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Discrimination between Tropical Bed Bug *Cimex hemipterus* and Common Bed Bug *Cimex lectularius* (Hemiptera: Cimicidae) by PCR-RFLP

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Abstract

Bed bugs, *Cimex hemipterus* and *Cimex lectularius*, are common blood-sucking ectoparasites of human and currently found in many countries around the world. In Thailand, both species have been found mostly in hotels in tourist attraction areas and the insecticide resistance of these insects was also documented. To date, identification of these two bed bug species is based on morphological taxonomy, a technique which requires expertise and in some instance is difficult especially for immature bed bugs or incomplete bed bug samples. In this study, we analyzed the *cytochrome c oxidase subunit I (COI)* gene of bed bugs, *C. hemipterus* and *C. lectularius* collected from various regions of Thailand. PCR-RFLP and phylogenetic analysis demonstrated that the *COI* gene could significantly differentiate between the two bed bug species. Moreover, the phylogenetic tree could separate clusters of insecticide resistant from insecticide susceptible *C. lectularius* strains. However, sequence analysis of *C. hemipterus* showed no significant intra-specific variation from different geographical regions of Thailand. Data obtained from this study will be valuable for epidemiological distribution of bed bugs in Thailand and subsequently for the most effective control of these insects.

Keywords: bed bug, *cytochrome c oxidase subunit I* gene, phylogenetic tree, PCR-RFLP

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บทคัดย่อ

การจำแนกตัวเรือดสายพันธุ์ *Cimex hemipterus* และ *Cimex lectularius* โดยวิธี PCR-RFLP

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ตัวเรือดชนิด *Cimex hemipterus* และ *Cimex lectularius* เป็นปรสิตภายนอก ที่ดูดเลือดรบกวนมนุษย์และพบได้ในหลายประเทศทั่วโลก ในประเทศไทยจะพบตัวเรือดทั้งสองชนิดนี้ในโรงแรมในพื้นที่ที่เป็นแหล่งท่องเที่ยวที่สำคัญและมีรายงานว่าตัวเรือดดังกล่าวมีการติดต่อสารเคมีกำจัดแมลง ปัจจุบันการจำแนกชนิดของตัวเรือดทั้งสองชนิดนี้อาศัยลักษณะทางสัณฐานวิทยา ซึ่งเทคนิคนี้ต้องการผู้เชี่ยวชาญและในบางกรณีเกิดความยุ่งยาก เช่นในระยะตัวอ่อน หรือตัวอย่างที่ไม่สมบูรณ์ รายงานนี้ได้ทำการศึกษายีนในตำแหน่งไซโตโครม ซี ออกซิเดส หน่วยย่อยที่ 1 (COI) ในตัวเรือดชนิด *C. hemipterus* และ *C. lectularius* ที่เก็บตัวอย่างมาจากภูมิภาคต่างๆ ของประเทศไทย และพบว่าวิธี PCR-RFLP และ แผนภูมิต้นไม้ในยีนตำแหน่ง COI สามารถแยกความแตกต่างระหว่างตัวเรือดสองชนิดได้อย่างมีนัยสำคัญ นอกจากนี้แผนภูมิต้นไม้สามารถแยกตัวเรือดสายพันธุ์ *C. lectularius* กลุ่มที่ติดต่อสารเคมีกำจัดแมลงออกมาจากกลุ่มที่ไวต่อสารเคมีกำจัดแมลงได้ อย่างไรก็ตามพบว่าตัวเรือดสายพันธุ์ *C. hemipterus* มีความหลายหลายทางพันธุกรรมอย่างไม่มีนัยสำคัญในภูมิภาคต่างๆของประเทศไทย ข้อมูลที่ได้จากการศึกษานี้จะมีประโยชน์ในด้านระบาดวิทยาของตัวเรือดในประเทศไทยและช่วยให้การควบคุมตัวเรือดดำเนินไปอย่างมีประสิทธิภาพสูงสุด

คำสำคัญ: ตัวเรือด ยีนไซโตโครม ซี ออกซิเดส หน่วยย่อยที่ 1 แผนภูมิต้นไม้ PCR-RFLP

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Introduction

Bed bugs (Hemiptera: Cimicidae) are important blood-sucking ectoparasites of human. Two major bed bug species feed on human blood; *Cimex hemipterus*, the tropical bed bug and *Cimex lectularius*, the common bed bug (Harlan et al., 2008). The first report of bed bug was from England in 1583 (Kemper, 1936). During the second half of the 20th century bed bugs were rare in North America and Western Europe (Ryan et al., 2002); however, in recent years they have increased in many parts of the world (Krueger and Paul, 2000; Bates, 2000; Potter et al., 2010; Criado et al., 2011). This may be due to the increase in human migration especially tourism industry and development of insecticides resistance of the insects (Romero et al., 2007; Krause-Parello and Sciscione, 2009). It is likely that the bed bugs were transported on clothes in luggages of travelers (Delaunay and Pharm, 2012).

