Diet Analysis of Wrinkle-Lipped Free-Tailed Bat (*Chaerephon plicatus* Buchannan, 1800) Using Direct-PCR DGGE Technique

Kantima Thongjued

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Molecular Biology and Bioinformatics

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ชื่อวิทยานิพนธ์ การวิเคราะห์อาหารของค้างคาวปากย่น (*Chaerephon plicatus* Buchannan, 1800) โดยใช้วิธี direct PCR-DGGE

ผู้เขียน นางสาวกานติมา ทองจีด

สาขาวิชา ชีววิทยาโมเลกุลและชีวสารสนเทศ

ปีการศึกษา 2561

บทคัดย่อ

การศึกษาที่ผ่านมาพบว่า ค้างคาวกินแมลงหลายชนิดทั่วโลกสามารถช่วยควบคุมแมลงศัตรูพืชได้ ในประเทศไทยค้างคาวปากย่น (*Chaerephon plicatus*) ช่วยควบคุมแมลงศัตรูพืชในนาข้าว การศึกษาอาหารของค้างคาวจะทำให้เข้าใจนิเวศบริการของค้างคาวเหล่านี้ได้ งานวิจัยที่ผ่านมาศึกษาอาหารที่คงเหลือจากการย่อยในมูลค้างคาวโดยอาศัยกล้องจุลทรรศน์ ซึ่งไม่สามารถระบุรายละเอียดถึงระดับชนิดพันธุ์ของแมลงที่ค้างคาวกินเป็นอาหารได้ ในการศึกษาครั้งนี้ผู้วิจัยมีความมุ่งหวังในการพัฒนาวิธีการ รวมถึงทวนสอบวิธีการตรวจระบุชนิดแมลงโดยอาศัยวิธีการเพิ่มปริมาณดีเอ็นเอด้วยเทคนิคลูกโซ่โพลีเมอเรสโดยตรง (direct PCR) ที่จะสามารถนำไปใช้ในการระบุชนิดแมลงครอบคลุมแมลงหลากหลายกลุ่ม อีกทั้งสามารถนำไปประยุกต์ใช้ในตัวอย่างแมลงที่ถูกเก็บรักษาสภาพด้วยวิธีการต่าง ๆได้ นอกจากนี้วิธีการที่พัฒนาขึ้นถูกนำไปใช้ร่วมกับเทคนิคอิเล็กโทรโฟรีซิส เรียกว่า เทคนิค direct PCR-DGGE เพื่อคัดแยกและระบุชนิดแมลง จากดีเอ็นเอผสมที่เพิ่มปริมาณได้จากกองมูลค้างคาว ซึ่งช่วยให้สามารถประเมินนิเวศบริการของค้างคาวปากย่นที่มีต่อพื้นที่การเกษตรที่ล้อมรอบถ้ำอาศัยของค้างคาวได้ ผลการศึกษาพบว่าวิธีการเตรียมตัวอย่างแบบใช้สารละลาย PBS สามารถให้อัตราความสำเร็จในการเพิ่มปริมาณดีเอ็นเอได้ถึง 100 เปอร์เซ็นต์ ใน 6 อันดับของแมลง ได้แก่ อันดับ Mantodea, Phasmatodea, Neuroptera, Odonata, Blattodea และ Orthoptera อัตราความสำเร็จปานกลางถึงระดับสูงพบใน 5 อันดับของแมลง ได้แก่ Lepidoptera (97.3%), Coleoptera (93.8%), Diptera (90.5%), Hemiptera (81.8%) และ Hymenoptera (75.0%) อีกทั้งลำดับเบสจากผลิตภัณฑ์พีซีอาร์ ที่ได้จากวิธีการข้างต้นนี้ มีคุณภาพดี ทำให้สามารถระบุชนิดพันธุ์แมลงได้อย่างน่าเชื่อถือ วิธีการที่พัฒนาขึ้นนี้มีความไววิเคราะห์สูง สามารถใช้วิเคราะห์ตัวอย่างขา หรือ ลำตัวแมลงที่มีขนาดเล็กเพียงเศษหนึ่งส่วนสี่ของชิ้นเนื้อเยื่อขนาดหนึ่งตารางมิลลิเมตรได้ และยังสามารถนำไปใช้ในการตรวจระบุชนิดจากตัวอย่างแมลงอบแห้ง, แมลงที่ดองในสารละลายเอธิลแอลกอฮอล์, แมลงที่ถูกปรุงเป็นอาหาร, ซากแมลงในกองมูลค้างคาว และตัวอย่างแมลงที่เก็บรักษาในพิพิธภัณฑ์เป็นเวลานานได้ ด้วยอัตราความสำเร็จ 100, 98.6, 90.0, 84.0 และ 30.0 เปอร์เซ็นต์ ตามลำดับ ในกองมูลค้างคาวจำนวน 207 จากทั้งหมด 240 กองมูลที่รวบรวมเป็นประจำทุกเดือนเป็นเวลา 1 ปี จากถ้ำที่ล้อมรอบด้วยพื้นที่นาข้าวและพื้นที่การเกษตร ถูกนำมาเพิ่มปริมาณ

ดีเอ็นเอด้วยวิธีการที่พัฒนาขึ้น พบว่ามีอัตราความสำเร็จมากถึง 86.3 เปอร์เซ็นต์ เมื่อนำผลิตภัณฑ์พีซีอาร์ที่ได้นี้มาวิเคราะห์ด้วยอิเล็กโทรโฟรีซิสแบบ DGGE พบว่าดีเอ็นเอผสมสามารถแยกออกได้เป็น 325 แถบดีเอ็นเอ ผลการหาลำดับเบสจากแถบดีเอ็นเอเหล่านี้พบว่าประกอบด้วย 42 รูปแบบของลำดับเบสที่สามารถระบุชนิดได้ด้วยเกณฑ์ที่กำหนดไว้ ผลการตรวจระบุชนิดพบว่าอาหารของค้างคาวประกอบด้วยแมลงอย่างน้อย 7 อันดับ, 25 วงศ์, 24 สกุล และ 26 ชนิดพันธุ์ ในจำนวนนี้แมลงส่วนหนึ่งเป็นแมลงที่อาศัย และใช้พื้นที่การเกษตรเป็นแหล่งอาหาร แสดงให้เห็นว่าอาหารของค้างคาวปากย่นฝูงนี้อาจจะถูกกำหนดโดยลักษณะพื้นที่การเกษตรที่ล้อมรอบถ้ำ ซึ่งเป็นปัจจัยที่มีผลโดยตรงต่อการปรากฏชนิดพันธุ์ของแมลงที่กระจายในบริเวณดังกล่าว จากชนิดพันธุ์แมลงที่ตรวจพบในครั้งนี้พบว่ามีแมลงศัตรูพืชที่สำคัญคือ เพลี้ยกระโดดสีน้ำตาล (*Nilaparvata lugens*) ประกอบอยู่ด้วย อีกทั้งยังพบยุงชนิดที่มีความสำคัญทางการแพทย์ (*Culex* sp.) แสดงให้เห็นว่าค้างคาวปากย่นสามารถทำหน้าที่ช่วยในการควบคุมแมลงศัตรูพืชและแมลงนำโรคเหล่านี้ได้ การศึกษาครั้งนี้เป็นการค้นพบชนิดพันธุ์ของแมลงที่เป็นอาหารของค้างคาวปากย่นโดยอาศัยเทคนิคทางอณูพันธุศาสตร์ได้เป็นครั้งแรก ซึ่งขยายให้เห็นภาพรวมของอาหาร รวมถึงพฤติกรรมการหาอาหารของค้างคาวชนิดนี้ได้ดีกว่าที่ผ่านมา ข้อมูลที่ได้ยังเป็นพื้นฐานที่จะนำไปใช้ในการร่างแผนงานอนุรักษ์ และการจัดการถ้ำค้างคาวอย่างมีประสิทธิภาพและยั่งยืน เพื่อที่การคงอยู่ของค้างคาวชนิดนี้จะได้เอื้อประโยชน์ต่อภาคการเกษตรในแง่ของการช่วยกำจัดแมลงศัตรูพืชให้กับพื้นที่เพาะปลูกที่ล้อมรอบถ้ำต่อไป

Thesis Title	Diet analysis of wrinkle lipped free-tailed bat (*Chaerephon plicatus* Buchannan, 1800) using direct PCR-DGGE technique
Author	Miss Kantima Thongjued
Major Program	Molecular Biology and Bioinformatics
Academic Year	2018

ABTRACT

Globally insectivorous bats have been reported as a biological pest control agent. *Chaerephon plicatus* may play an important role for rice pest suppression. Diet analysis is used to reveal this ecosystem service. However, fecal examination using microscopic method have never provided reliable species prey list due to the possibility of thorough mastication for some insects. In this study, first, we developed and validated a direct PCR protocol for fast and effective universal insect species identification. Second, we tested applicability of the well-optimized protocol in various sample types regularly encountered in ecological studies. Third, we employed direct PCR protocol together with Denaturing Gradient Gel Electrophoresis (DGGE) (called direct PCR-DGGE technique) to identify insect preys in bat guano samples, and fourth, the ecosystem service of *C. plicatus* in regulating insect pest and also its foraging behavior in the surrounding agricultural landscapes was assessed. The developed direct PCR protocol that incorporates a 2-min sample preparation in PBS-buffer step achieved 100% success rates for amplification in six insect orders: Mantodea, Phasmatodea, Neuroptera, Odonata, Blattodea, and Orthoptera. High and moderate success rates were obtained for five other groups: Lepidoptera (97.3%), Coleoptera (93.8%), Diptera (90.5%), Hemiptera (81.8%), and Hymenoptera (75.0%). High-quality sequencing data were obtained from these amplifiable products, allowing confidence in species identification. The method was sensitive down to ¼ of a 1-mm^2 fragment of leg or body and its success rates with oven-dried, ethanol-preserved, food, bat guano, and museum specimens were 100%, 98.6%, 90.0%, 86.3%, and 30.0%, respectively. Two hundreds and seven of 240 bat guano pellets collected monthly from bat caves surrounded by rice fields were successfully amplified and provided 325 bands on DGGE gel. Sequencing confirmed that these bands comprised 42 identified OTU of insects and could be assigned to 7 orders, 25 families, 24 genera, and 26 species. The results

showed that *C. plicatus* diet was shaped by agricultural landscape, and also relied on availability of insect preys in their foraging range. Potential rice pest species, e.g. brown planthoppers (*Nilaparvata lugens*), and medical important insects, e.g. mosquitoes (*Culex* sp.) were consumed by *C. plicatus*, indicating its function as pest suppressing agent. This is the first time direct PCR-DGGE has been successfully used to analyze bat diet from guano samples. Diet of the bat was revealed genetically down to species level resulting in a more complete picture of ecosystem service, which allows further understanding of predator-prey interaction. These findings also provide basic data which could further benefit conservation and sustainable management of bat caves adjacent to the farmland to protect their habitat and prevent population decline, which may help to improve productivity, profitability of the agriculture industry, and consequently promote human well-being.

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Kantima Thongjued

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LIST OF ABBREVIATIONS

BOLD	Barcode of Life Database
COI	Cytochrome C oxidase subunit I
^{o}C	Degree Celsius
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
FOO	Frequency of occurrence
GC	Guanine (G) and Cytosine (C),
h	Hour
M	Mole per litre
m	Metre
µl	Microlitre
min	Minute
ml	Millilitre
mm	Millimetre
mm^2	Square millimetre
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NS	Nucleic acid similarity
OTU	Operational taxonomic unit
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
T_m	Melting temperature
V	Volt

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CHAPTER 1

Introduction

1.1 Background and rationale

Bats play an important role in the ecosystem, including pest regulation (Kunz *et al.* 2011). Globally, insectivorous bat have significantly contributed to human well-being by help suppressing pest insect consequently beneficial for food security, fiber production, or even in preventing emergence of disease pathogen (Cleveland *et al*. 2006; Gonsalves *et al*. 2013; Kemp *et al*. 2019; Puig-montserrat *et al*. 2015; Reiskind and Wund 2009; Wanger *et al*. 2014; Wray *et al*. 2018), particularly the free-tailed bats that form large assemblage colonies could be responsible for the massive services serve to mankind due to the ecosystem service they provide (Boyles *et al*. 2013; Kunz *et al*. 2011; Russo *et al*. 2018). The wrinkle-lipped free-tailed bats (*Chaerephon plicatus*) have been reported to habitually consume insect pests in rice field in central Thailand, particularly planthoppers, one of the most detrimental rice pests in Asia (Leelapaibul *et al*. 2005; Srilopan *et al*. 2018). Currently, analysis in foraging activity of *C. plicatus* in vertical stratification suggested that this bat species actively follows migratory planthoppers in the air 100-200 m above ground. Therefore, *C. plicatus* possibly play an important role in insect pest regulation in agricultural landscape where is adjacent to their roosting cave (Nguyen *et al.* 2019). Economically, the rice yield protection by the bat colony at Khao Chong Phran, Ratchaburi, is valued as high as 1.2 million USD each year (Wanger *et al*. 2014). Therefore, in order to evaluate the role *C. plicatus* play in controlling pest species, their foraging behavior, as well as their impact on the environment, diet analysis must be performed.

