50	3	-
	Aug-2018	Ayutthaya	33	33	33	-	-
	Sep-2018	Ayutthaya	34	34	34	-	-
	Oct-2018	Ayutthaya	24	24	24	-	1
	Oct-2018	Saraburi	65	65	65	5	-
	Nov-2018	Ayutthaya	57	57	57	4	2
	Dec-2018	Ayutthaya	30	30	30	-	-
			588	576	591	24	6
2019	Jan-2019	Ayutthaya	30	30	30	5	-
	Jan-2019	Saraburi	60	60	60	3	-
	Feb-2019	Ayutthaya	49	49	49	4	-
			139	139	139	12	0
Total			730	715	730	36	6

Table 6 Detailed description of bat sample collection by location of sample collection (Ayutthaya and Saraburi)

Location (province)	Month	No. of Bats	Oral Sample	Rectal Sample	Urine Sample	Fecal Sample
Ayutthaya	Mar-2018	49	49	49	-	-
	Apr-2018	51	51	51	-	-
	May-2018	48	35	48	7	3
	Jun-2018	50	49	50	3	-
	Jul-2018	50	49	50	3	-
	Aug-2018	33	33	33	-	-
	Sep-2018	34	34	34	-	-
	Oct-2018	24	24	24	-	1
	Nov-2018	57	57	57	4	2
	Dec-2018	30	30	30	-	-
	Jan-2019	30	30	30	5	-
	Feb-2019	49	49	49	4	-
		502	490	505	26	6
Saraburi	Apr-2018	50	50	50	2	-
	Jul-2018	50	50	50	-	-
	Oct-2018	65	65	65	5	-
	Jan-2019	60	60	60	3	-
		225	225	225	10	-
Total		730	715	730	36	6

4.2 Bat species identification

4.2.1 Description of Bats

To identify the species of bats in this thesis, we recorded the demographic and morphological information of each bat to identify the type of bat. Then the species of bats was confirmed by using PCR specific to mitochondrial cytochrome B gene. The record of bat's data including weight, sex, average age, body length, fore-arm length and special marking via (scars, deformation, etc.) was carried out in each individual bat. The examples of data recording including sample collection worksheet and procedures during recording of bat characteristics, body-length and fore-arm length are shown in Figure 8.

4.2.2 PCR for detection of bat's mitochondrial cytochrome B gene

To confirm the species of bat sampled in this thesis, we performed morphological characteristic identification and cytochrome B gene sequencing. Based on the morphological characteristics, bat from both colonies were identified as flying fox or fruit which identified as Magabat. For cytochrome B gene sequencing, our result showed that bats from both colonies were identified as *Pteropus Lylei*. BLAST analysis of cytochrome B gene sequence confirmed the species of bats from both colonies as *Pteropus Lylei* with 100% identities (Table 7 and Figure 9).

(A)

วันที่เก็บ_____ ผู้เก็บตัวอย่าง_____

Sample Collection Worksheet Bat

สถานที่_____

ที่อยู่_____หมู่_____ตำบล_____อำเภอ_____จังหวัด_____

Location: ☐ village (วัด ฯลฯ) ☐ park (วนอุทยาน) ☐ Forest ☐ Other_____

Latitude:_____Longitude:_____

ชื่อผู้ติดต่อของสถานที่_____โทร_____

ลักษณะสถานที่_____

จำนวน ชนิด ลักษณะการหากิน และรายละเอียดอื่นๆของค้างคาว _____

ผู้บันทึก_____

Version update : 12-12-2016

(B.)

วันที่เก็บ_____ ผู้เก็บตัวอย่าง_____

Sample Collection Worksheet Bat

No.	Common name	species	Sex	Age	Forearm length	Body length	น้ำหนัก โดยประมาณ	Type of specimen									
								B		OS		RS		F		U	

Notes : - Sex : M=male, F=female -Age: J=juvenile, A=Adult

- Specimen type : B=blood, OS=oral swab, RS=rectal swab, F=feces U= Urine

Version update : 12-12-2016

Figure 7 Sample Collection Worksheet (A). Location data recording sheet, (B). Bat data recording sheet during bat sample collection

(A)

(B)

Figure 8 Recording of bat characteristics, (A) bat's body length recording and (B) bat's forearm length recording

Table 7 Result of bat species identification by morphological characteristics and cytochrome B characterization

Sample	Location	Morphological characteristic	Cytochrome B characterization
BT21369R	Saraburi	*Pteropus* spp.	*Pteropus giganteus*
BT21376R		*Pteropus* spp.	*Pteropus lyei*
BT21396R	Ayutthaya	*Pteropus* spp.	*Pteropus giganteus*
BT21402R		*Pteropus* spp.	*Pteropus lyei*