Bed bugs require blood for development of nymphs to the next developmental stages (Johnson, 1941) and for reproduction of adults. Female bed bug produces 5-7 eggs per week with approximately 200-500 eggs in her lifetime, and adults can survive for up

to a year without feeding (Pinto et al., 2007). Bed bugs live in cracks and crevices around bed or wooden furniture in hotels, hostels, private homes, trains, and cruise ships (Delaunay et al., 2011) and they can spread easily from shelter to shelter (Stephen et al. 2005). They are notorious as pests that crawl out at night to bite and feed on human blood. Although there has been no scientifically-based evidence showing that bed bugs transmit diseases (Dolling, 1991), people who are bitten may suffer from intense itch, inflammation, allergic symptoms and psychological effects (Usinger, 1966; O'Neill et al., 1997; Doggett and Russell, 2009).

In Thailand, bed bugs had disappeared from the country for decades. Until recently, bed bugs were found in hotels in tourist attraction areas in different regions of the country and these bed bugs were resistant to various insecticides, especially those in the pyrethroid group (Tawatsin et al., 2011). In fact, *C. hemipterus* was resistant to DDT since 1970s (WHO, 1976) and *C. lectularius* showed resistance to bifenthrin and α -cypermethrin recently (Suwannayod et al., 2010).

Identification of bed bugs in Thailand has been based on insect morphology. Although this

procedure can identify adult stage easily, it is very difficult in immature stages or eggs (Kolb et al., 2009). Moreover, taxonomic identification requires highly experienced person and complete samples of bed bugs. Nowadays, molecular techniques have been developed for taxonomic identification such as nucleotide sequence analysis, phylogenetic tree, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kress and Erickson, 2008). These techniques are fast, accurate and highly sensitive; moreover, it can be performed for species identification in immature stages, cast skins or incomplete samples of bed bugs from the fields. In this study, we demonstrate the utility of mitochondrial *cytochrome c oxidase* subunit I (*COI*) gene for discrimination by PCR-RFLP and phylogenetic analysis of these two main important human bed bugs. Comparisons of the *COI* gene among the bed bugs collected from different regions and between insecticide resistant and susceptibility strains were also reported.

Materials and Methods

Bed bug collections and rearing: Bed bugs, *C. hemipterus* and *C. lectularius*, in this study were collected from hotels in different parts of Thailand; Central (Bangkok), Northern (Chiang Mai and Phitsanulok), North-Eastern (Ubon Ratchathani), and Southern (Phuket and Krabi). Insecticide susceptible strain (from Tokyo) and insecticide resistant strain (from Chiba) of *C. lectularius* (provided by Dr. Mamoru Watanabe) were also used in this study. The bed bugs were identified using morphological keys described by Pratt and Stojanovich (1967). The insects were maintained in laboratory of the Biology and Ecology Section, National Institute of Health, Department of Medical Sciences, Thailand. Environmental conditions of the rearing room were set at 26-28°C, 60-80% RH, and a photoperiod of 12 : 12 (L : D) hour. The bed bugs were reared in plastic cups covered with fine mesh chiffon cloth. A piece of cardboard (4 x 8 cm) was put inside the cups for the bed bugs to crawl up and insert their mouthparts through the mesh top to feed. For blood feeding, the bed bugs had access to artificial feeding unit for 30 minutes, using almost expired donated-blood received from Blood Bank, Thai Red Cross. This method was modified from that developed by Montes et al. (2002).

DNA extraction: Individual bed bug of each sample was lysed by lysis buffer and placed in liquid nitrogen for 1 minute and then ground with a sterile plastic pestle. Genomic DNA was isolated using DNA extraction kits: Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 100 µl of elution buffer; the fraction of extracted DNA was spectrophotometrically quantitated using a Nanodrop 2000c (Thermo-scientific, USA). The extracted DNA samples were kept at -80°C for long term storage.