Methods for bat diet analysis employed both directly visual observation and DNA-based techniques. Nonetheless, direct observation of foraging behavior is sophisticated, whereas microscopic examination of gut contents or fecal matter requires expertise and intensive labor. Also, because of thorough digestion, soft-bodied preys were often missed (Bohmann *et al*. 2011; Hope *et al*. 2014; Srilopan *et al*. 2018; Whitaker *et al*. 2009; Wray *et al*. 2018; Zeale *et al*. 2011). DNA barcoding-based

approaches have been employed to overcome these limitations. Either group-specific PCR primers or universal primers can be used. With specific primers, one or a few targeted prey DNAs can be recovered. For universal primers, cloning to discriminate mixed-community amplicons followed by sequencing selected clone is employed to identify species (Pompanon *et al*. 2012; Zeale *et al*. 2011). However, this approach cost both massive budget and effort. Currently, next generation sequencing (NGS) is becoming the most powerful tool in diet analysis area, as it allows faster processing with lower cost per sequence when performs in a large batch of samples, but public bioinformatics pipelines required for analyzing a large number of information are still limited and interpretation can be complicated (Pompanon *et al*. 2012). The high cost of establishing and maintaining an NGS facility and the lack experts also hinder NGS accessibility, especially for developing countries (Helmy *et al*. 2016). Therefore, other alternative approaches for diet analysis should be developed

An improved DNA-based method called direct PCR, which bypasses the DNA extraction step, has been successfully used for species identification from feces (Kitpipit *et al*. 2014). The technique saves analysis time and cost, and it has high efficiency with degraded samples. Without prior DNA extraction, it also obviates complex procedure and toxic chemicals (Mercier *et al*. 1990; Panaccio *et al*. 1993). The method is achieved using genetically modified DNA polymerases, which have higher tolerance to inhibitors, and proprietary additives, such as PCR enhancers, to the reaction buffer (Śpibida *et al*. 2017). Direct PCR was first introduced to the entomology community about two decades ago (Grevelding *et al.* 1996). It has been successfully used to identify some insects mainly from order Diptera (e.g. fruit flies, nonbiting midges and mosquitoes) and Lepidoptera (e.g. fall armyworm) (Grevelding *et al*. 1996; Loto *et al*. 2013; Werblow *et al*. 2016; Wong *et al*. 2014). Direct PCR also shows promising results in amplifying DNA from ethanol-preserved samples (Loto *et al*. 2013; Shokralla *et al*. 2010). However, the numbers of samples in these studies were limited and many taxonomic groups still failed to amplify, especially hard-bodied insects such as Coleoptera and Odonata (Wong *et al.* 2014). More recently, direct PCR has been used with next-generation sequencing (NGS) to barcode hundreds and thousands of samples at a very low cost per sample (less than 1 USD per barcode)

(Baloğlu *et al.* 2018; Meier *et al*. 2016; Wang *et al.* 2018). However, the success rates of these NGS-based studies can still be improved (e.g. 60%–80% in Baloğlu *et al*. (2018) and 82% in Wang *et al*. (2018)), and some potentially problematic insect sample types have not been assessed. A direct PCR assay or a simple and effective pre-PCR step that can be universally applied across most insect taxa, sample types, and detection techniques will greatly benefit conservation, epidemiological, agricultural studies.

Denaturing gradient gel electrophoresis (DGGE) is another option successfully used for dietary study (Deagle *et al.* 2005; Lee *et al*. 2013; Martin *et al.* 2006). This electrophoretic system allowed mixed PCR products from various preys eaten by a predator to separate on denaturant-integrated polyacrylamide matrix based on the nucleotide composition (Murray *et al*. 1996; Muyzer *et al*. 1993). Technically, using universal primer in PCR amplification of a fecal matter resulting in PCR products of several preys in identical size. PCR products amplicon of each prey species has unique individually GC content or melting temperature (T_m). In a DGGE gel, double-strand amplicons migrate along an increased denaturing environment. Once these DNA molecules move through increased denaturant concentration, some molecules with lower T_m then partially break that create zipper-like structure of DNA molecules allowing them to stop mobility while other molecules with higher T_m can still be migrated, the low T_m molecules are therefore separated from the whole community first, and follow by higher T_m molecules (Andersen and Larsen 2004). The advantages of this method are non-invasive approach, accurate identification and not dependent on prey morphological structure left in feces. This method is commonly used when study of microbial community, characterization of gut flora (Regensbogenova *et al.* 2004; Simpson *et al.* 1999), characterization of eukaryote community diversity, discrimination among mixed-species in food product (Noh *et al.* 2017; Zhang *et al.* 2007) and especially in diet analysis (Deagle *et al*. 2005; Lee *et al.* 2013; Martin *et al.* 2006), PCR-DGGE is modified targeting variety of genetic makers such as cytochrome *B*, 16S rDNA, 18S rDNA and 28S rDNA. In addition, this option can be applied in a wide range of predators such as sea lion (Deagle *et al*. 2005), krill (Martin *et al.* 2006), and leopard cats (Lee *et al.* 2013). However, this PCR-based method never been applied to diet analysis of insectivorous bat species.

In this study we thus aimed to first examine whether direct PCR could be used to identify insect species spanning a wide range of taxonomic groups, the direct PCR protocol were then developed and fully validated for DNA barcoding. The reproducibility and sensitivity test were performed for this purpose. Second, the well-optimized direct PCR protocol was applied in various insect sample types typically encountered in study ecology including five different sample types (oven-dried, ethanol-preserved, museum, cooked insect and bat guano samples). Third, the developed direct PCR protocol with DGGE technique was applied for insect species identification from bat guano samples to study diet of the wrinkled-lipped free-tailed bats (*C. plicatus*), and fourth, to assess the role of *C. plicatus* play in regulating pest species and also their foraging behavior in the surrounding agricultural landscapes through insect prey species revealed using direct PCR-DGGE.

1.2 Literature review

1.2.1 Study species

Kingdom Animalia
Phylum Chordata
Class Mammalia
Order Chiroptera
Family Molossidae
Genus *Chaerephon*
Species *C. plicatus*

They are smallest of species in the genus *Chaerephon* with forearm length between 43.1-50.2 mm. They have an obvious stout tail process beyond the narrow interfemoral membrane. The fleshy ears are connected by a membrane across the forehead. Their skull is small with an average condylocanine length of 16.6 mm (15.9-17.1 mm). Their braincase is rounded and not flattened above. The rostrum is also rounded and narrow. Their pre-maxillary bones are filled on the palatal side. Pre-maxillae are fused with the surrounding bones, leaving two small foramina at the end of the palate, or a very small notch in front of the incisors (Utthammachai 2009). Their pelage is soft, dense and very short. Their fur are usually dark brown on the dorsal side and paler on the ventral surface (Utthammachai 2009).

Chaerephon is a genus of bats that form very large colonies. The largest colony of *Chaerephon plicatus* in Thailand has approximately 2.6 million bats. It is located at Khao Chong Phran cave (Boonpha *et al*. 2019; Hillman 1999; Leelapaibul *et al*. 2005). The total number of *C. plicatus* in Thailand is approximately 8 million individuals in 18 caves spread across the country except the northeast part (Boonkerd and Wanghongsa 2002). The previous study claimed that free-tailed bats may forage at high altitude up to several kilometers, and as far as 25 km from their caves (Leelapaibul *et al*. 2005; Williams *et al*. 1973). Previous study about the habitat use in central of Thailand concluded that *C. plicatus* activity was highest within 0-5 km from roosting

caves. However, bat activity varied significantly with breeding status and temperature (Utthammachai 2009).

Normally, an insectivorous bat consumes preys of amount about half its body mass per night. They eat and defecate rapidly after meal to reduce body weight and save energy for flying (Boonkerd and Wanghongsa 2002). A previous study indicates that *C. plicatus* consumes a wide range of insect taxa; Lepidoptera, Homoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Odonata and Orthoptera (Leelapaibul *et al.* 2005; Srilopan *et al.* 2018). Their meal varies between development stages. Leelapaibul *et al*. (2005) found that lactating females eat more varied taxa than pregnant females and feed significantly more on Coleopterans and Lepidopterans. In addition, their meal may also vary between feeding bouts. Whitaker *et al.* (1996) has suggested that Mexican free-tailed *Tadarida brasiliensis*, a similar species of *C. plicatus*, consumed a bigger meal in the evening feeding bout than morning and that evening meal contains highly chitinous prey. These insects appeared in fecal matter more than soft-body preys.

1.2.2 Insects

Insects are classified in class Insecta of phylum Arthropoda, the largest group of invertebrate animals having segmented legs. It is estimated that 820,000 species exist around the world, consisting 31 orders. Among that, the 5 highest diverse orders are: Coleoptera (beetles) as high as 300,000 species, Diptera (flies) more than 150,000 species, Lepidoptera (butterflies and moths) as high as 150,000 species, Hymenoptera (ants) as high as 115,000 species and Hemiptera (bugs) as high as 35,000 species (Triplehorn *et al*. 2005).

The body of an insect is cylindrical and bilaterally symmetric. Its body is divided into three parts; the head, within it are the neural integration such as brain, ocelli, antennae, compound eyes; the thorax, origin of three pair of legs and two pair of wings; the abdomen, housing most of the visceral organs, pheromone gland and trachea for respiration. For insects, legs generally work well for molecular-based techniques because these appendages are enriched with muscular tissue that support their

locomotion. A leg composes of five parts: coxa, trochanter, femur, tibia and tarsi. Insect's exoskeleton (sclerites) compose mainly of chitin (polymer of sugar N-acetyl-glucosamine).

In paddy areas, there are several common insect pests such as yellow rice borer (*Scirpophaga incertulas*), leaf-folder (*Cnaphalocrocis medinalis*), brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), zigzag leafhopper (*Recilia dorsalis*), rice green leafhopper (*Nephotettix* sp.), rice Gundhi bug (*Leptocoriza acuta*), southern green stink bug (*Nezara viridula*), grasshopper (*Valanga nigricornis*), and black bug (*Scotinophara* sp.) (Pathak and Khan 1981; Shepard *et al*. 1995). However, planthoppers play a major role economically whereby they harbor rice viruses thus bringing devastating effects to rice plants (Heong and Hardy 2009; Wanger *et al*. 2014). Brown planthopper (BPH; *Nilaparvata lugens*) are also a potential pest in which they feed by phloem abstraction causing hopper-burn disease (Catindig *et al*. 2009; Heong *et al*. 2015; Sogawa 2015); they are also responsible for transmitting economically important viral diseases, including stunt virus, rice ragged stunt virus and grassy stunt virus. Crop failure is also exacerbated by the prevalence of monoculture. This resulted in almost 100% yield losses in 1992 and 1993 of the Thai rice strain SP60 harvests, predominantly due to rice ragged stunt virus transmitted by BPH (Ou 1985; Sogawa 2015; Tinjuangjun *et al*. 2000; Zhang 2007).

1.2.3 DNA barcoding

DNA barcoding is one way of identifying organisms using genomic approach by revealing DNA sequence by means of acting as a barcode unique among species based on the various combinations of the four nucleotides. For a wide range of animals, genomic marker or region that can be used to discriminate among species usually are within the mitochondrial gene-coding regions such as cytochrome C oxidase subunit I (*COI*). Barcoding based on the *COI* have two important advantages: it is easy to design a universal primer based on this region due to conservation of nucleotide sequences (low to no intraspecies variation) in most phyla; also, compared to other mitochondrial regions this gene contains only slight nucleotide bases difference

allowing discrimination of closely related species. On the other hand, *COI* region is not suitable for plant species identification but two genes within the chloroplast: *matK* and *rbcL*, are more appropriate for use as marker due to low mutation rate.

DNA barcode analysis protocol is proposed by a group of researchers, led by Paul Hebert, researcher at the University of Guelph in Ontario, Canada. The overview of standard pipeline includes three main parts; specimen collection and tissue sampling, laboratory operation, and database and data interpretation. For specimen collection, the approach for killing and storage condition should be considered. Using either formalin or ethyl acetate can cause DNA degradation thus posing difficulty in the barcode recovery. The best way for specimen killing are freezing, cyanide fuming and emersion in ethanol. For tissue sampling, it is done on clean surface with sterile equipment to avoid cross-contamination among species.

For laboratory operation, tissues can be extracted using various methods, e.g., Phenol-Chloroform method, Chelex-based extraction, silica membrane-based extraction, magnetic bead-based method (Asghar *et al*. 2015). Extracted genomic DNA is quantified and amplified at the barcode region using PCR amplification. In this step, primer design and usage is a critical factor in barcode recovery. Suboptimal primer condition can cause failure to recover barcode region due to it preferentially amplifying nuclear pseudogene when single-base-pair mismatches at 3'end of the primer. PCR products provided by PCR amplification will be checked on agarose gel and cleanup prior sequencing via capillary sequencer (e.g. ABI PRISM® 3100 Avant Genetic Analyzer, Agilent Technologies 7100 Capillary Electrophoresis System, Lumex Instruments Capel-205 Capillary Electrophoresis System).

In data analysis part, the *COI* electropherograms are edited using DNA analysis software. Two effective software widely used are Sequencher™ (Gene Codes Corporation) and SeqScape® (Applied Biosystems) that can be used for checking base calling, primer trimming and evaluate sequence quality. Good quality sequence will be compare against reference sequence for identification of species that is available on either BOLD (http://www.boldsystems.org/) or NCBI database (http://www.ncbi.nlm.nih.gov/).

DNA barcoding can be used to overcome morphological problems in identification process of insect taxonomy study. Using morphological characteristics for identification is restricted when done in large batch due to high cost & time consumption and requiring specialist. Species identification using cytochrome oxidase subunit I (*COI*) as molecular marker for barcoding is very useful in this case because this system provides reliable and economic solution. Hebert *et al*. (2003) studied potential of *COI* as a discrimination tool for animals by creating *COI* profile of 55 representative species in seven animal phyla and then examined the assay in eight orders of Hexapod including 200 closely related species of Lepidopteran. The results indicated that 53 out of 55 animal species were correctly identified and the others two species that failed to be identified were Annelid and Bivalve. Hexapod and 200 individuals of Lepidopteran were correctly identified (100 percent). Hebert *et al*. (2004) also studied cryptic species in the Neotropical skipper butterfly (*Astraptes fulgerator*). Four hundred and eighty-four individual museum specimens and 30 wild-caught pupae were collected and either 658 bp or 350 bp of *COI* fragments was amplified for reconstruction of phylogenetic tree. Analysis of DNA barcode with color pattern of caterpillars, food plant of caterpillar, habitat distribution showed that within *A. fulgerator* were hidden at least 10 separable species. These taxa have different caterpillar food plants, distinctive color pattern of caterpillars and different ecosystem preferences. However, their similarity indicated common ancestor and succession of mimic fashion to survive in nature.