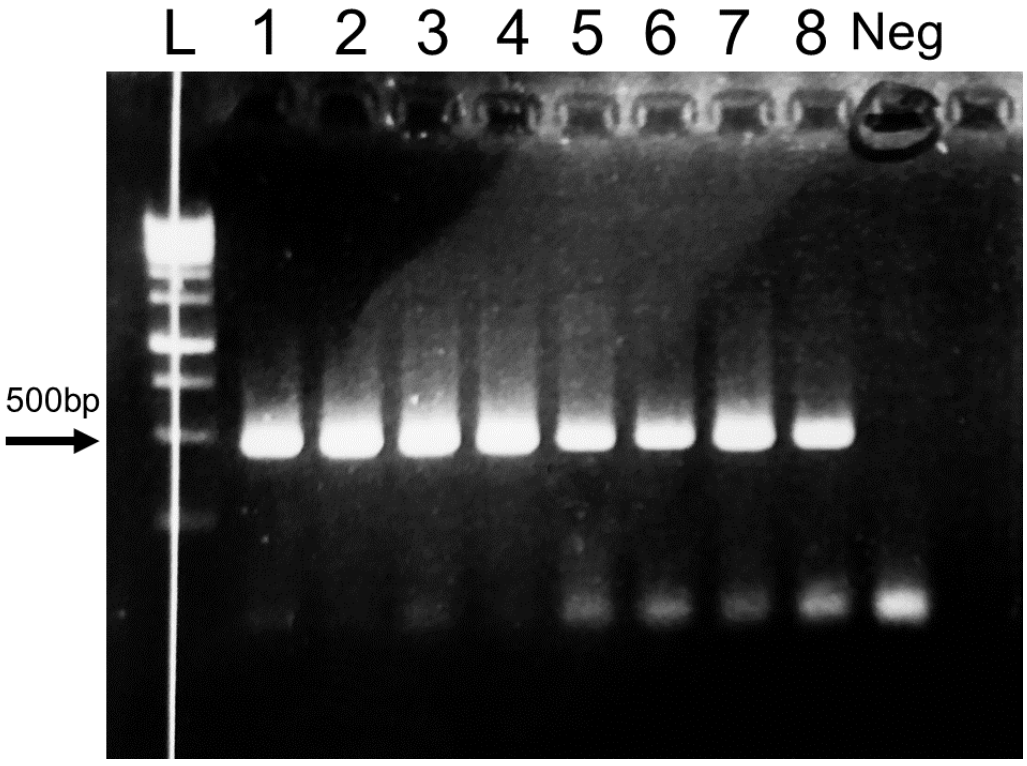

Figure 9 Bat species identification by PCR specific to cytochrome B and gene sequencing. (A). Gel electrophoresis of cytochrome B specific PCR, (B). cytochrome B sequences identities by BLAST analysis

4.3 Coronavirus detection in bats

During March 2018 – February 2019, total 1,487 samples were collected from two bat colonies in central Thailand. All the samples were subjected to RNA extraction. Then the samples were tested by one step RT-PCR using specific primers detecting RdRp gene of coronavirus. Our results showed that 4.30% (64/1,487) of samples were positive with RdRp gene of CoV. In detail, in Ayutthaya province in 2018, Coronavirus (CoV) can be detected in March (8.16% (8/98)), April (3.92% (4/102)), May (7.53%, (7/93)), July (3.92% (4/102)), September (2.94% (2/68)) and December (11.67% (7/60). While in Saraburi province in 2018, CoV can be detected in April (18.63% (19/102)) and July (13.00% (13/100)) (Table 8). It is noted that no positive CoV samples detected in 2019. By colonies of bats, in Ayutthaya, the coronaviruses could be detected at 3.11% (32/1,027). In Saraburi, the coronaviruses could be detected at 6.96% (32/460) (Table 8 and Figure 10). By type of samples, CoV could be detected in oral swab 3.36% (24/715), rectal swab 5.48% (40/730), urine 0% (0/36) and feces 0% (0/6). It is noted that there were no positive oral and rectal swabs from the same bats in this study (Table 4.5 and Figure 10).

Table 8 Results of coronavirus detection in bats by time of sample collection.

Month	Species	Location	Total samples	CoV positive (No.)	CoV positive (%)
Mar-18	*P. lylei*	Ayutthaya	98	8	8.16
Apr-18	*P. lylei*	Saraburi	102	19	18.63
Apr-18	*P. lylei*	Ayutthaya	102	4	3.92
May-18	*P. lylei*	Ayutthaya	93	7	7.53
Jun-18	*P. lylei*	Ayutthaya	102	-	-
Jul-18	*P. lylei*	Saraburi	100	13	13
Jul-18	*P. lylei*	Ayutthaya	102	4	3.92
Aug-18	*P. lylei*	Ayutthaya	66	-	-
Sep-18	*P. lylei*	Ayutthaya	68	2	2.94
Oct-18	*P. lylei*	Saraburi	135	-	-
Oct-18	*P. lylei*	Ayutthaya	49	-	-
Nov-18	*P. lylei*	Ayutthaya	120	-	-
Dec-18	*P. lylei*	Ayutthaya	60	7	11.67
			1,197	64	5.34
Jan-19	*P. lylei*	Saraburi	123	-	-
Jan-19	*P. lylei*	Ayutthaya	65	-	-
Feb-19	*P. lylei*	Ayutthaya	102	-	-
			290	0	0
Total			1,487	64	4.30

Table 9 Results of coronavirus detection in bats by type of samples

Virus	Time	Location	No. CoV positive	Type of sample		
				Oral swab	Rectal swab	Urine & Feces
BT20956R	Mar-18	Ayutthaya	8	-	1	-
BT20957R	Mar-18	Ayutthaya		-	1	-
BT20958R	Mar-18	Ayutthaya		-	1	-
BT20959O	Mar-18	Ayutthaya		1	-	-
BT20978O	Mar-18	Ayutthaya		1	-	-
BT20980O	Mar-18	Ayutthaya		1	-	-
BT20982O	Mar-18	Ayutthaya		1	-	-
BT20982R	Mar-18	Ayutthaya		-	1	-
BT21332R	Apr-18	Saraburi	19	-	1	-
BT21333R	Apr-18	Saraburi		-	1	-
BT21334R	Apr-18	Saraburi		-	1	-
BT21335R	Apr-18	Saraburi		-	1	-
BT21336R	Apr-18	Saraburi		-	1	-
BT21337O	Apr-18	Saraburi		1	-	-
BT21338O	Apr-18	Saraburi		1	-	-
BT21340O	Apr-18	Saraburi		1	-	-
BT21342O	Apr-18	Saraburi		1	-	-
BT21344O	Apr-18	Saraburi		1	-	-
BT21344R	Apr-18	Saraburi		-	1	-
BT21346O	Apr-18	Saraburi		1	-	-
BT21346R	Apr-18	Saraburi		-	1	-
BT21347O	Apr-18	Saraburi		1	-	-
BT21352O	Apr-18	Saraburi		1	-	-
BT21352R	Apr-18	Saraburi		-	1	-
BT21353O	Apr-18	Saraburi		1	-	-
BT21353R	Apr-18	Saraburi		-	1	-

Table 9 Results of coronavirus detection in bats by type of samples (con.)