PCR amplification: Sequences of the *COI* gene of *C. hemipterus* and *C. lectularius* were obtained from

GenBank with Accession number GU985538.1 and GU985525.1, respectively (Balvin and Vilimova, 2010). The sequences were aligned using the multiple alignment programs ClustalX version 1.81 (Thompson et al., 1997). Degenerate oligonucleotide primers were designed as forward primer (5' GMCAACCTGGCTCATTTATTG 3') and reverse primer (5' ATAAGTGTGTYAWAGWARAGG 3'). Primers were synthesized by 1st BASE Oligonucleotide (Oligo) Synthesis services company (1st BASE Laboratories, Malaysia). The amplification reaction was set up in a final volume of 25 µl, containing approximately 100 ng of extracted DNA. Polymerase chain reactions (PCR) were performed in a GeneAmp PCR system 2400; Applied Biosystems®, USA. The reaction conditions are as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min and final extension at 72°C for 7 min. Aliquots of the amplicons were analyzed on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

DNA sequencing and RFLP patterns prediction: The PCR amplicons were ligated into pGEM-T Easy Vector (Promega, USA). The ligated vectors were transformed into DH5α strain competent cells, and then the chimeric plasmids were screened by blue-white colony selection system. The suspected positive colonies were cultured and used for further plasmid DNA extraction by using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH, Germany) following the manufacturer's instructions. Purified plasmids were sent to sequence by 1st BASE DNA sequencing services (1st base laboratories, Malaysia) using universal forward T7 primer. Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) and the consensus sequences were BLAST search (available at <http://www.ncbi.nlm.gov/BLAST>) for species identification. The nucleotide sequences of *COI* gene obtained from this study were submitted to the GenBank database. The resulting sequences were used for prediction of species-specific restriction sites by using NEBcutter V2.0 web-based program (available at <http://tools.neb.com/NEBcutter2/index.php>). From restriction prediction data, *BfaI* restriction enzyme recognizes 5'...C↓T A G...3' sites were chosen for PCR-RFLP.

PCR-RFLP: The PCR products were digested in separate reaction with *BfaI* (Thermo-scientific, USA). The reaction mixture was incubated at 37°C for 15 min followed by heat inactivation at 65°C for 5 min. The restriction products were electrophoresed through 8% native polyacrylamide gel electrophoresis run at 100 V for 70 min (MiniProtein 3 cell; Bio-Rad®, USA), followed by ethidium bromide staining and visualized on a Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

Sequence variation and Phylogenetic tree construction: The nucleotide sequences of each

species from various regions were aligned for variation positions. Phylogenetic tree were constructed by Maximum-likelihood method using Kimura's 2-parameter model implemented in MEGA[®] version 5.1 (Tamura et al., 2011). The reliability of an inferred tree was tested by 1000 bootstrap. *Triatoma dimidiata* (Kissing bug) accession no. JQ575031 as outgroup.

Results

In this study, 15 bed bugs were collected from 6 different regions of Thailand and 2 samples were provided from Japan. PCR amplicons of the *COI* gene from *C. hemipterus* and *C. lectularius* were approximately 580 bp in size (Fig 1). Amplified *COI* gene sequences obtained from this study varied from 576 to 581 bp. Consensus *COI* gene sequences of *C. hemipterus* and *C. lectularius* were blast in the GenBank database and showed the percentage identity range from 99 to 100. The nucleotide sequences showed maximum intra-specific variation approximately 0.8% in *C. hemipterus* and 0.6% in *C. lectularius*; nevertheless, the minimum inter-specific variation showed approximately 19.6% (data not shown). The nucleotide sequences of *COI* gene from the bed bugs were submitted to GenBank and accession numbers of JX826468 to JX826482 were assigned (Table 1).

The *COI* sequences of *C. hemipterus* collected from various parts of Thailand could be grouped into three groups (CH1, CH2 and CH3). The sequences variation was found at position 380 and deletion of three bases of *COI* gene between positions 445-447 were found in CH1 and CH3 (Fig 2). *COI* gene sequence of *C. lectularius* collected from Ubon Ratchathani was 100% identical to the insecticide susceptible *C. lectularius* from Tokyo isolated. Variations of *COI* gene sequences of *C. lectularius* between insecticide resistant and susceptible strains were found at position 96, 146 and 204 in this study (Fig 3).