In addition to full-length barcode (658 bp), 'mini-barcode' was also proposed as a potential marker instead of the 658 bp full-length *COI* barcode for routine identification in degraded museum specimens. The developed short fragments targets (~100 and ~200 bp) were analyzed both *in silico* and experimentally. Using MEGA software for comparison of percentage of variable and parsimony informative site in fishes and Lepidopteran insects indicated that 93% and 92% of the species were correctly identified with the 218 bp and 109 bp mini-barcodes, respectively, compared to 95% with the 658 bp *COI* full-length barcode. For the experimental test, two pairs of primer were designed from the 3' end of original full-length barcode to amplify 221 bp and 134 bp amplicon. Amplification performed in 2 to 21 years oven-dried Sphingid

moths demonstrated 94% and 97% success rates in species-level discrimination respectively. Furthermore, mini marker of 407 bp from the 3′ end and 135 bp from the 5′ end of the original full-length *COI* barcode were designed for amplifying 1 to 14 years ethanol-preserved Braconid insects. The results showed 84% and 98% success rate from recovering 407 bp and 135 bp mini-barcodes respectively. This supports the advantage of DNA barcoding method for quick and reliable routine identification even if DNA degradation occur (Hajibabaei *et al*. 2006).

From agricultural aspect, accurate identification is essentially for pest management. DNA barcoding plays an important role whereby it helps distinguishing species without considering development stages or lifeform of animals. This approach therefore profits in monitoring of introduced taxa and facilitates to establish quarantine plan and control them immediately (Armstrong and Ball 2005; Waugh 2007; Wilson *et al*. 2017).

1.2.4 Direct PCR

Direct PCR is an approach of amplifying target DNA via polymerase chain reaction (PCR) by placing samples directly into the reaction without prior DNA extraction. This method offers rapid, low cost and high sensitivity due to omission of extraction process that can cause up to 70% DNA loss through the multiple wash steps and transferring during extraction process. In the previous decade, direct PCR has been applied for detection of microorganism from polluted environment, food and clinical sample including human forensic samples.

Applications of direct PCR in insect species have been neglected because of low amplification success rate. In 1996, Grevelding *et al.* demonstrated that direct PCR is possible for application in multicellular organisms, DNA amplification is possible to be done directly from intact tissue without DNA isolation. In this study, fruit flies (*Drosophila melanogaster*) and blood flukes (*Schistosoma mansoni*) were used as model organisms. PCR amplification was performed on embryos, first and third instar larvae, pupae and adults of fruit flies, using initial incubation at 95 ºC for 5 minutes with samples in PCR master mix prior beginning of normal PCR cycling. The expected

368 bp PCR product was obtained from most of the life stages and also provided high quality PCR product, results similar to using purified DNA template, with only exception from pupae samples. These results indicated that DNA isolation process is not essential as initial incubation can lyse cells leading to the release of ample DNA as template for PCR amplification. However, the amplification success rates were not reported in this study.

Shokralla *et al*. (2010) proposed that the preservative medium of specimen can be used as a source of DNA template for PCR amplification. For proving the hypothesis, the liquor containing caterpillars were incubated at 56 ºC for the alcohol to evaporate. The residue was dissolved with molecular grade water and extracted with column extraction (NucleoSpin®kit) for eliminating impurities from subsequent PCR amplification and sequencing. PCR was successful and good quality sequence was obtained from this process. Accurate taxon identification was obtained based on the BOLD database similarly with that using extracted DNA from caterpillar tissue. In parallel, fresh insect specimens (caddisflies and mayflies) were preserved in 95% ethanol for 24 hours then the preserved medium was transferred and evaporated. Residue was dissolved and use as DNA template in direct PCR amplification. The result indicated 100% amplification success rate (N=25) of full-length barcodes were obtained by this method thus supported that preservative medium can be used as a source of DNA template for direct amplification without invasive procedure on samples and useful in cases with lacking of tissue for starting material.

Direct PCR can be used not only for fresh tissue, but it can be also successfully be used to amplify ethanol-preserved samples. Previous study has examined the efficiency of direct PCR amplification from fall armyworm samples (*Spodoptera frugiperda*). To test the performance of the method eggs and neonate larvae were used, amplified using either fresh or preserved samples (freezing and ethanol preserved) as DNA template source. High initial temperature (97 ºC for 6 min) were also used in the incubating process prior normal PCR cycling. The presence of 569 bp-barcode band indicated that in the case of using egg for DNA template source, one or two eggs were enough to provide successful amplification as extracted genomic DNA. However no PCR product was obtained when three eggs were used as template

as increasing the number of eggs subsequently increase the degree of PCR inhibition either by template overload or inhibitor action. The result also indicated that preserving of eggs by both freezing and ethanol provide poor PCR success rate. Conversely in neonate larvae, the samples preserved by freezing or ethanol provided better result than fresh ones hence suggesting that preserved tissues can be used as DNA template source; but the low success rate obtained could be due to suboptimal amount of tissue used for the reactions (Loto *et al*. 2013).

Optimal protocol for direct PCR has been firstly proposed for use with Chironomid insects (Diptera): the effective bioindicator taxa. In this study, four key factors for high amplification success rate was reported: tissue quantity, body part (source of tissue), primer pair and type of *Taq* polymerase used. In the experiment, tissue source and quantity of tissue are the critical factors. Chironomid species of larvae and adults were separated to three classes depending on its size. For mature insect, a single whole body was used for smallest class, three legs for medium class and two legs for biggest class. While for larvae, ~1 mm of anterior segment of all classes were used as DNA template. The result showed that amplification success rates using suggested amount of tissue were high, ranging from 90-100% of all classes and gave good sequence quality, comparable to the sequences obtained using purified DNA template. However, the developed protocol was unsuccessful for use with heavily sclerotized taxa and glandular bodies (Wong *et al*. 2014).

1.2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a method that could separate similar or same-sized PCR amplicons based on their melting temperature (T_m) along a denaturant concentration gradient within an electrophoresed polyacrylamide matrix. DGGE has given promising results for diversity studies in environmental microbial community (e.g. Muyzer *et al*. 1993, Murray *et al.* 1996), and gut flora community (Simpson *et al*. 1999, Regensbogenova *et al.* 2004). For the last two decades, DGGE has been employed to characterize eukaryote community diversity (Díez *et al*. 2001; Gast *et al*. 2004; van Hannen *et al*. 1998), to assess dietary diversity

or identify prey species consumed by potential predators (Deagle *et al*. 2005; *et al*. 2006; Pompanon *et al*. 2012; Lee *et al*. 2013). This method has also been applied to characterize animal species in food products (Noh *et al*. 2017; Zhang *et al.* 2007)

This technique could be employed to address sequence heterogeneity in complex mixture for various applications based on using both group-specific and universal primers (Martin *et al.* 2006; Pompanon *et al.* 2012). Molecular markers used in PCR-DGGE technique can be varied depending on target taxa and purpose of each study. Based on study for species identification and phylogenetic analysis, species-specific information among different organism can be given by analyzing distinguished genes (Kocher *et al*. 1989). The primer target used in previous studies therefore varied: 16S rDNA was targeted for bacteria community study (Simpson *et al*. 1999; Regensbogenova *et al.* 2004); 18S rDNA for zooplankton (Martin *et al*. 2006); cytochrome oxidase subunit I for fishes (Noh *et al.* 2017); and chloroplast *rbcL* gene for plant species (Irwin and Orrego 1998).

1.3 Objectives

1.3.1 To develop and validate a direct PCR protocol for various insect species identification spanning a wide range of taxonomic groups. Full validation of the developed protocol was also performed to test its robustness in terms of reproducibility and sensitivity test.

1.3.2 To examine applicability of the developed protocol in various ecological sample types including oven-dried, ethanol-preserved, museum, cooked insect and bat guano samples.

1.3.3 To apply the developed direct PCR workflow with DGGE for insect species identification to analyze diet using guano samples of wrinkled-lipped free-tailed bats (*C. plicatus*).

1.3.4 To study diet of *C. plicatus* for assessing their ecology in foraging behavior, seasonal variation of their prey species and ecosystem service they contributed to agricultural landscape adjacent to the roosting cave.

CHAPTER 2

Methodology

The research was divided into two parts. The first part was conducted to develop and fully validate a direct PCR protocol in order to use as an identification tool for a wide range of insect species. The experiment began from optimization of the protocol comprising seven steps as shown in Figure 1. This well-optimized protocol was then validated with three tests: reproducibility test, sensitivity test, and applicability test. The testes were done to examine efficiency that the developed protocol can be universally used with variable tissue amounts from various tissue sources, to investigate optimum and minimum amount of tissue used to prepare pre-PCR solution, and to test if the developed protocol was able to be used in various ecological sample types (e.g. oven-dried, ethanol-preserved, museum, food, and bat guano samples). In the second part, the well-developed protocol was used with DGGE to analyze the wrinkle-lipped free-tailed bat diet (Figure 1) to reveal their feeding behavior and seasonal variation of their insect prey species, as well as to assess ecosystem service this bat species contributed to agricultural landscape as a potential biological pest control agent.

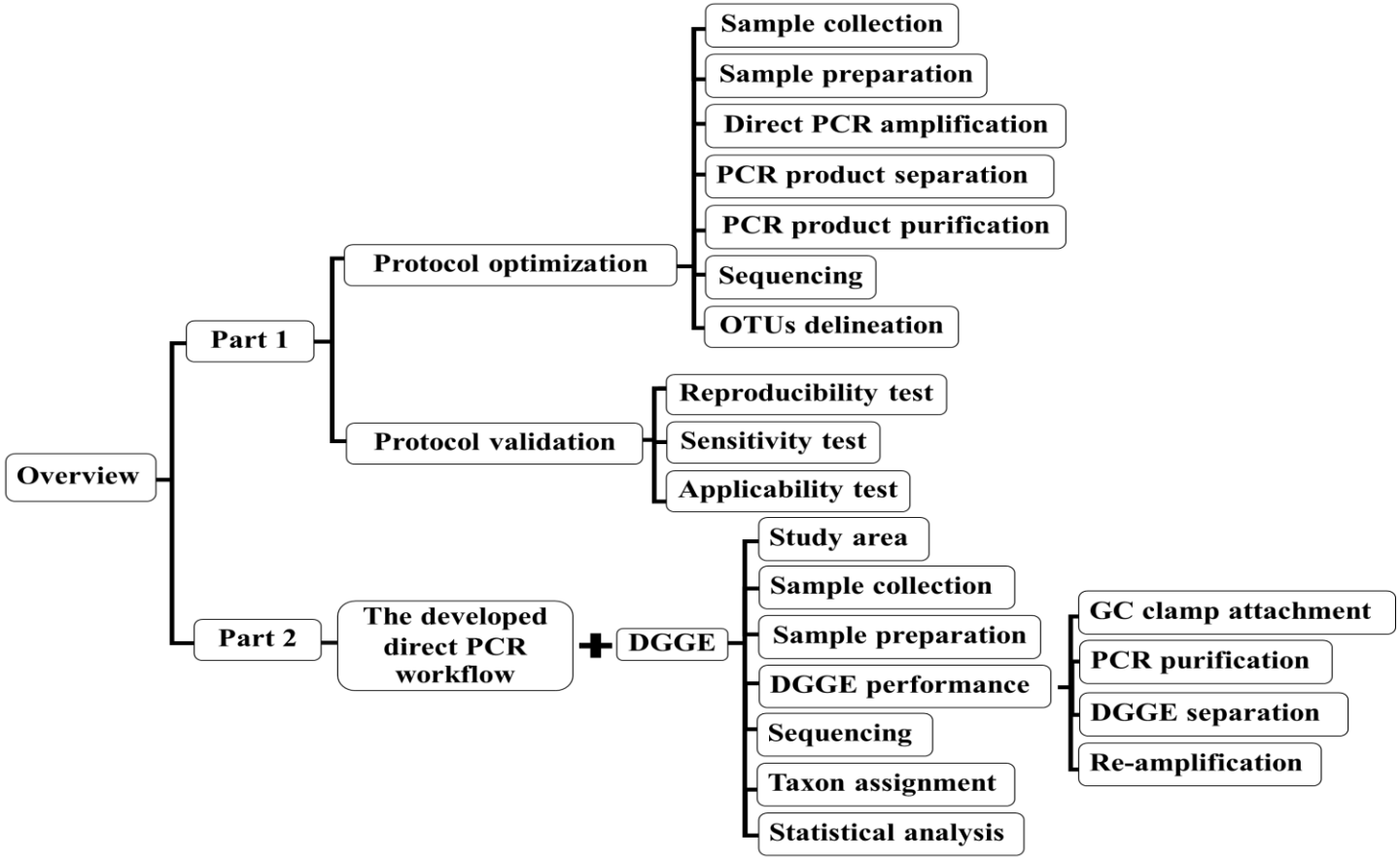

Figure 1 Flow chart showing overview of the experiments conducted in the present study. Part 1 experiment comprises two sub-parts: protocol optimization and validation. Part 2 experiment was conducted to analyze the wrinkle-lipped free-tail bat diet using the successfully developed workflow (from part 1) with DGGE.

2.1 Study area

C. plicatus guano pellet samples were collected from 2 caves in central Thailand. First, Khao Wongkot Cave in Lop Buri province (15°01’06.04”N, 100°32’42.81”E) where is home to approximately a million individuals of *C. plicatus.* Within 20-km radius around this cave, rice fields contribute the most proportion of land use (70%) follows by human settlement, sugarcane, and corn and cassava plantations accounted for 20, 8, and 2%, respectively (Srilopan *et al.* 2018). The second study site located at Khao Chakan cave, Sa Kaeo province (13°39’44.86”N, 102°05’25.50”E), in which around 300,000 bat individuals have been estimated. Land use in this area within 20-km radius around bat roosting cave encompasses cassava plantation (26%) follows by human settlement, sugar cane plantation, rice field, and rubber plantation accounts for 25, 24, 22, and 3%, respectively (Srilopan *et al.* 2018).