Virus	Time	Location	No. CoV positive	Type of sample		
				Oral swab	Rectal swab	Urine & Feces
BT21354O	Apr-18	Saraburi		1	-	-
BT21417O	Apr-18	Ayutthaya	4	1	-	-
BT21420O	Apr-18	Ayutthaya		1	-	-
BT21424O	Apr-18	Ayutthaya		1	-	-
BT21428O	Apr-18	Ayutthaya		1	-	-
BT21521R	May-18	Ayutthaya	7	-	1	-
BT21524R	May-18	Ayutthaya		-	1	-
BT21525R	May-18	Ayutthaya		-	1	-
BT21527R	May-18	Ayutthaya		-	1	-
BT21529R	May-18	Ayutthaya		-	1	-
BT21531R	May-18	Ayutthaya		-	1	-
BT21532R	May-18	Ayutthaya		-	1	-
BT21843R	Jul-18	Saraburi	13	-	1	-
BT21853R	Jul-18	Saraburi		-	1	-
BT21855R	Jul-18	Saraburi		-	1	-
BT21858R	Jul-18	Saraburi		-	1	-
BT21859R	Jul-18	Saraburi		-	1	-
BT21860R	Jul-18	Saraburi		-	1	-
BT21862R	Jul-18	Saraburi		-	1	-
BT21872R	Jul-18	Saraburi		-	1	-
BT21876R	Jul-18	Saraburi		-	1	-
BT21880O	Jul-18	Saraburi		1	-	-
BT21882R	Jul-18	Saraburi		-	1	-
BT21886R	Jul-18	Saraburi		-	1	-
BT21887R	Jul-18	Saraburi		-	1	-

Table 9 Results of coronavirus detection in bats by type of samples (con.2)

Virus	Time	Location	No. CoV positive	Type of sample		
				Oral swab	Rectal swab	Urine & Feces
BT21889R	Jul-18	Ayutthaya	4	-	1	-
BT21901R	Jul-18	Ayutthaya		-	1	-
BT21910R	Jul-18	Ayutthaya		-	1	-
BT21913O	Jul-18	Ayutthaya		1	-	-
BT22247R	Sep-18	Ayutthaya	2	-	1	-
BT22259R	Sep-18	Ayutthaya		-	1	-
BT23332O	Dec-18	Ayutthaya	7	1	-	-
BT23334O	Dec-18	Ayutthaya		1	-	-
BT23334R	Dec-18	Ayutthaya		-	1	-
BT23336R	Dec-18	Ayutthaya		-	1	-
BT23337O	Dec-18	Ayutthaya		1	-	-
BT23351R	Dec-18	Ayutthaya		-	1	-
BT23361O	Dec-18	Ayutthaya		1	-	-
Total			64	24	40	0