Table 1 Collection site, isolated code, species, and accession number of each bed bug in this study

Location	Isolated code	Bed bug species	Accession no.
Chiang Mai	C.H.agri1	<i>C. hemipterus</i>	JX826468
	C.H.gate2	<i>C. hemipterus</i>	JX826469
	C.H.sarin3	<i>C. hemipterus</i>	JX826470
	C.H.cmu4	<i>C. hemipterus</i>	JX826471
	C.H.cmu1-5	<i>C. hemipterus</i>	JX826472
	C.H.cmu2-6	<i>C. hemipterus</i>	JX826473
Phuket	C.H.phu7	<i>C. hemipterus</i>	JX826474
Krabi	C.H.kb8	<i>C. hemipterus</i>	JX826475
Bangkok	C.H.bk 9-1	<i>C. hemipterus</i>	JX826476
	C.H.bk 10-2	<i>C. hemipterus</i>	JX826477
	C.H.12	<i>C. hemipterus</i>	JX826479
Phitsanulok	C.H.pl11	<i>C. hemipterus</i>	JX826478
Tokyo (susceptibility)	C.L.Tokyo	<i>C. lectularius</i>	JX826480
Chiba (resistance)	C.L.Chiba	<i>C. lectularius</i>	JX826481
Ubon Ratchathani	C.L.2	<i>C. lectularius</i>	JX826482

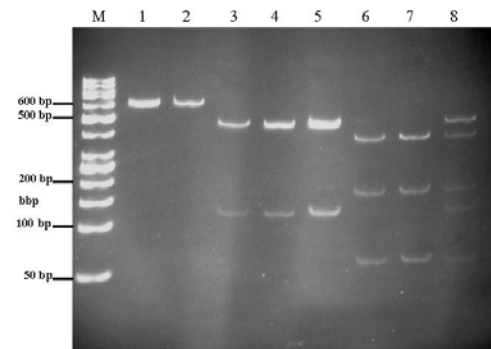


Figure 1 8% native polyacrylamide gel electrophoresis shows PCR-RFLP patterns of *COI* product digested with *BfiI* restriction enzyme. Lane1-2: undigested PCR products amplified from *C. lectularius* and *C. hemipterus*, respectively. RFLP patterns of *C. lectularius*: 120 and 459 bp (lane 3-5); *C. hemipterus*: 57, 168, and 351 bp (lane 6-7) and mixed DNA of both species: 57, 120, 168, 351 and 459 bp (lane 8) from *BfiI* digestion. Lane M: 25 bp DNA standard marker.

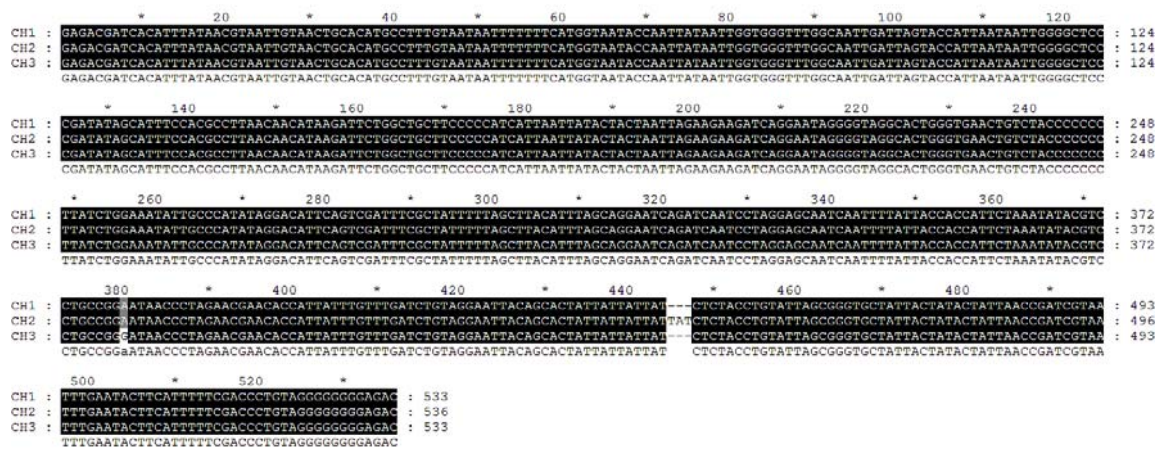


Figure 2 Nucleotide sequence comparison of *COI* genes of *C. hemipterus*, based on these sequences they can be classified into three groups: **CH1** represented sequence of *C. hemipterus* isolates C.H.sarin3, CMU4 and agri1 from Chiang Mai; **CH2** represented sequence of *C. hemipterus* isolates C.H.cmu2-6, gate 2 and C.H.cmu 1-5 from Chiang Mai, *C. hemipterus* isolates C.H.kb8 from Krabi, *C. hemipterus* isolate C.H.bk9-1 from Bangkok, and *C. hemipterus* isolates C.H.phu7 from Phuket; **CH3** represented sequence of *C. hemipterus* isolates C.H.bk10-2 and C.H.12 isolate from Bangkok and *C. hemipterus* isolate C.H.pl11 from Phitsanulok.