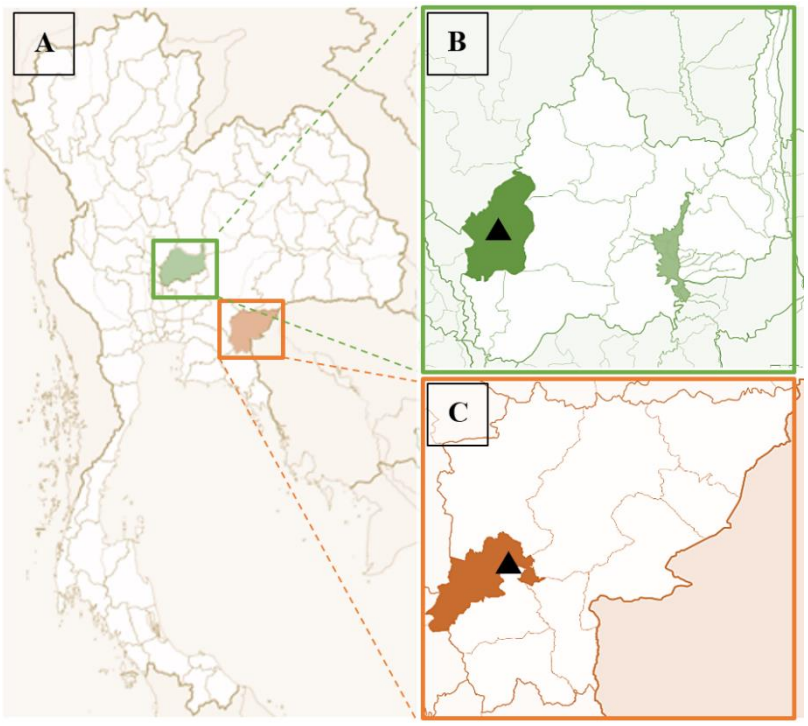

Figure 2 Maps showing study areas (A; left) Thailand, (B; right; top) Lop Buri province, and (C; right; bottom) Sa Kaeo province. Black triangles indicate locations in which the study areas are took place.

2.2 Sample collection

In this study we collected 593 insect samples, including 160 fresh, 30 dried, 10 museum, 143 ethanol-preserved, 240 bat guano, and 10 food samples. Taxonomic details and location where the samples were collected are shown in Table 1. Fresh insect specimens were used to optimize and test the performance of the workflow, and the remaining 433 non-fresh samples (dried, museum, ethanol-preserved, bat guano, and food samples) were used to validate the workflow and diet analysis of wrinkle-lipped free-tailed bats.

Table 1 A total number of specimens used in the present study. Details including taxonomic categories (order of insect, families, genera, and species), sample size, and location that collected each sample (Latitude, Longitude). The number before the slash (n/) indicates the number of samples that were amplified and sequenced successfully and the number after the slash (/n) indicates the total number of samples. (#Y) indicates the age of the specimens in number of years (e.g. 5Y means 5-year-old specimen).

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Blattodea	Ectobiidae	*Blattella*	*Blattella lituricollis* (Walker, 1868)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Blattodea	Blattidae	*Periplaneta*	*Periplaneta americana* (Linnaeus, 1758)	14/14	14	-	-	-	-	7.01N, 100.52E
Coleoptera	Cerambycidae	*Aeolesthes*	*Aeolesthes aurifaber* (White, 1853)	1/1	1	-	-	-	-	7.01N, 100.52E
Coleoptera	Carabidae	*unknown*	*unknown*	1/1	1	-	-	-	-	7.01N, 100.52E
Coleoptera	Curculionidae	*Hypomeces*	*Hypomeces squamosus* (Fabricius, 1792)	2/2	1	1(3Y)	-	-	-	18.82N, 98.88E
Coleoptera	Chrysomelidae	*Micraspis*	*Micraspis discolor* (Fabricius, 1798)	6/6	6	-	-	-	-	7.80N, 100.24E
Coleoptera	Coccinellidae	*Nephus*	*Nephus ryuguus* (Kamiya, 1966)	1/1	1	-	-	-	-	14.01N, 99.97E
Coleoptera	Curculionidae	*Ophionae*	*Ophionea nigrofasciata* (Schmidt-Gobel, 1846)	0/1	1	-	-	-	-	7.01N, 100.52E
Coleoptera	Cerambycidae	*Orthosoma*	*Orthosoma brunneum* (Forster, 1771)	1/1	1	-	-	-	-	7.01N, 100.52E
Coleoptera	Staphilinidae	*Paederus*	*Paederus fuscipes* (Curtis, 1840)	8/8	4	1(3Y)	3(5Y)	-	-	7.80N, 100.24E
Diptera	Tephritidae	*Bactrocera*	*Bactrocera dorsalis* (Hendel, 1912)	25/26	2	2(3Y)	22(3Y)	-	-	7.01N, 100.52E
Diptera	Calliphoridae	*Chrysomya*	*Chrysomya rufifacies* (Macquart, 1843)	1/1	1	-	-	-	-	7.01N, 100.52E

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Diptera	Calliphoridae	*Lucilia*	*Lucilia cuprina* (Wiedemann, 1830)	0/1	1	-	-	-	-	7.01N, 100.52E
Diptera	Calliphoridae	*Sarcophaga*	*Sarcophaga peregrina* (Robineau-Desvoidy, 1830)	1/1	1	-	-	-	-	7.01N, 100.52E
Diptera	Tipulidae	*unknown*	*Tipulidae* sp. (Latreille, 1802)	0/1	1	-	-	-	-	7.01N, 100.52E
Diptera	Culicidae	*Mansonia*	*Mansonia bonneae* (Edwards, 1930)	15/15	15	-	-	-	-	7.01N, 100.52E
Hemiptera	Coreidae	*Anoplocnemis*	*Anoplocnemis phasiana* (Fabricius, 1781)	1/1	1	-	-	-	-	7.01N, 100.52E
Hemiptera	Cicadellidae	*Bothrogonia*	*Bothrogonia* sp. (Melichar, 1926)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Hemiptera	Cicadellidae	*unknown*	*unknown*	2/2	2	-	-	-	-	7.80N, 100.24E
Hemiptera	Cicadellidae	*Recilia*	*Recilia dorsalis* (Motschulsky, 1859)	23/23	1	-	22(5Y)	-	-	7.80N, 100.24E
Hemiptera	Cicadellidae	*Nephotettix*	*Nephotettix virescens* (Distant, 1908)	31/31	7	-	24(5Y)	-	-	7.80N, 100.24E
Hemiptera	Cicadidae	*unknown*	*unknown*	2/2	2	-	-	-	-	7.80N, 100.24E
Hemiptera	Delphacidae	*Nilaparvata*	*Nilaparvata lugens* (Stål, 1854)	27/27	1	-	26(5Y)	-	-	7.80N, 100.24E
Hemiptera	Delphacidae	*Sogatella*	*Sogatella furcifera* (Horváth, 1899)	20/20	1	-	19(5Y)	-	-	7.80N, 100.24E
Hemiptera	Cicadidae	*Dundubia*	*Dundubia nagarasingna* (Distant, 1881)	8/8	1	4(3Y)	3(3Y)	-	-	7.01N, 100.52E
Hemiptera	Pentatomidae	*Eocanthecona*	*Eocanthecona furcellata* (Wolff, 1811)	3/4	1	1(3Y)	2(3Y)	-	-	14.01N, 99.97E
Hemiptera	Flatidae	*unknown*	*unknown*	0/1	1	-	-	-	-	7.01N, 100.52E
Hemiptera	Reduviidae	*Leptocorisa*	*Leptocorisa oratoria* (Fabricius, 1764)	0/1	1	-	-	-	-	7.01N, 100.52E
Hemiptera	Pentatomidae	*unknown*	*unknown*	0/1	1	-	-	-	-	7.01N, 100.52E

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Hemiptera	Reduviidae	*unknown*	*unknown*	0/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Formicidae	*Anoplolepis*	*Anoplolepis gracilipes* (Smith, 1857)	1/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Braconidae	*Bracon*	*Bracon hebetor* (Say, 1857)	0/1	1	-	-	-	-	14.01N, 99.97E
Hymenoptera	Formicidae	*Camponotus*	*Camponotus rufoglaucus* (Jerdon, 1851)	0/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Braconidae	*Cotesia*	*Cotesia flavipes* (Cameron, 1891)	1/1	1	-	-	-	-	14.01N, 99.97E
Hymenoptera	Formicidae	*Diacamma*	*Diacamma rugosum* (Le Guillou, 1842)	1/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Formicidae	*Monomorium*	*Monomorium destructor* (Jerdon, 1851)	1/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Formicidae	*Oecophyla*	*Oecophylla smaragdina* (Fabricius, 1775)	1/1	1	-	-	-	-	7.01N, 100.52E
Hymenoptera	Formicidae	*Tapinoma*	*Tapinoma melanocephalum* (Fabricius, 1793)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Abraxas*	*Abraxas lugubris* (Prout, 1925)	4/4	1	2(3Y)	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Arctiidae	*Amata*	*Amata* sp. 1 (Fabricius, 1807)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Amathusia*	*Amathusia friderici* (Fruhstorfer, 1904)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Amerila*	*Amerila* sp. (Walker, 1855)	4/4	1	2(3Y)	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Sphingidae	*unknown*	*unknown*	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Asota*	*Asota caricae* (Fabricius, 1775)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Celerena*	*Celerena signata* (Warren, 1898)	3/3	1	1(3Y)	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Arctiidae	*Amata*	*Amata* sp. 2 (Fabricius, 1807)	1/1	1	-	-	-	-	7.01N, 100.52E

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Lepidoptera	Arctiidae	*Creatonotos*	*Creatonotos gangis* (Linnaeus, 1763)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Arctiidae	*Creatonotos*	*Creatonotos transiens* (Walker, 1855)	3/3	1	1(3Y)	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Arctiidae	*Cyana*	*Cyana coccinea* (Moore, 1878)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Arctiidae	*Cyana*	*Cyana cruentata* (Talbot, 1926)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Sphingidae	*Daphnis*	*Daphnis nerii* (Linnaeus, 1758)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Eudocima*	*Eudocima* sp. (Billberg, 1820)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Xantodes*	*Xanthodes transversa* (Guenée, 1852)	1/1	1	-	-	-	-	18.82N, 98.88E
Lepidoptera	Erebidae	*Euplocia*	*Euplocia membliaria* (Cramer, 1780)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia alpheda* (Godart, 1824)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia evelina* (Stoll, 1790)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia evelina* (Stoll, 1790) †	0/1	-	1(6Y)	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia malaccana* (Fruhstorfer, 1889)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia monina* (Fabricius, 1787)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Hesperiidae	*unknown*	*unknown*	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Hypolimnas*	*Hypolimnas bolina* (Linnaeus, 1758)	3/3	1	1(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Hypolimnas*	*Hypolimnas bolina* (Linnaeus, 1758) †	1/1	-	1(3Y)	-	-	-	7.21N, 100.38E
Lepidoptera	Nymphalidae	*Hypolimnas*	*Hypolimnas bolina* (Linnaeus, 1758) †	0/1	-	1(39Y)	-	-	-	7.89N, 98.38E

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Lepidoptera	Noctuidae	*Marumba*	*Marumba* sp. (Moore, 1882)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Melanitis*	*Melanitis leda* (Linnaeus, 1758)	5/5	2	2(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Mycalesis*	*Mycalesis janardana* (Fruhstorfer, 1908)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Papilionidae	*Papilio*	*Papilio memnon* (Linnaeus, 1758)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Mycalesis*	*Mycalesis mineus* (Linnaeus, 1758)	2/2	1	-	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Geometride	*Ornithospila*	*Ornithospila esmeralda* (Hampson, 1895)	2/2	1	-	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Papilionidae	*Papilio*	*Papilio polytes* (Linnaeus, 1758)	2/2	1	1(3Y)	-	-	-	7.01N, 100.52E
Lepidoptera	Arctiidae	*Pareuchaetes*	*Pareuchaetes insulata* (Walker, 1855)	2/2	1	1(3Y)	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Polyura*	*Polyura athamas* (Drury, 1773)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Arctiidae	*Psilogramma*	*Psilogramma* sp. (Rothschild & Jordan, 1903)	1/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Noctuidae	*Spodoptera*	*Spodoptera litura* (Fabricius, 1775)	2/2	1	1(3Y)	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Tanaecia*	*Tanaecia julii* (Lesson, 1837)	6/6	2	3(3Y)	1(3Y)	-	-	7.01N, 100.52E
Lepidoptera	Arctiidae	*Trigonodes*	*Trigonodes hyppasia* (Cramer, 1779)	3/3	1	1(3Y)	1(3Y)	-	-	18.82N, 98.88E
Lepidoptera	Uranidae	*Lyssa*	*Lyssa zampa* (Butler, 1773)	0/1	1	-	-	-	-	7.01N, 100.52E
Lepidoptera	Nymphalidae	*Parthenos*	*Parthenos sylvia* (Cramer, 1776) †	1/1	-	1(5Y)	-	-	-	6.99N, 100.15E
Lepidoptera	Nymphalidae	*Melanocyma*	*Melanocyma faunula* (Westwood, 1850) †	0/1	-	1(6Y)	-	-	-	7.36N, 99.96E
Lepidoptera	Nymphalidae	*Doleschallia*	*Doleschallia bisaltide* (Cramer, 1777) †	1/1	-	1(6Y)	-	-	-	7.01N, 100.52E