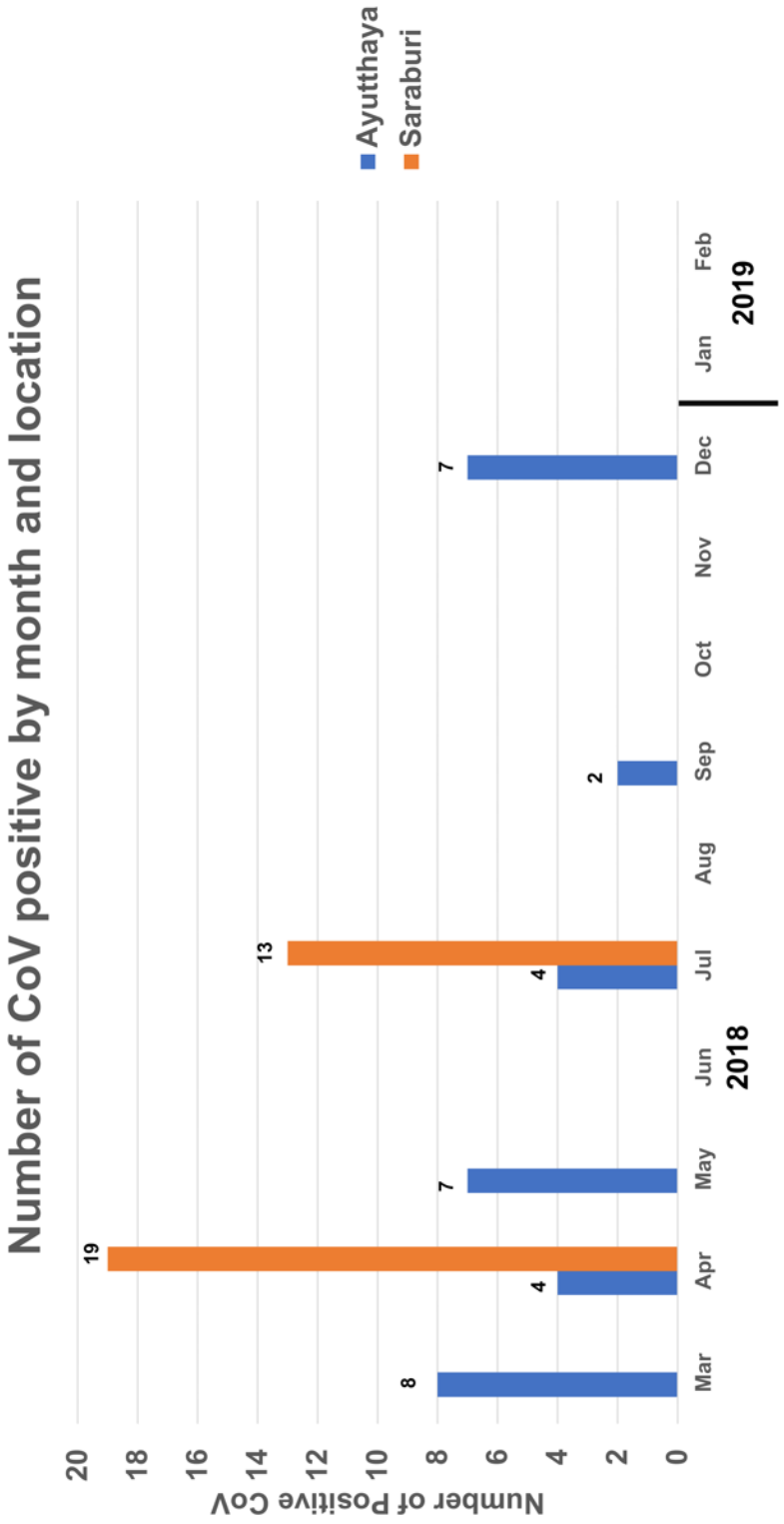

Figure 10 Results of coronavirus detection in bats by time and location of sample collection.

4.4 Genetic characterization of coronavirus from bats

4.4.1 Coronavirus genetic characterization

In this thesis, 34 CoV positive samples were selected for RdRp gene sequencing by Sanger's sequencing (Table 10). The samples (n=34) submitted for sequencing were from Ayutthaya (n=22) and Saraburi (n=12). By type of sample, the samples were selected from oral swab (n=9) and rectal swab (n=25). Of 34 selected samples, we are able to acquire completed RdRp sequences from 6 out of 34 positive samples. All CoV with completed RdRp gene sequences were came from rectal swab sample (6/6). There is no oral swab sample were successfully sequenced (0/13). By province, 5 sequences were from bats in Ayutthaya province and 1 sequence from bats in Saraburi province. The sequences from Ayutthaya were from May (n=3), July (n=1) and September (n=1). Only one sequence from Saraburi was from July (n=1). All nucleotide sequences of 6 CoVs characterized in this study is shown in Figure 11.

The results of nucleotide BLAST analysis of RdRp gene sequences of CoV showed that all CoVs detected from Ayutthaya and Saraburi had 98-100% similarities to bat CoV previously reported in fruit-eating bat from Thailand (*Pteropus lylei)*. In detail, RdRp sequences of the CoV from Ayutthaya (n=5) posed 98-100% nucleotide identities to Bat- CoV recovered from *Pteropus lylei* from Eastern part of Thailand. The RdRp sequences of the CoV from Saraburi, one sequence (n=1) had less nucleotide identity (94%) to bat CoV from *Pteropus lylei* in Ayutthaya (Table 4.8). There is no similarity between bat CoV detected in this study and bat CoVs founded in insectivore bats.

Table 10 CoV positive samples selected for RdRp gene sequencing by location and type of samples

Virus	Location	Month	Type of Sample		
			Oral swab	Rectal swab	Urine & Feces
BT20957R	Ayutthaya	Mar-18	-	1	-
BT20958R	Ayutthaya	Mar-18	-	1	-
BT21417O	Ayutthaya	Apr-18	1	-	-
BT21420O	Ayutthaya	Apr-18	1	-	-
BT21428O	Ayutthaya	Apr-18	1	-	-
BT21531R	Ayutthaya	May-18	-	1	-
BT21521R	Ayutthaya	May-18	-	1	-
BT21524R	Ayutthaya	May-18	-	1	-
BT21525R	Ayutthaya	May-18	-	1	-
BT21527R	Ayutthaya	May-18	-	1	-
BT21529R	Ayutthaya	May-18	-	1	-
BT21531R	Ayutthaya	May-18	-	1	-
BT21532R	Ayutthaya	May-18	-	1	-
BT21901R	Ayutthaya	Jul-18	-	1	-
BT21889R	Ayutthaya	Jul-18	-	1	-
BT21913O	Ayutthaya	Jul-18	1	-	-
BT22259R	Ayutthaya	Sep-18	-	1	-
BT23332O	Ayutthaya	Dec-18	1	-	-

Table 10 CoV positive samples selected for RdRp gene sequencing by location and type of samples (con.)