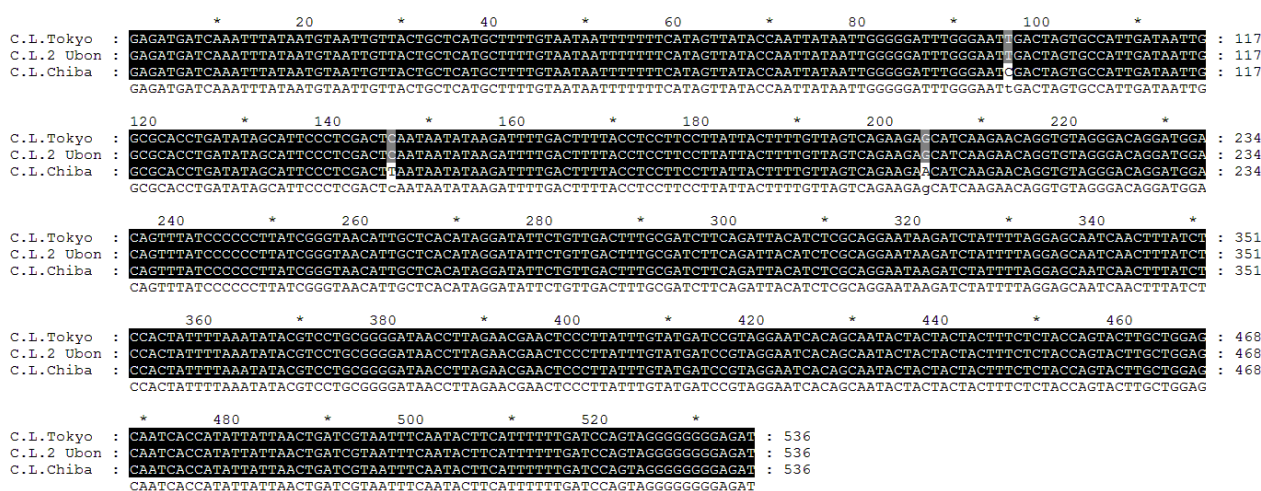


Figure 3 Nucleotide sequence comparison of COI genes of insecticide resistant and susceptible *C. lectularius* strains; C.L.Tokyo: *C. lectularius* isolate C.L.Tokyo (susceptibility); C.L.2 Ubon: *C. lectularius* isolate C.L.2 from Ubon Ratchathani; C.L.Chiba: *C. lectularius* isolate C.L.Chiba (resistant).

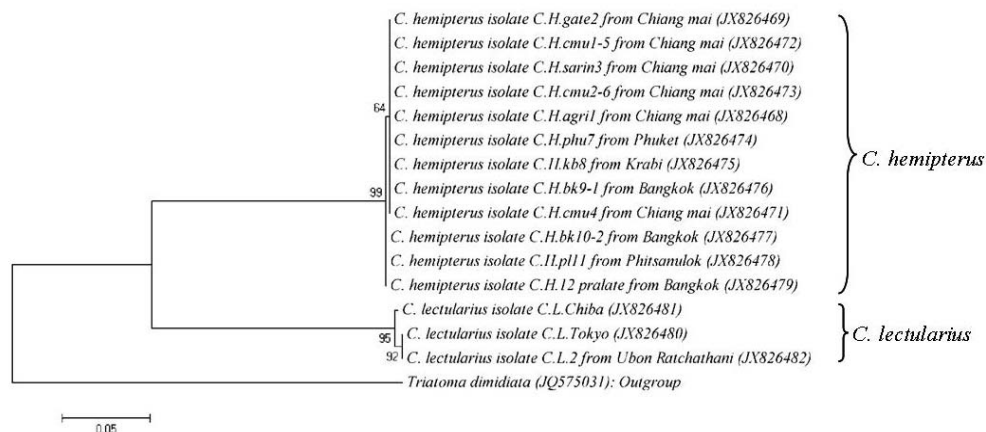


Figure 4 Maximum-likelihood trees were constructed using GTR + G + I evolution model of COI gene in 12 isolates of *C. hemipterus* and 3 isolates of *C. lectularius*. *T. dimidiata* accession no. JQ575031 sequences were used as outgroup.