Order	Family	Genus	Species	Amplifiable samples/Total sample tested						Location
				Overall	Fresh	Dry	Ethanol	Fecal	Food	
Lepidoptera	Nymphalidae	*Euthalia*	*Euthalia dunya* (Doubleday, 1848) †	0/1	-	1(19Y)	-	-	-	6.95N, 100.23E
Lepidoptera	Nymphalidae	*Junonia*	*Junonia lemoias* (Linnaeus, 1758) †	0/1	-	1(33Y)	-	-	-	6.95N, 100.23E
Lepidoptera	Nymphalidae	*Euploea*	*Euploea mulciber* (Cramer, 1777) †	0/1	-	1(35Y)	-	-	-	6.99N, 100.15E
Lepidoptera	Nymphalidae	*Euploea*	*Euploea modesta* (Butler, 1866) †	0/1	-	1(38Y)	-	-	-	6.95N, 100.23E
Neuroptera	Chrysopidae	*unknown*	*unknown*	1/1	1	-	-	-	-	7.01N, 100.52E
Neuroptera	Chrysopidae	*Plesiochrysa*	*Plesiochrysa ramburi* (Schneider, 1851)	5/5	4	-	1(3Y)	-	-	14.01N, 99.97E
Odonata	Libellulidae	*Neurothemis*	*Neurothemis fulvia* (Drury, 1773)	4/4	4	-	-	-	-	7.01N, 100.52E
Odonata	Libellulidae	*Neurothemis*	*Neurothemis tullia* (Drury, 1773)	2/2	2	-	-	-	-	7.01N, 100.52E
Odonata	Libellulidae	*Tholymis*	*Tholymis tillarga* (Fabricius, 1798)	6/6	6	-	-	-	-	7.01N, 100.52E
Odonata	Coenagrionidae	*Coenagrionidae*	*Coenagrionidae* sp. (Kirby, 1890)	1/1	1	-	-	-	-	7.01N, 100.52E
Orthoptera	Tettigoniidae	*unknown*	*Tettigoniidae* sp. 1 (Krauss, 1902)	4/4	4	-	-	-	-	7.01N, 100.52E
Orthoptera	Tettigoniidae	*unknown*	*Tettigoniidae* sp. 2 (Krauss, 1902)	6/6	6	-	-	-	-	7.01N, 100.52E
Phasmatodea	Heteropterygidae	*Heteropteryx*	*Heteropteryx* sp. (Parkinson, 1798)	8/8	8	-	-	-	-	8.72N, 99.70E
Mantodea	Mantidae	*Mantidae* sp.	*Mantidae* sp. (Burmeister, 1838)	4/4	4	-	-	-	-	7.01N, 100.52E
Unidentified	*unidentified*	*unidentified*	*unidentified*	207/240	-	-	-	240	-	15.22N, 100.55E
Lepidoptera	*unidentified*	*unidentified*	*unidentified*	9/10	-	-	-	-	10	7.15N, 100.59E
			Total	540/593	160	40	143	240	10	

† **refers to specimens obtained from the museum**

Fresh insect samples were either collected from the forestry area of Prince of Songkla University, Thailand using various collecting methods (swipe net, light trapping, fruit trapping and pit fall trapping), or donated by the National Biological Control Research Center and His Majesty the King Insects Park, Kasetsart University, Thailand (Table 1). All specimens were classified to various taxonomic levels before further processing by morphological characteristics following classification keys (Kononenko and Pinratana 2005; Pinratana and Černy 2009; Triplehorn *et al*. 2005). Individual samples were kept in sterile plastic bags and stored at -20 °C until further analysis. When the study was done, the samples were arranged to deposit according to the suggestion of the Princess Maha Chakri Sirindhorn Natural History Museum, Thailand.

To test the efficiency of workflow on stored samples and ecological samples, four sample types were used (total N = 218), including dried specimens (N = 40), ethanol-preserved specimens (N = 143), pellets of bat droppings (N = 25) and food specimens (N = 10) (see Table 1 for details). Forty dried specimens were collected from two sources. First, thirty insects were dried from pinned specimens by drying in a hot air oven at 50 °C for 2 weeks. Second, ten dried specimens (age between 3 and 39 years) were provided by the Princess Maha Chakri Sirindhorn Natural History Museum. For ethanol-preserved specimens, 143 wholly preserved specimens (age between 3 and 5 years) were donated by Small Mammals, Bird, and Spiders Research Unit, Department of Biology, Faculty of Science, Prince of Songkla University. These specimens were preserved individually in a 1.5 ml microcentrifuge tube (or 50 ml vial for large insect) containing 70% ethanol and were preserved immediately after sampling in the field. Twenty-five pellets of insectivorous bat guanos were also donated by the same group. Bat guano pellets samples were collected every month from October 2015 until September 2016 by placing plastic baskets underneath roosting position. For each collected position, pellets were kept dry in 1.5 ml tube with silica gel in-field before transfer to -20 ºC for long-term storage. A single pellet in each collecting position was randomly chosen for insect DNA analysis. A total of ten samples per month was analyzed which accounted for 240 guano pellets (10 pellets*12 months*2 caves). Nonetheless, in the initial study, only 25 of the collected bat guano pellet samples were

used to examine whether the developed protocol is applicable to fecal matters analysis. All of the bat guano pellet samples collected were used for addressing diet of *C. plicatus* in the present study. In addition, 10 food samples collected from street food markets were included for testing in this study.

2.3 Sample preparation

Fresh samples and dried insect samples were prepared by putting a few pieces of approximately 1×1 mm^2 leg (for large insect) or whole body (for small insect) in a 1.5 ml microcentrifuge tube. Twenty micro litres of 1X phosphate buffer saline (PBS) was added to the tube before briefly mixing at room temperature and incubating at 98 °C for 2 min. The supernatant, called pre-PCR solution, was then added directly to a PCR mastermix instead of purified DNA.

For ethanol-preserved specimens, the samples were dip-rinsed in sterile distilled water, briefly shaken using vortex mixer, wiped dry with a filter paper and prepared as same as fresh specimens (i.e. dissected, added to 20 µl PBS, mixed and incubated for 2 min).

For bat guano samples, a single pellet was ground in a 1.5 ml sterile tube into fine powder using sterile plastic pestle. The powder was then mixed with 1,000 µl of PBS and briefly centrifuged. Twenty micro litres of clear supernatant was transferred to a new tube, incubated for 2 min and used as pre-PCR solution.

For food samples, insect tissue was dissected to 1×1 mm^2 in cross section surface and prepared like the fresh specimens (i.e. dissected, added to 20 µl PBS, mixed and incubated for 2 min).

2.4 Direct PCR amplification

PCR amplification was carried out using the Phire® Hot Start II DNA polymerase kit (Thermo Fisher Scientific, USA). PCRs were prepared in total volume of 20 µl comprising 1X PCR buffer, 0.2 mM dNTPs, 1.0 unit Phire® Hot Start II DNA polymerase, 1 µl of pre-PCR solution, sterile distilled water and primers shown in Table 2. PCR was performed using the T100™ Bio-Rad thermal cycler (Bio-Rad, USA) using the PCR conditions listed in Table 2. In case of failure to amplify initially, a second amplification was performed by using a freshly prepared pre-PCR solution made from a different starting tissue of the same specimen.

Table 2 The seven selected primer pairs that is targeted to *COI* gene of different taxonomic groups are shown. Details of each primer used in this study including primer name, sequence, expected amplicon size (bp), targeted taxa, thermal steps condition, and references. For sequences used to design primer Planthopper F/R, is additional shown in Table 3.

Primer name	Sequence (5' to 3')	Size (bp)	Target species	Thermal steps		References
				Typical PCR	Touch down PCR	
UEA7 UEA10	TACAGTTGGAATAGACGTTGATAC TCCAATGCACTAATCTGCCATATTA	700	Diptera	Initial denature: 94 ºC, 5 min Denaturation: 94 ºC, 40 s Annealing: 55 ºC, 60 s Extension: 72 ºC, 40 s Final extension: 72 ºC, 2 min Number of cycles: 35		Lunt *et al*. 1996
tRWF1 LepR	AAACTAATARCCTTCAAAG TAAACTTCTGGATGTCCAAAAAATCA	700	Orthoptera Mantodea Phasmatodea	Initial denature: 95 ºC, 2 min Denaturation: 94 ºC, 40 s Annealing: 45 ºC, 40 s Extension: 70 ºC, 70 s Number of cycles: 5	Denaturation: 94 ºC, 40 s Annealing: 51 ºC, 40 s Extension: 72 ºC, 70 s Final extension: 72 ºC, 2 min Number of cycles: 40	Park *et al*. 2010
LepF LepR	ATTCAACCAACCAATCATAAAGATATTGG TAAACTTCTGGATGTCCAAAAAATCA	658	Universal primer for insect taxa	Initial denature: 94 ºC, 1 min Denaturation: 94 ºC, 30 s Annealing: 45 ºC, 40 s Extension: 72 ºC, 60 s Number of cycles: 5	Denaturation: 94 ºC, 30 s Annealing: 55 ºC, 40 s Extension: 72 ºC, 60 s Final extension 72 ºC, 2 min Number of cycles: 35	Hebert *et al*. 2004
CI-J-1632 CI-N-2191	TGATCAAATTTATAAT GGTAAAATTAAAATATAAACTTC	570	Coleoptera	Initial denature: 95 ºC, 3 min Denaturation: 95 ºC, 30 s Annealing: 45 ºC, 60 s Extension: 72 ºC, 60 s Final extension: 72 ºC, 2 min Number of cycles: 35		Simon *et al*. 1994
ShortF ShortR	CAATTTCCAAATCCNCCAAT GGTCAACAAATCATAAAGATATTGGAA	220	Coleoptera	Initial denature: 98 ºC, 30 s Denaturation: 98 ºC, 5 s Annealing: 50 ºC, 5 s Extension: 72 ºC, 10 s Final extension: 72 ºC, 1 min		Gilbert *et al*. 2007

Primer name	Sequence (5’ to 3’)	Size (bp)	Target species	Thermal steps		References
				Typical PCR	Touch down PCR	
				Number of cycles: 35		
ZBJ-ArtF ZBJ-ArtR	AGATATTGGAACWTTATATTTTATTTTTGG WACTAATCAATTWCCAAATCCTCC	211	Universal primer for insect taxa	Initial denature: 94 ºC, 3 min Denaturation: 94 ºC, 30 s Annealing: 61 ºC, 30 s Extension: 72 ºC, 30 s Number of cycles: 16	Denaturation: 94 ºC, 30 s Annealing: 53 ºC, 30 s Extension: 72 ºC, 30 s Final extension: 72 ºC, 2 min Number of cycles: 24	Zeale *et al*. 2011
Planthopper F Planthopper R	TTAATAATTGGTGCACCAGATATAG AWAGGGGGGGATAAAYDGTTC	145	Hemiptera			Self-developed primer

Table 3 Detail of species used for primer designed

Order	Family	Species	Common name	Accession number
Hemiptera	Delphacidae	*Nilaparvata lugens*	Brown planthopper	JN563997.1
		Nilaparvata lugens	Brown planthopper	JX880069.1
		Nilaparvata muiru	Planthopper	JN563998.1
		Nilaparvata bakeri	Planthopper	NC_033388.1
		Sogatella furcifera	White-backed planthopper	NC_021417.1
		Sogatella furcifera	White-backed planthopper	KC512915.1
		Laodelphax striatella	Small brown planthopper	FJ360695.1
		Laodelphax striatella	Small brown planthopper	JX880068.1
	Cicadellidae	*Nephotettix cincticeps*	Green leafhopper	NC_026977.1
		Nephotettix virescens	Green leafhopper	AB976528.1
		Nephotettix virescens	Green leafhopper	KU324170.1
		Nephotettix virescens	Green leafhopper	HM160144.1
		Nephotettix virescens	Green leafhopper	KU324167.1
		Recilia dorsalis	Zigzag leafhopper	KU324164.1
		Recilia dorsalis	Zigzag leafhopper	KU324166.1
		Recilia dorsalis	Zigzag leafhopper	KU324165.1
		Recilia dorsalis	Zigzag leafhopper	KU324163.1

2.5 PCR product separation and visualization

2.5.1 Agarose gel electrophoresis

PCR products were checked using agarose gel electrophoresis. To do this, 2% agarose gel stained with ethidium bromide was prepared and loaded with 20 µl PCR products along with 100 bp DNA ladder (Thermo Fisher Scientific, USA). Both PCR products and 100 bp DNA ladder were mixed well with loading dye (Thermo Fisher Scientific, USA) in proportion 5:1 before loaded in each gel's well. The system was run in 1X Tris-Borate-EDTA (TBE buffer) at 120 V for 30 min then visualized under Bio-Rad Gel Doc™ EZ system (Bio-Rad, USA).

2.5.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE was employed for diet analysis from bat guano. To do this four steps were performed; amplification of target region and GC-clamp attachment using nested PCR, PCR product purification, DGGE separation and re-amplification each isolated amplicon.

For amplification of target region (Figure 3) and GC-clamp attachment, second PCR amplification was operated by using 0.2 µl of successfully amplified products as DNA templates in the second PCR amplification for DGGE analysis. In this step, PCR reagents and conditions remained the same as those used in section 2.4 except the forward primer. GC-clamped forward primers (GGGGCGGGGCGGGG CGGGGCGGGGGGGCAGATATTGGAACWTTATATTTTATTTTTGG and CCG CCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCG CTTAATAATTGGTGCACCAGATATAG) were used instead of ZBJ-ArtF1c and Forward planthopper-specific primer, respectively.

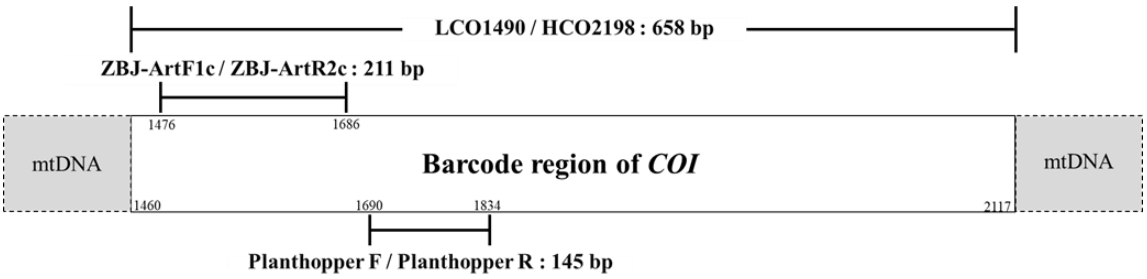

Figure 3 The target regions of the candidate primers which were used in analyzing the wrinkle-lipped free-tailed bat guano are shown. In this figure, the primers are localized on the reference mitochondrion genome of JF905446.1: *Melanitis leda* (Lepidoptera; Nymphacidae). These primers are on the *COI* standard barcode region which has been successfully used to identify insect species.