Virus	Location	Month	Type of Sample		
			Oral swab	Rectal swab	Urine & Feces
BT23334O	Ayutthaya	Dec-18	1	-	-
BT23334R	Ayutthaya	Dec-18	-	1	-
BT23351R	Ayutthaya	Dec-18	-	1	-
BT23361O	Ayutthaya	Dec-18	1	-	-
			7	15	0
BT21333R	Saraburi	Apr-18	-	1	-
BT21334R	Saraburi	Apr-18	-	1	-
BT21352O	Saraburi	Apr-18	1	-	-
BT21855R	Saraburi	Jul-18	-	1	-
BT21858R	Saraburi	Jul-18	-	1	-
BT21859R	Saraburi	Jul-18	-	1	-
BT21860R	Saraburi	Jul-18	-	1	-
BT21862R	Saraburi	Jul-18	-	1	-
BT21880O	Saraburi	Jul-18	1	-	-
BT21882R	Saraburi	Jul-18	-	1	-
BT21886R	Saraburi	Jul-18	-	1	-
BT21887R	Saraburi	Jul-18	-	1	-
			2	10	0
Total			9	25	0

Table 11 Detail of coronaviruses characterized in this study

Virus	Location	Month	Species	Type of samples
BT21524R	Ayutthaya	May-18	*P. lylei*	Rectal swab
BT21531R	Ayutthaya	May-18	*P. lylei*	Rectal swab
BT21532R	Ayutthaya	May-18	*P. lylei*	Rectal swab
BT21901R	Ayutthaya	Jul-18	*P. lylei*	Rectal swab
BT22259R	Ayutthaya	Sep-18	*P. lylei*	Rectal swab
BT21886R	Saraburi	Jul-18	*P. lylei*	Rectal swab

```
LOCUS       BT21524R/AY/May-18       301 bp    DNA     linear   MAM 26-NOV-2020
DEFINITION  UNVERIFIED: Pteropus lylei.
ACCESSION   BT21524R/AY/May-18
VERSION
KEYWORDS    UNVERIFIED.
SOURCE      Pteropus lylei
  ORGANISM  Pteropus lylei
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
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  AUTHORS   Moolthep,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2020) Department of Public Health, Faculty of
            Veterinary Science, Chulalongkorn University, 39 Henry Dunant Rd.,
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  TITLE     Direct Submission
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JOURNAL unpublished
REFERENCE 2 (bases 1 to 196)
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TITLE Direct Submission
JOURNAL Submitted (26-NOV-2020) Department of Public Health, Faculty of
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  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2020) Department of Public Health, Faculty of
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Figure 11 Detail of nucleotide sequences of 6 CoVs characterized in this study

4.4.2 Phylogenetic analysis and genetic analysis of coronaviruses

In this thesis, 6 sequences from bat CoV were included for phylogenetic analysis. The phylogenetic tree was constructed by using MEGA1.0 program to identify genetic relatedness of Thai bat CoVs. To identify the genetic diversity of bat CoV, 6 bat CoVs recovered in this thesis were compared with reference sequences of coronaviruses including Alpha, Beta, Gamma and Delta coronaviruses. Percentages of nucleotide identities among bat CoV (n=6) in this thesis were 98.4-100%. Thai bat CoV had highest nucleotide identities with Thai bat CoV of Betacoronavirus lineage D (BtCoV/CB1_THA/Bat/Thailand/2012) at 98.9% but not Betacoronavirus lineage A (62.1%), B (67.9%) and C (67.4%) (Table 12). Phylogenetic analysis, our result showed that bat CoVs were clustered with Batacoronavirus lineage D. The bat CoVs (n=6) in this thesis were closely related to bat CoVs previously reported in Thailand. It is noted that bat CoVs from Thailand did not group into the same lineage with SARS-CoV (causing SARS) (lineage B) and SARS-CoV-2 (causing COVID-19) (lineage B) (Figure 12).

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Table 12 Comparison of nucleotide sequences among Thai bat CoVs and reference bat CoVs.

Virus	**Genus**	**Lineage**	**% nucleotide RdRp gene (190 bp)**
BT21524R/AY/May-18	BetaCoV	D	100
BT21531R/AY/May-18	BetaCoV	D	98.9
BT21532R/AY/May-18	BetaCoV	D	100
BT21901R/AY/Jul-18	BetaCoV	D	98.4
BT22259R/AY/Sep-18	BetaCoV	D	99.5
BT21886R/SB/Jul-18	BetaCoV	D	94.2
Reference			
HCoV/OC43/VR-759/USA/196	AlphaCoV	-	68.9
BtCov/HKU8/AFCD77/China/2005	BetaCoV	A	62.1
BtCoV/Yunnan/RaTG13/China/2013	BetaCoV	B	67.9
HCoV/MERS/Al-Hasa1/Saudi_Arabia/2013	BetaCoV	C	67.4
BtCoV/CB1_THA/Bat/Thailand/2012	BetaCoV	D	98.9

Table 13 Percentage of similarity (upper triangle) and percentage of divergence (lower triangle) between bat CoV in this thesis and reference sequences

	BT21524R/ AY/May-18	BT21531R/ AY/May-18	BT21532R/ AY/May-18	BT21901R/ AY/Jul-18	BT22259R/ AY/Sep-18	BT21886R/ SB/Jul-18	HCoV/OC43/USA/1960	BtCoV/RaTG13/China/2013	BtCoV/HKU8 /China/2005	HCoV/MERS/Saudi/2013	BtCoV/ /Thailand/ 2012
BT21524R/ AY/May-18	***	98.9	100	98.4	99.5	94.2	68.9	62.1	67.9	67.4	98.9
BT21531R/ AY/May-18	98.9	***	98.9	99.5	99.5	95.3	69.5	62.6	68.9	67.9	100
BT21532R/ AY/May-18	100	98.9	***	98.4	99.5	94.2	68.9	62.1	67.9	67.4	98.9