Discussion

RFLP pattern of these two species were predicted by the NEBcutter V2.0 web-based program and *Bfa*I restriction was selected. PCR products of each species were digested with the *Bfa*I restriction enzyme and the fragments separated by 8% native polyacrylamide gel electrophoresis. The results demonstrated the PCR product of approximate 579 bp in length for both species, *C. hemipterus* (57, 168, and 351 bp) and *C. lectularius* (120 and 459 bp) and mixed DNA of both species (57, 120, 168, 351 and 459 bp) (Fig 1).

The constructed phylogenetic tree could clearly separate two major clades of *C. hemipterus* and *C. lectularius* although they belonged to the same genus. However, all of the *C. hemipterus* isolates clustered together showing no significant difference between different regions because of minor nucleotide variations between the same species. On the contrary, in *C. lectularius*, the insecticide resistant strain from Chiba was separated from Tokyo (insecticide susceptibility) and Ubon Ratchathani strains by the phylogenetic construction (Fig 3).

Nowadays, there are increasing reports of bed bug infestation and resistance to various insecticides has also been documented. Two species of bed bugs, *C. hemipterus* and *C. lectularius*, are found in Thailand and they become resistant to various insecticides (Tawatsin et al., 2011). Ghauri (1973) revealed that two species of bed bugs can be distinguished by looking at the first segment of the thorax, which expanded more laterally and of which the extreme margins are more flattened in *C. lectularius* than *C. hemipterus*. Several reports suggested that the type of insecticide resistance were different between bed bug strains (Karunaratne et al., 2007; Romero et al., 2007; Kilpinen et al., 2011). Therefore, taxonomic identification is important with necessary specialized taxonomic expertise. Molecular techniques are commonly used to apply in research labs worldwide for species identification such as sequence, phylogenetic tree analysis and PCR-RFLP in order to identify reliably and practically. This study used DNA-based identification by application of COI sequences for differentiation of bed bugs. COI is a mitochondrial gene which is conserved in arthropods,

species specific and has relatively high degree of genetic variation. We demonstrated the value of PCR-RFLP to differentiate two bed bug's species. This result showed that intra-specific polymorphism was not observed here by digestion with *Bfal* restriction enzyme as well as RFLP could be used for mix samples of two bed bugs. This benefit can help the survey of the bed bugs, when only cast skins or eggs as well as carcass damage of bed bugs are found. The PCR-RFLP can potentially lead to supersede taxonomic misidentification errors. In addition, phylogenetic tree showed the monophyletic clade in each species. According to intra-specific variation analysis, we found intra-variation in *C. hemipterus* (approximately 0.8%) because the sequence showed indeling of TAT base position, which could be confirmed by pick individual 10 colonies for sequencing. *C. hemipterus* can be grouped into 3 groups based on sequence variations. CH1 was found only in Chiang Mai, CH 2 was found in Chiang Mai, Bangkok, Krabi and Phuket, and CH3 was found in Bangkok and Phitsanulok (Fig 2). The study of *C. lectularius* indicated minor nucleotide variations between the insecticide resistant and susceptible strains in Japan as well as insecticide resistant isolates single branch of insecticide susceptible and *C. lectularius* in Thailand. This study is the first to report that the *COI* gene sequences were different between insecticide resistant (Chiba) and susceptible (Tokyo) strains of common bed bugs. *C. lectularius* collected from Ubon Ratchathani used in our study was susceptible to various insecticides (unpublished data) and the *COI* sequences were 100% identical to the insecticide susceptible isolates from Tokyo (Fig 3). Susceptible strain of *C. hemipterus* was unavailable in our laboratory, so it was not included in this study. Furthermore, this study investigated only 2 cosmopolite species, therefore, studies in other species such as *C. columbarius*, *C. pipistrelli*, *C. dissimilis*, and *Oeciacus* should be conducted as well as more collected samples from different geographical regions.

In conclusion, we demonstrated the ability of PCR-RFLP to discriminate between common bed bug and tropical bed bug. The sequence data obtained from the study showed minor variation between the same bed bug species. However, *COI* sequences of *C. lectularius* were different between insecticide resistant and insecticide susceptible strains. The sequence data from this study will be useful for epidemiological studies and for proper planning for effective bed bug control in the future.

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