Amplicons obtained from PCR amplification were then loaded in agarose gel to separate target amplicon from non-specific bands (see section 2.5.1 for details). The selective amplicons were excised from agarose gel and PCR product purification was performed subsequently to select only target band to separate in DGGE system. The method of PCR product purification was stated in section 2.6.

For DGGE separation, purified amplicons that amplified using the universal primers (GC-clamped ZBJ-ArtF1c and non-clamped ZBJ-ArtR2c) were loaded onto 20-25% denaturant gradient (5.6 M urea and 30% deionized formamide v/v) in 10% polyacrylamide whereas 20-50% denaturant gradient (7 M urea and 40% deionized formamide v/v) in 10% polyacrylamide was prepared for PCR products amplified using the GC-clamped Forward planthopper-specific primer and non-clamped Reverse planthopper-specific primer (See Table 4 for details). The DGGE separation was performed using OmniPAGE VS20WAVE-DGGE (Cleaver Scientific, Warwickshire, United Kingdom) at a constant voltage of 50 V and temperature of 55 ^{o}C for 18 h. After electrophoresis, the gels were stained in 0.5 mg/ml ethidium bromide solution for 15 min, soaked to de-stain for 30 minutes, visualized and photographed using UVIdoc HD2 (UVITEC, Cambridge, United Kingdom).

Table 4 List of chemicals and their quantity use to prepare DGGE gel for separating mixed-insect DNAs obtained from bat guano pellet. Formula 1 reagent (5.6 M urea and 30% deionized formamide v/v) can be prepared DGGE gel for amplicons obtained from universal primer (Zeale *et al*. 2011) while formula 2 reagents (7 M urea and 40% deionized formamide v/v) was used for another primer set

Chemical	Denaturing solution		
	0%	100%	
		Formula 1	Formula 2
40% Acrylamide/ Bis	25 ml	25 ml	25 ml
50x TAE buffer	2 ml	2 ml	2 ml
Formamide (deionized)	-	30 ml	40 ml
Urea	-	33.6 g	42 g
Distilled water	73 ml	to 100 ml	to 100 ml
Total volume	100 ml	100 ml	100 ml

After DGGE performance was employed, mixed-DNA of insect prey species was isolated. In this step, amplicons bands that appeared on DGGE gel theoretically contained DNA of a single species, these separated bands were excised from the gel, and incubated in sterile distilled water for an hour to extracted PCR product from gel. Diffused PCR product were removed GC-clamp by re-amplification using reagents and conditions according to section 2.4. Amplified PCR products were checked on agarose gel and purified as mention as section 2.6. before sequencing.

2.6 PCR product purification and sequencing

Successfully amplified products were purified using illustra™ ExoProStar™ (GE Healthcare Life Sciences, USA). In case of presence of nonspecific DNA bands, expected PCR products were cut from the gel and purified using QIAquick® Gel Extraction Kit (Qiagen, Germany). Purified PCR products were then quantified using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific) and kept at -20 °C until further analysis. Purified PCR products were sequenced at First BASE Laboratories SDN BHD, Malaysia. Ambiguous bases were checked and corrected using the software Finch TV Version 1.4.0 (Geospiza Inc, USA).

2.7 OTUs delineation and taxon assignment

Good quality sequences from samples were matched with known reference sequences in the NCBI database using the program BLASTn. Doing this insect species could be successfully identified to species if 98-100% nucleotide similarity score was matched with the references sequences (Waugh 2007). Scientific name based on morphological identification was cross checked with taxon assignment subsequently to examine whether the given specie by two identification method was corresponded or at least belonged to the common higher taxonomic group in case could not identify down to species level.

To identify prey insect species in bat diet, good quality sequences were examined using 3 criteria which was modified from OTUs identification method of Wray *et al*. (Collins and Cruickshank 2013; Wray *et al*. 2018). First, each OTU matched with ≥ 99% nucleic acid similarity and 99% query cover with reference sequences from a single taxon accumulated in GenBank, or each OTU hit ≥ 99% match with reference sequences from a single taxon deposited in the BOLD system (Wilson *et al*. 2017). Second, taxa assigned to OTUs were from taxa that have been previously found in Thailand or Southeast Asia. The OTUs that could not pass the mentioned criteria or in case multiple species shared the highest matched score, identification was downgraded to higher taxonomic level e.g. family or order (Aizpurua *et al*. 2018).

2.8 Validation of the developed direct PCR workflow

2.8.1 Reproducibility test

The reproducibility test was employed to determine the applicability and robustness of the developed workflow to samples which were collected from different sources of tissue, body parts or amount of tissue. Since variations in DNA availability of insects from different sources and in different stages, sizes, or body parts, may affect to amplification success rate, we wanted to manifest that the optimized tissue amounts were robust to these variations. Therefore, 63 out of 160 fresh samples of specimens used for the optimization experiment mentioned in section 2.3-2.7 were reused to prepare pre-PCR solution. These samples spanned 13 species from 8 orders (see Table 1).

2.8.2 Sensitivity test

The sensitivity test was employed to determine the optimal and minimum amount of insect tissue that could be detected by the developed protocol. To prepare pre-PCR solution, the legs of large-bodied insects (e.g. Swallowtail butterflies) or the whole body of small insects (e.g. parasitoid wasp) were dissected. Collective number of 1-mm^2 fragments (8, 6, 4, 2, 1, $1/2^{nd}$, $1/4^{th}$, and $1/8^{th}$ pieces) were used in pre-PCR solution preparing. Ten replication were amplified using the methods mentioned in section 2.3-2.5 for each number of fragments. The amplification success rates were scored from a present of bands on Agarose gel electrophoresis.

2.8.3 Applicability to various ecological sample types

Applicability test was performed to examine whether the developed protocol can be used in difficultly amplified samples which is typically encounter in Ecology study. Five sample types included oven-dried, ethanol-preserved, museum, bat guano and cooked insect samples were therefore collected (further details in Table 1) and amplified using the method from section 2.3-2.7.

2.9 Statistical analysis

Since ecosystem service assessment could be more obvious with information involved both prey species incidence and quantitative estimation of those preys. To answer the question how many insects being eaten by bats, percentage frequency of occurrence has been the only suggested for quantifying the information given by molecular technique (Boyles *et al*. 2013; Razgour *et al*. 2011). This number could be calculated accordingly the following equation.

$$\%\boldsymbol{FOO} = \frac{\boldsymbol{N_a \times 100}}{\boldsymbol{N}}$$

When %FOO means the percentage frequency of occurrence. N_a means the number of occurrences of particular species (number of pellets containing the given prey taxon), and N means the total occurrences for all taxa.

A Chi-square contingency test was employed to determine whether the percentage frequency of the given insect prey species differed between the two rice growing season (which was encompassed active and inactive duration in the year of study). Also, Bayesian Estimation Supersedes the t-test (BEST) was used to investigate whether %FOO of each insect prey species was different between rice growing season. Data were analyzed using available online Kruschke's platform (Kruschke 2013) at http://www.sumsar.net/best_online/.

CHAPTER 3

Results and Discussion

This study aimed to evaluate ecosystem service contributed by wrinkle-lipped free-tailed bats (*Chaerephon plicatus* (Buchannan, 1800)) in regulating pest population around their roosting caves where adjacent to rice fields and other croplands in central Thailand. To achieve the goal, diet analysis from guano of this bat species was investigated using direct PCR and DGGE technique. Firstly, we developed and fully validated direct amplification workflow to identify a wide range of prey taxa based on DNA analysis. Secondly, bat guano was analyzed by using the developed direct PCR protocol with DGGE to amplify and identify insect prey's DNA for ecosystem service interpretation. The overview of results is shown in Figure 4.

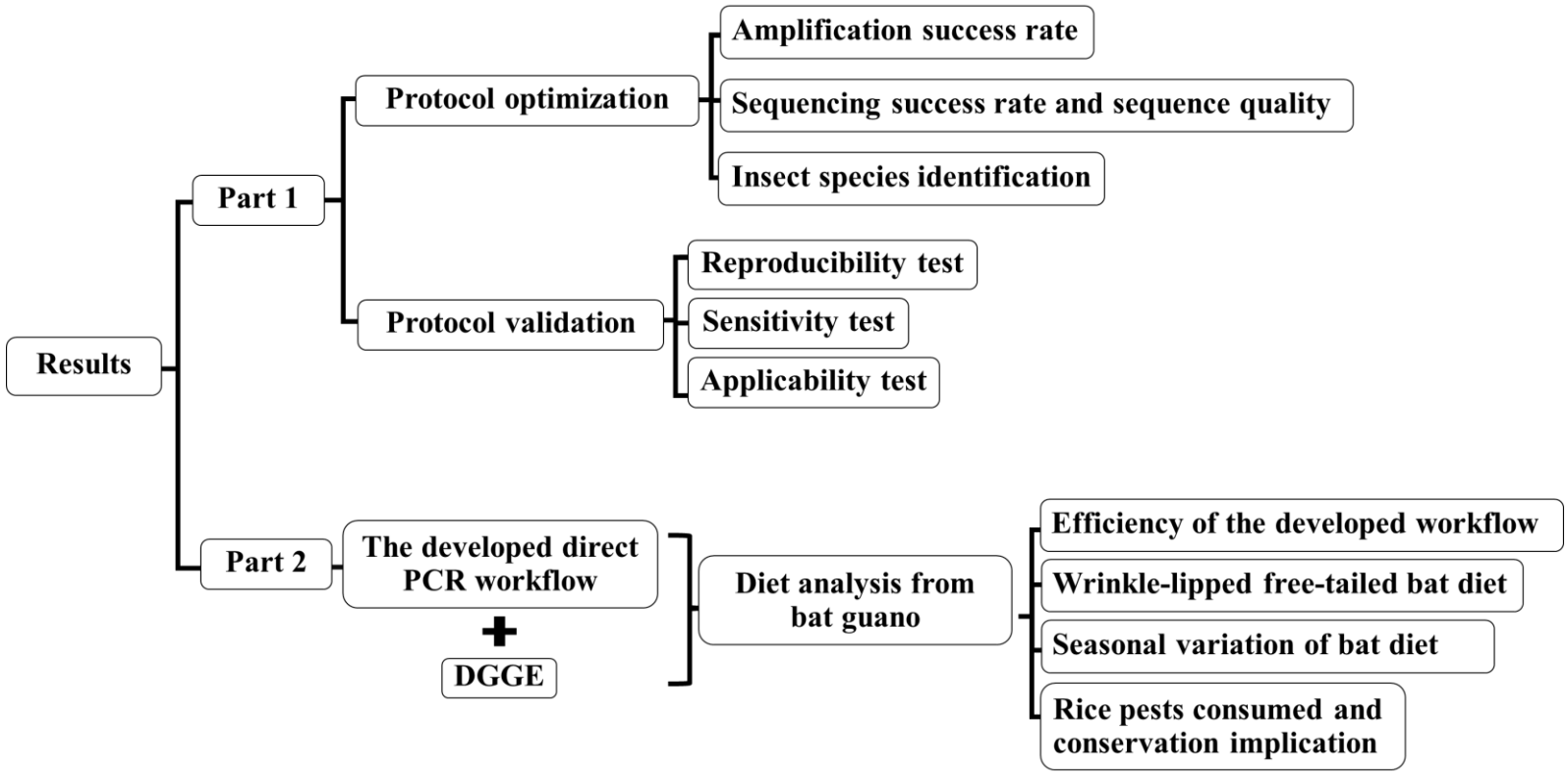

Figure 4 Flow chart showing overview of the results that are divided into two parts. Part 1 comprises two sections namely protocol optimization and protocol validation. Part 2 focuses on the analysis of the wrinkle-lipped free-tail bat diet using the developed direct PCR-DGGE workflow. Further details are provided in subsequent sections.

3.1 Development of direct PCR workflow

In this section, we successfully developed a direct PCR workflow for amplification of DNAs from a wide range of insect species. The workflow incorporates with pre-PCR preparation step in which the protocol was shown in section 2.3. One hundred sixty fresh insect samples from 11 orders including Lepidoptera (N=38), Hemiptera (N=22), Diptera (N=21), Coleoptera (N=16), Blattodea (N=15), Odonata (N=13), Orthoptera (N=10), Hymenoptera (N=8), Phasmatodea (N=8), Neuroptera (N=5), and Mantodea (N=4) were analyzed using the developed workflow. Results are shown in terms of amplification success rate, sequencing success rate, and species identification.

3.1.1 Amplification success rate

Overall, result showed 75-100% first pass amplification success rate for all 11 insect orders (Figure 5). One hundred percent amplification success rate was observed in six orders: Mantodea, Phasmatodea, Neuroptera, Odonata, Blattodea, and Orthoptera. High to moderate amplification success rate ranging between 75-92% was observed in the other five orders: Lepidoptera (92.1%), Diptera (85.7%), Hemiptera (81.8%), Coleoptera (75%) and Hymenoptera (75%). A second amplification trial was employed for all samples that failed to amplify in the first round. The second amplification success rate was improved in three orders: Lepidoptera (92.1 to 97.3%), Diptera (85.7 to 90.5%), and Coleoptera (75 to 93.8%). As a result, an overall amplification success rate accounted 93.8%.