BT21901R/ AY/Jul-18	98.4	99.5	98.4	***	98.9	94.7	68.9	62.1	68.4	68.4	99.5
BT22259R/ AY/Sep-18	99.5	99.5	99.5	98.9	***	94.7	69.5	62.1	68.4	67.9	99.5
BT21886R/ SB/Jul-18	94.2	95.3	94.2	94.7	94.7	***	69.5	62.1	68.4	66.8	95.3
HCoV/OC43/ USA/1960	68.9	69.5	68.9	68.9	69.5	69.5	***	62.1	67.9	66.8	69.5
BtCoV/HKU8 /China/2005	62.1	62.6	62.1	62.1	62.1	62.1	62.1	***	65.8	62.1	62.6
BtCoV/RaTG13/China/2013	67.9	68.9	67.9	68.4	68.4	68.4	67.9	65.8	***	66.8	68.9
HCoV/MERS/ Saudi/2013	67.4	67.9	67.4	68.4	67.9	66.8	66.8	62.1	66.8	***	67.9
BtCoV/Thailand/2012	98.9	100	98.9	99.5	99.5	95.3	69.5	62.6	68.9	67.9	***

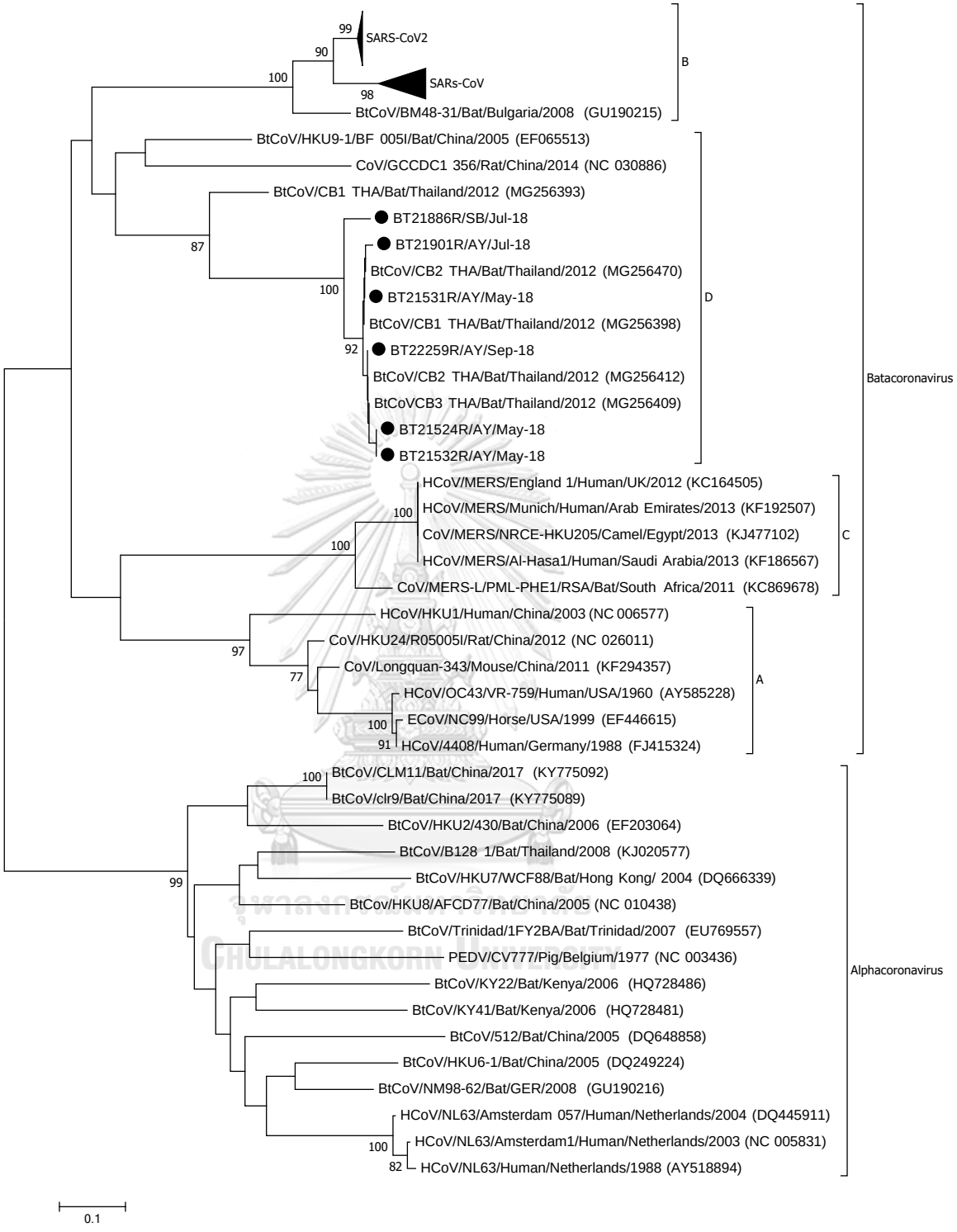

Figure 12 Phylogenetic tree of RdRp gene of Thai bat CoVs and reference CoV. Phylogenetic tree was constructed by using MEGA v.7.0 (Tamura et al., 2013) with neighbor-joining method applying parameter with 1,000 bootstrap replicates. The black circle indicates Thai bat CoVs in this study

Chapter 5

Discussion

5.1 Longitudinal surveillance of Coronaviruses in bats of Thailand

In this thesis, we investigated coronavirus circulating in two colonies of fruit-eating bats in Ayutthaya and Saraburi provinces. In Ayutthaya, we conducted monthly longitudinal sample collection at Tan-en temple for one year, in total 12 visits. In Saraburi, we collected bat sample at Nong Sida temple every 3 months, in total 4 visits. It should be noted that Nong sida temple is one of the tourist attractions, so we could not perform monthly sample collection at this location. These bats have been roosting and living nearby human villages for generations. Thus, there is a close encounter between bats and human (villagers & tourists). A colony of flying fox or fruit bat (*Pteropus* spp.) can consist of several hundreds to thousands of bats in one colony (Pierson and Rainey, 1992). In Thailand, coronaviruses in bats had been reported in Eastern part of Thailand (Wacharapluesadee et al., 2015; Wacharapluesadee et al., 2018). However, these two bat colonies selected in this thesis have never been surveyed for CoVs. Thus, this thesis was focused on longitudinal survey of CoVs in bats from two bat colonies in central provinces (Ayutthaya and Saraburi) of Thailand.

5.2 Bat species

Bats are varying in types and species. There are over 1,400 species of bats existing in the world. There are many methods for bat species identification. Bat identification by morphological characteristics including type of faces, shape of ears and nose-leaf. These morphological characteristics are the easiest ways to identify bat species at the Family or Genus levels (Dietz, 2005). Bat identification by frequency of bat's echolocation can be used to identify the species of insectivore bats (Fenton and Bell, 1981). However, Megachiroptera, or frugivorous bat, which forage by visual and scents (Raghuram et al., 2009), so the acoustic method cannot

be applied to Megabats in this study. The external appearance measurement such as body weight, body length and forearm length can help grouping bats into Family or genus levels. In average *Pteropus lylei*'s body weight range in adult bat is 390 to 480 g. The forearm length is approximately 145 – 160 mm in adult bat (Francis, 2019). Based on the appearances and measurement of forearm length, we can categorize the bats in this thesis as fruit bat or flying fox in the genus *Pteropus* spp.

To identify bats as species level, cytochrome B sequencing is a DNA based method that can characterize the eukaryotic cells at sub-species level (Bradley and Baker, 2001). Cytochrome B gene is the housekeeping gene that very unique in each organism. Since morphological data and measurement of the bat's bodies could not help indicating the exact species of animals. Our result showed that we can identify bat species as *Pteropus lylei* or (Lyle's flying fox) and *Pteropus giganticus* (Indian flying fox) in both bat colonies in this thesis. Because cytochrome B gene locus may be about 1,140 bp, while our sequences were around 350 - 400 bp. The diversity in the same species is around 0.25 - 2.74%, while the variation between species of animal can be 5.97 - 34.83% (Hsieh et al., 2001). Lacking of the information of the whole gene and less number of sequences, the results of cytochrome B detection might be shifted. Besides, it is noted that there are two species of *Pteropus* spp. living in Thailand which are *Pteropus lylei* and *Pteropus vampyrus* (Large flying fox). And there is only one Flying fox species living and distributing in central part of Thailand. While *Pteropus vampyrus* distributes in the tropical area in the Southern part of Thailand (Wanghongsa and Boongird, 2003; Hillman, 2005). *Pteropus giganticus*, on the other hand, forages and distributes in India, Bangladesh, Bhutan, Nepal, Pakistan, Sri Lanka and Myanmar (List, 2004). Combinding between morphological measurement, bat distribution and cytochrome B detection we can conclude that the bat in two bat colonies is *Pteropus lylei*.