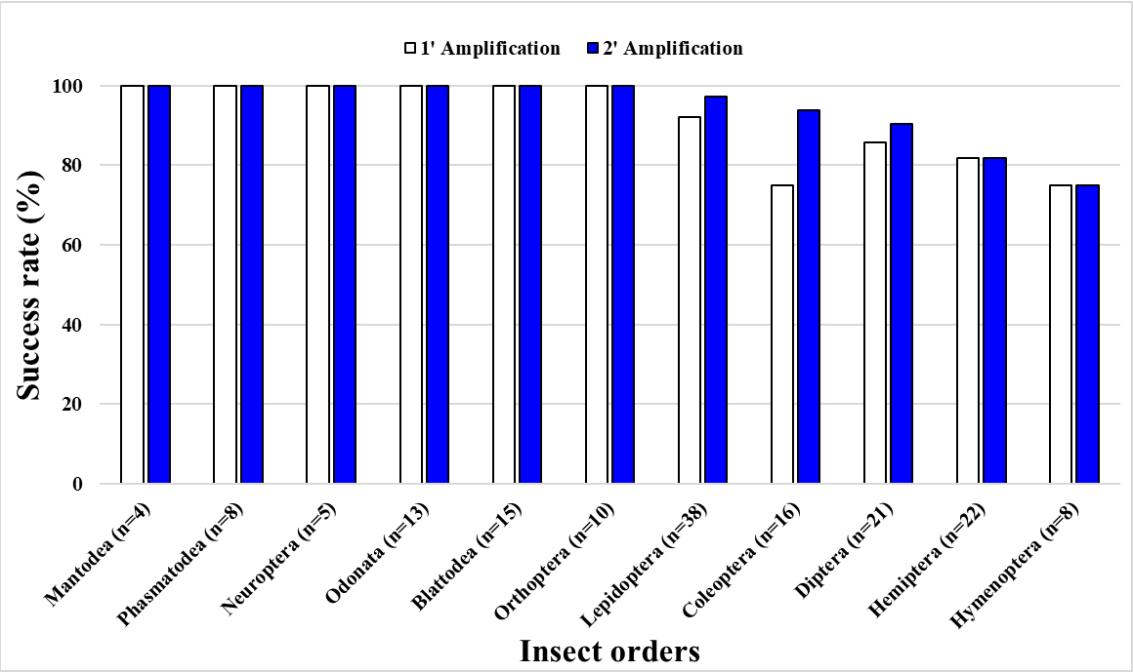

Figure 5 The efficiency of the developed workflow categorized by insect order is shown. Numbers in parentheses (X) on the x-axis indicate the number of samples in each order. Samples that did not produce any visible PCR product in the first trail (1st amplification) were re-amplified using a new pre-PCR solution (2nd amplification).

We attributed the achievement to many factors. First, this developed workflow incorporated a dilution step using PBS solution (pH 7.4) which assisted to dilute the potential PCR inhibitors carried by insect tissues and maintain the proper pH of the reaction (Kitpipit *et al*. 2014). Second, the boiling step broke down cell membrane which released DNA for the PCR reaction and denatured proteins that could affect DNA or interfere enzymatic reaction (Grevelding *et al*. 1996). Third, the DNA polymerase used in this study was more tolerant of PCR inhibitors which allowed amplification of a wide range of insect species although, varying degrees of inhibitors were presented in the reaction (Wang *et al*. 2004). Fourth, the suitable primers helped to avoided primer-template mismatches (Varadinova *et al*. 2015; Waugh 2007), which increase success rates for some taxa that the previous study reported primers could not amplify.

The taxa that contributed high success rates in this study agreed with previous studies which used non-modified direct amplification technique to amplify these insects (Ball and Armstrong 2008; Loto *et al*. 2013). However, this result was the first time Phasmatodea and thick exoskeleton taxa (e.g. Coleoptera and Odonata) were successfully amplified using direct PCR or the technique that DNA extraction was omitted. Prior to this study, these thick exoskeleton taxa had been considered fail to amplify using direct PCR (Wong *et al*. 2014). The slightly lower success rates noticed in Hymenoptera, Hemiptera, Diptera, and Coleoptera could be due to inhibitor problems. For example, Hymenopteran species (e.g. ants and parasitoid wasps) could contain various PCR inhibitors in their tissue, such as melanin in their compound eyes, haemocyanin, and formic acid. As a result, enzymatic reaction was possible interfered in various mechanisms. For instance, melanin binds with DNA templates and hinders the activity of DNA polymerase (Boncristiani *et al*. 2011; Opel *et al.* 2010). Haemocyanin, due to its structural similarity to haemoglobin, may act as a chelating agent to prevent enzymatic functions. Glandular legs also contain other secretions which could inhibit PCR (Billen 2009; Wong *et al.* 2014). Similarly, light-colored exoskeleton contained phenolic compound (e.g. arterenone, dopamine, and noradrenaline) in their integuments (Kramer *et al*. 2001). These compounds are precursors in many metabolic pathways and may act as PCR inhibitors by chelating metal ions (Schrader *et al.* 2012). Diptera is one of the most abundance taxa and therefore it is hard to share a common universal primer among subgroup (Waugh 2007). For Coleoptera, these insects were responsible for the lowest amplification success rate in the first trial using primers we selected from previous studies (CI-J-1632/CI-N-2191). Even with conventional PCR using a purified DNA template, no PCR product was observed using these universal primers. This could be due to reaction specific factor such as primer-mismatch (Castalanelli *et al*. 2010). Once the other universal primers were used, amplification success rate was improved. These results indicated that for the most abundance taxa, primer specificity is an issue that need attention.

3.1.2 Sequencing success rate and sequence quality

A total of 150 out of 160 amplifiable PCR products obtained from fresh samples were sequenced**.** Overall, result showed that sequencing success rate was as high as 90% (144 in 160) which is shown in Figure 6**.** One hundred forty-four out of 150 samples (96%) provided good quality electropherograms which showed single peak in each called base position and minimal background noise**.** The rest of six samples failed to sequence**.** These failed samples were from various taxa included Coleoptera, Diptera, Hemiptera, Hymenoptera, Odonata, and Orthoptera.

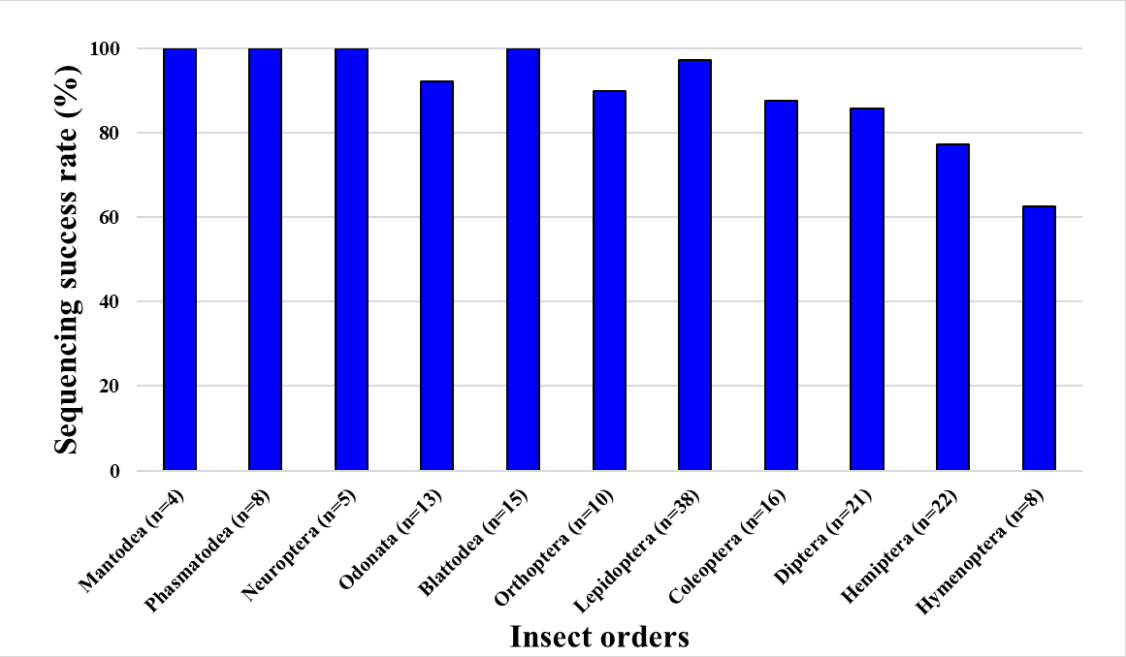

Figure 6 The sequencing success rate categorized by insect order is shown. Numbers in parentheses (X) on the x- axis indicate the number of samples in each order. Sequencing success rates were calculated by dividing number of good quality sequences by a total of samples in that group. For example, Odonata, there are 12 good quality sequences obtained from 13 samples which were amplified and sent to sequence, the sequencing success rate therefore equates 92.3%.

We attributed unsuccessful sequencing to contamination during sample collection**.** Since these samples were collected for morphological study, therefore concern of cross-contamination of DNA were limited. The sequencing success rates of insect orders correlate moderately with its amplification success rates (maximal information coefficient (MIC) = 0.40) which means amplifiable PCR products could consistently give successful sequencing results.

3.1.3 Insect species identification

From 150 amplifiable PCR products, 144 samples were successfully sequenced and provide high quality sequences. These sequences were then queried against reference sequences available on GenBank for insect species identification. Figure 7 shows the category level (species, genus, family, and order) for all quality profile.

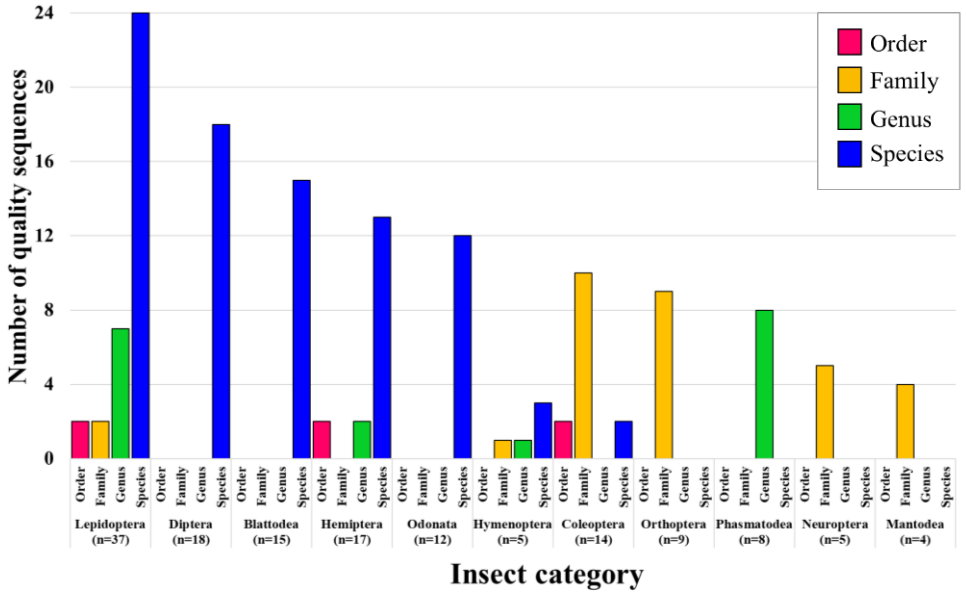

Figure 7 High quality sequences obtained from amplification using the developed workflow were classified in 4 categories (Order, Family, Genus and Species) based on matching against NCBI GenBank database. Numbers in parentheses (X) represent the number of sequences in each Order.

For example, all 15 sequences from Order Blattodea were classified to species level. The matching results showed that all 144 sequences could be identified with the nucleotide similarity of 85-100% (Table 5). However, only sequences that met 98-100% nucleotide similarity were considered accurately species. The results showed that these samples were classified into four taxonomic levels, which are order (4.2%), family (21.5%), genus (12.5%), and species (61.8%). The reason that almost half of sequences obtained could not be identified down to species level is the lack of voucher insect sequences in GenBank itself (Jinbo *et al.* 2011). In comparison, DNA-based identification results agreed for all samples which were prior identified based on morphological features (Table 5). This indicates that the developed protocol could be used as a tool to successfully amplify DNA for insect species identification.

Table 5 List of successfully sequenced insect specimens including insect order, family, genus and scientific name obtained from conventional identification, percent of nucleic acid similarity (%NS), accession number on NCBI database and closest related species available on NCBI database based on sequencing data obtained from the fresh samples (see Table 1 for the number of samples in each insect species) in this study. Only one entry is listed per species, as all the sequences obtained from the specimens of each species were 100% similar and thus only one representative sequence was queried against the Genbank NCBI database.