5.3 Coronaviruses circulating in bat colonies

In this thesis, we conducted longitudinal survey of CoVs in bats from 2 bat colonies in the Ayutthaya and Saraburi provinces, Thailand, during March 2018 to February 2019. Our result showed the prevalence of CoV was 4.30% (64/1,487). By provinces, CoV positivity in Ayutthaya was 3.11% (32/1,027), which was lower than that of Saraburi (6.96%; 32/460). The area that the bat colony in Saraburi are living is smaller than the area of bat colony in Ayutthaya. This might be one of the reasons that percentage of CoV positive sample in Saraburi was higher than in Ayutthaya. More density of bats in the area promoted the transmission of CoV between bats within the colony. By type of sample, CoV positivity in rectal swab sample was the highest 5.48% (40/730) followed by oral swab sample, urine and feces samples at 3.36% (24/715), 0% (0/36) and 0% (0/6) respectively. Our result in agreement with previous study that bat CoVs were mostly detected from both rectal swab and fecal samples (Poon et al., 2005; Wacharapluesadee et al., 2015; Wacharapluesadee et al., 2018) . While CoVs could be detected from Oral swab in some studies (Shehata et al.; Dominguez et al., 2007; Groschup and Balkema-Buschmann, 2016). On the other hand, there was no report of CoVs detection in urine sample from bats. Even though some studies showed the evidence of SARS-CoV-2 detected in COVID-19 human patient's urine (Brönimann et al., 2020). Furthermore, there is no detection of CoV in fecal samples due to natural inhibitors in fecal samples.

5.4 Genetic characteristics of Coronaviruses from bats

From 64 CoV positive samples, we selected 34 samples for RdRp gene sequencing by Sanger's sequencing. These 34 samples represented the time and location of each sample collection. However only 6 out of 34 nucleotides of CoV could be elucidated. This due to the low quality of RNA samples or low concentration of RNA template. In this thesis, all sequences (n=6) were from rectal swab samples. The result of nucleotide BLAST showed that all samples from Ayutthaya (n=5) were closely related with high percentages of nucleotide identities to the bat CoV sequences from previous study in Lyle's flying fox colony in Chonburi province; Eastern part of Thailand (Wacharapluesadee et al., 2018). For the samples from Saraburi, there is one (n=1) sample had nucleotide identities to the sequences from Chonburi.

Phylogenetic analysis of RdRp gene of CoV, shows that there are two major groups of CoVs. The first group is Alphacoronavirus including corovirus in pigs (PED-CoV), coronavirus in human (HCoV) and coronavirus in bats (bat CoV). Second group is Betacoronavirus which can be further divided to lineage A, B, C and D. The Betacoronavirus group consists of coronavirus in bats (bat CoV) and coronavirus in humans (SARS-CoV, MERS-CoV and SARS-CoV2). In this thesis, all 6 sequences were clustered into Betacoronavirus lineage D. The sequences were grouped together with bat CoV found in bat in the same species (*Pterpus lylei)* related to the previous study in Chonburi province (Wacharapluesadee et al., 2018). Comparing to the other contagious zoonotic CoV, MERS-CoV belongs to Betacoronavirus lineage C. While the SARS-CoV and SARS-CoV-2 are in the branch Betacoronavirus lineage B, it should be noted that the bat CoVs in this thesis were not relate to the pandemic CoVs. For genetic analysis, the genetic comparison showed that all the 6 sequences from two bat colonies had high nucleotide identities to Bat CoV of Betacoronavirus lineage D detected in Thailand (BtCoV/CB1_THA/Bat/Thailand/2012) (Wacharapluesadee et al.,

2018). Percentage of nucleotide identities between our sequences and Thai bat CoV are very high at 98.4 – 100%. Thus, they were very closely related. The percentage identities between CoVs from Ayutthaya and Saraburi are less similar at 94.2%. Due to the CoV were from bats of different locations and bat colonies. In contrast, comparing to the others reference sequences, CoVs in this thesis had low percentage of nucleptide identities to Betacoronavirus lineage A, B and C (62.1 – 68.9%). Our phylogenetic analysis and genetic analysis showed that bat CoV circulating in Lyle's flying fox in this study are closely related to previous bat CoV reported in Thailand but grouped into separated lineages from pandemic CoVs or emerging CoV (SARS-CoV-2). However, bat CoV in Thailand should be routinely monitored to understand the dynamic of CoV infection as well as the genetic characteristics the viruses.

Conclusion

Coronavirus (CoV) is a highly contagious virus. CoV can infect in many species of mammals and avian including human. Bats are the natural reservoir for many genera of CoVs. Bat distribution covers in every parts of Thailand, including caves, tourist attractions, human villages. The aims of this thesis were to detect coronavirus circulating in bats of Thailand and to identify viral genetics and diversity of CoVs in bat colonies in two provinces of Thailand. Bat sample collection was performed in two bat colonies in Ayutthaya and Saraburi provinces during March 2018 – February 2019. Total 1,487 samples were collected from 730 bats. The samples consisted of 715 oral swabs, 730 rectal swabs, 36 urines and 6 fecal samples. The bat species identification via morphological measurement and cytochrome B detection showed that the bats from two colonies are Lyle's flying fox (*Pteropus lylei*). For CoV detection, we detected the viruses using one step RT-PCR specific to RdRp gene. The results showed that 4.30% (64/1,487) samples from bat were positive to CoVs. Then, 34 positive samples were selected to RdRp gene sequencing. However only, RdRp nucleotide sequences of 6 CoVs could be elucidated. The phylogenrtic tree was constructed by comparing the bat CoV with references nucleotide sequences of Alphacoronavirus, Betacoronavirus. Gammacoronavirus and Deltacoronavirus. Pylogenetic analysis showed the bat CoVs in this thesis were clustered with Thai bat CoV of BetaCoV lineage D from *Pteropus lylei* in Chonburi province. Moreover, bat CoVs were grouped into different subgroups/lineages of pandemic CoVs (MERS-CoV, SARS-CoV and SARS-CoV-2 (COVID-19)). For genetic analysis, bat CoV in this thesis had very high percentage of nucleotide identities to Thai bat CoV at 98-100%. On the other hands, bat CoVs had low percentage identity (<70%) to others reference sequences of AlphaCoV, BetaCoV lineage A B and C. Our results confirmed that CoVs are circulating in two bat colonies in Ayutthaya and Saraburi provinces and the viruses are closely genetic related to bat CoV circulating in bat in the same species in Eastern part of Thailand. Although the bat CoVs in this thesis were far related with

pandemic CoVs, but routinely surveillance of CoVs in bats should be performed due to bat CoV could be a potential zoonotic virus.

Based on the results of this thesis, the recommendations for the surveillance and monitoring CoVs in bats and other animals are as following:

- **Bat**
    - Demographic information and distribution of bat colonies in Thailand should be systemically surveyed and recorded for future database and research.
    - Surveillance and monitoring of CoVs in bats should be conducted and expanded to represent every parts of Thailand.
    - Surveillance and monitoring of CoVs in both Megachiroptera and Microchiroptera bats should be conducted to represent several species of bats in Thailand.
    - Whole genome characterizations should be performed to represent genetic information of CoV from bats in Thailand and the information should be shared for scientific communities.
- **Human**
    - Education about human-bat interface and risk of CoVs infection should be performed especially the population living nearby the bat colonies.
    - Information of human-bat interface, bat-related diseases and bat consumption should be studied in the future.
- **Domestic and farm animals**
    - CoVs surveillance on domestic and farm animals living nearby the bat colonies should be conducted to monitor the CoVs distribution in other animals.

- Role of domestic and farm animals as a potential intermediate host for CoVs should be further investigated.

Future study for prevention and control of coronaviruses in bat colonies should be focusing on the following:

- Monitoring the genetic diversity and evolution of CoVs circulating in bats.
- Expanding the genetic study at whole genome level especially on structural proteins (S-protein and N-protein).
- Developing the rapid diagnostic kit for CoVs detection in the fields.

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