Morphological identification			**DNA based identification**		
Order	**Family**	**Species**	**%Nucleic acid similarity**	**Accession No.**	**Closest species**
Blattodea	Blattidae	*Periplaneta americana*	100	KU379702.1	*Periplaneta americana*
Diptera	Calliphoridae	*Chrysomya rufifacies*	100	KT894980.1	*Chrysomya rufifacies*
Hemiptera	Cicadellidae	*Recilia dorsalis*	100	LN681350.1	*Recilia dorsalis*
	Delphacidae	*Nilaparvata lugens*	100	KC333654.1	*Nilaparvata lugens*
	Delphacidae	*Sogatella furcifera*	100	KC512915.1	*Sogatella furcifera*
Lepidoptera	Sphingidae	*unknown*	100	JQ344666.1	*Lepidoptera* sp.
	Sphingidae	*Daphnis nerii*	100	FJ485745.1	*Daphnis nerii*
	Nymphalidae	*Hypolimnas bolina*	100	KJ459843.1	*Hypolimnas bolina*
	Nymphalidae	*Melanitis leda*	100	KT880656.1	*Melanitis leda*
	Nymphalidae	*Mycalesis mineus*	100	KF226536.1	*Mycalesis mineus*
	Noctuidae	*Spodoptera litura*	100	KX863232.1	*Spodoptera litura*
	Nymphalidae	*Tanaecia julii*	100	HQ962116.1	*Tanaecia julii*
	Nymphalidae	*Papilio memnon*	100	HQ962218.1	*Papilio memnon*
	Arctiidae	*Trigonodes hyppasia*	100	KX863070.1	*Trigonodes hyppasia*
Odonata	Libellulidae	*Neurothemis tullia*	100	KT957503.1	*Neurothmis tullia*
	Libellulidae	*Tholymis tillarga*	100	AB709196.1	*Tholymis tillarga*
Blattodea	Ectobiidae	*Blattella* sp.	99	KY349765.1	*Blattella lituricollis*
Coleoptera	Chrysomelidae	*Micraspis discolor*	99	EU392417.1	*Micraspis discolor*
	Staphilinidae	*Paederus fuscipes*	99	KU188413.1	*Paederus fuscipes*
Diptera	Culicidae	*Mansonia bonneae*	99	HQ398879.1	*Mansonia bonneae*
Hemiptera	Cicadidae	*Dundubia nagarasingna*	99	GQ527074.1	*Dundubia nagarasingna*
	Reduviidae	*Eocanthecona furcellata*	99	KJ459922.1	*Eocanthecona furcellata*
Hymenoptera	Formicidae	*Anoplolepis gracilipes*	99	KX051605.1	*Anoplolepis gracilipes*
	Braconidae	*Cotesia flavipes*	99	JF865973.1	*Cotesia flavipes*

Morphological identification			DNA based identification		
Order	**Family**	**Species**	**%Nucleic acid similarity**	**Accession No.**	**Closest species**
	Formicidae	*Diacamma rugosum*	99	HQ619699.1	*Diacamma rugosum*
	Formicidae	*Oecophyla smaragdina*	99	JQ681064.1	*Oecophyla smaragdina*
Lepidoptera	Nymphalidae	*Amathusia friderici*	99	KF226268.1	*Amathusia friderici*
	Noctuidae	*Amerila* sp.	99	HQ921264.1	*Amerila alberti*
	Noctuidae	*Asota caricae*	99	GU828615.1	*Asota caricae*
	Arctiidae	*Creatonotos gangis*	99	KX863293.1	*Creatonotos gangis*
	Arctiidae	*Creatonotos transiens*	99	KX861984.1	*Creatonotos transiens*
	Noctuidae	*Eudocima* sp.	99	KY196412.1	*Eudocima phalonia*
	Noctuidae	*Xantodes transversa*	99	HQ951631.1	*Xantodes transversa*
	Nymphalidae	*Euplocia membliaria*	99	KC499520.1	*Euplocia membliaria*
	Nymphalidae	*Euthalia alpheda*	99	AB511407.1	*Euthalia alpheda yamuna*
	Nymphalidae	*Euthalia evelina*	99	HQ962345.1	*Dophla evelina*
	Nymphalidae	*Euthalia monina*	99	KF226457.1	*Euthalia monina*
	Nymphalidae	*Mycalesis janardana*	99	KX153938.1	*Telinga janardana*
	Papilionidae	*Papilio polytes*	99	KM215138.1	*Papilio polytes*
	Nymphalidae	*Polyura athamas*	99	KF226598.1	*Polyura athamas*
	Geometride	*Ornithospila esmeralda*	99	MG014811.1	*Ornithospila esmeralda*
Diptera	Tephritidae	*Bactrocera* sp.	98	KM359604.1	*Bactrocera dorsalis*
	Calliphoridae	*Sarcophaga* sp.	98	JX861412.1	*Sarcophaga peregrina*
Hemiptera	Cicadellidae	*Nephottix virescens*	98	KF371523.1	*Nephottix virescens*
Lepidoptera	Arctiidae	*Pareuchaetes insulata*	98	JQ556160.1	*Pareuchaetes insulata*
Odonata	Libellulidae	*Neurothemis fulvia*	98	KP835515.1	*Neurothmis fulvia*
Hemiptera	Coreidae	*Anoplocnemis phasiana*	97	HQ236471.1	*Anoplocnemis phasiana*
	Cicadidae	*unknown*	97	GQ527074.1	*Dundubia nagarasingna*
Lepidoptera	Arctiidae	*Amata* sp.	97	JF840300.1	*Amata hueneri*
	Noctuidae	*Marumba* sp.	97	KX861614	*Marumba dyras*
	Arctiidae	*Cyana cf. coccinea*	96	KC571061.1	*Cyana meyricki*
	Arctiidae	*Cyana cf. cruentata*	96	KC571061.1	*Cyana meyricki*
	Nymphalidae	*Euthalia malaccana*	95	AB511419.1	*Euthalia lubentina*
Hemiptera	Cicadellidae	*unknown*	94	JQ344801.1	Hemiptera sp.
Mantodea	Mantidae	Mantidae sp.	94	EF383858.1	*Rhomantis* sp.
Lepidoptera	Noctuidae	*Abraxas lugubris*	93	KF388367.1	*Abraxas sporocrossa*
Hemiptera	Cicadidae	*unknown*	92	GQ527089.1	*Dundubia spiculata*
Lepidoptera	Noctuidae	*Celerena signata*	92	HQ923877.1	*Celerena griseofusa*

Morphological identification			DNA based identification		
Order	**Family**	**Species**	**%Nucleic acid similarity**	**Accession No.**	**Closest species**
Neuroptera	Chrysopidae	*unknown*	92	AB981362.1	*Chrysoperla* sp.
Phasmatodea	Heteropterygidae	*Heteropteryx* sp.	92	AB477468.1	*Heteropteryx dilatata*
Coleoptera	Cerambycidae	*Aeolesthes aurifaber*	91	KY357573.1	*Laccobius striatulus*
Lepidoptera	Arctiidae	*Amata* sp.	91	JF854958.1	*Sthenognatha gentilis*
Neuroptera	Chrysopidae	*Plesiochrysa ramburi*	91	AB981362.1	*Chrysoperla* sp.
Coleoptera	Coccinellidae	*Nephus ryuguus*	89	GU073951.1	*Nephus includens*
Orthoptera	Tettigoniidae	*unknown*	89	AM886777.1	*Poecilimon hamatus*
Coleoptera	Cicindellidae	*unknown*	87	JX259884.1	*Cicindela splendida*
Hemiptera	Cicadellidae	*Bothrogonia* sp.	87	KC135907.1	*Bothrogonia japonica*
Hymenoptera	Formicidae	*Tapinoma melanocephalum*	87	KP232114.1	*Nylanderia* sp.
Lepidoptera	Hesperiidae	*unknown*	87	GU149788.1	*Nyctelius nyctelius*
Coleoptera	Curculionidae	*Hypomeces squamosus*	85	KR916789.1	*Sitona hispidulus*
Coleoptera	Cerambycidae	*Orthosoma brunneum*	85	JX987292.1	*Monochamus alternatus*
Orthoptera	Tettigoniidae	*unknown*	85	KX057733.1	*Holochlora fruhstorferi*

3.2 Validation of direct PCR workflow

3.2.1 Reproducibility test

In order to examine robustness and accuracy of the developed workflow for insect species identification, sixty-three samples were randomly selected from the pool of fresh samples and analyzed using the developed workflow. The results showed that all samples were consistently amplified accounting 100% amplification success rate. The sequences obtained from these amplifiable products could be used to identify insect species accurately (Table 5). These results demonstrated that the developed workflow is highly repeatable and accurate.

3.2.2 Sensitivity test

Sensitivity test was conducted to determine the range of optimal sample amounts adding in pre-PCR preparation step. To do this, number of 1-mm^2 pieces of insect leg or whole body were varied from 8-1/8th pieces for preparing pre-PCR solution. The experiment was replicated 10 times for each sample amount. The results showed that four to eight 1-mm^2 pieces dissected from the leg of large-bodied insect consistently yielded for all reaction accounting 100% amplification success rates (Figure 8). In comparison, small-bodied insects, only one 1-mm^2 piece dissected from the body yielded 100% success rate (Figure 8). The detectable PCR products which presented at least once in ten trials could be down to 1/4th and 1/8th of a piece for large- and small-bodied insects, respectively.

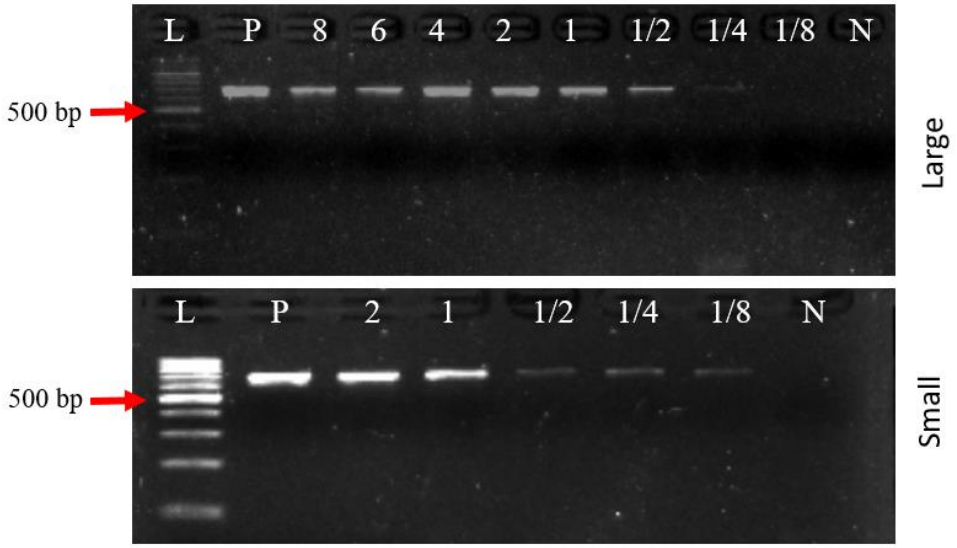

Figure 8 Sensitivity result from amplifying different amount of insect tissue using the developed protocol for large-bodied and small-bodied insects are shown. Eight to 1/8th of 1-mm^2 pieces were used to prepare pre-PCR solutions. A total of 10 replicates were performed to examine consistent results. Successful amplification was score if a detectable PCR product band was observed on agarose gel. L, P and N stand for 100 bp ladder, positive control, and negative control respectively.

These results demonstrated that the developed workflow had high sensitivity. In comparison, conventional extraction methods require at least a single leg for large insects (Foottit *et al.* 2014; Hebert *et al*. 2004; Oba *et al*. 2015) and multiple legs or whole bodies for smaller insects for identifying purposes (Gutiérrez *et al*. 2014; Hebert and Gregory 2005). This makes the developed workflow more advantage for cases where the samples are decomposed naturally (i.e. ecological specimens such as bat guano) and cases where starting material was limited. Also, it provides an alternative tool to employ DNA barcoding with less deconstruction of voucher specimens in museums.

3.2.3 Applicability to various ecological sample types and cooked samples

This experiment was conducted to determine whether the developed workflow can be used to amplify various insect sample types which typically encoutered in entomological study. To do this, a total of 218 insect samples were analyzed with the developed workflow. These samples included oven-dried samples (*N=30*), museum samples ($N = 10$), ethanol-preserved samples ($N = 143$), bat guano samples ($N = 25$), and food samples ($N = 10$). The results demonstrated that amplification success rates obtained from oven-dried samples, ethanol-preserved samples, food samples, and bat guano was as high as 100%, 98.6%, 90%, and 84% respectively while amplification success rate for museum samples was 30%. These results indicated that the developed workflow is applicable to almost ecological sample types.

Low amplification success rate was observed from museum samples. These could be due to several reasons which are highly degraded sample, inhibitor, and sample preservation process. We then extracted DNA from the certain specimens using a commercial extraction kit (QIAamp DNA mini kit, QIAGEN) and amplified using standard conventional PCR. The results showed that although standard protocol was used, amplifiable samples still remained low (only 50% success rate) and low PCR product concentrations (i.e. faint bands or no band observed on agarose gel) were observed in specimens aged over six years old (Figure 9).

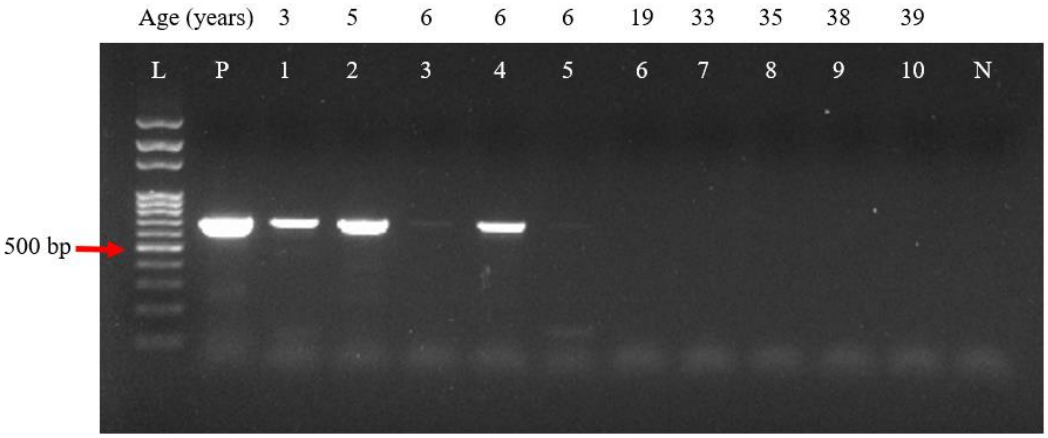

Figure 9 Applicability result of the developed workflow in museum specimens. A total of 10 samples in various age were tested. Successful amplification was score if a detectable PCR product band (expected 650 bp) was observed on agarose gel. L, P and N stand for 100 bp ladder, positive control, and negative control respectively. Numbers 1-10 are museum specimens, detail of these specimen can be found in Table 1.

These results indicated that the developed workflow may not be a source of problem. Age of sample and sample degradation level could be a main cause. Moreover, according to sample preservation method, the specimens were pinned and dried to study external characteristics; as such, the soft tissue inside rigid-external integument was dried and decomposed by time without chemical preservative treatment (Dick *et al*. 1993; Sutrisno 2012), intact DNA source e. g. muscle was therefore restricted and could not serve adequate amount in PCR amplification. Another possibility is if the specimens were treated by dichlorvos, a preservative chemical helps to prevent collection pest, this may negatively affect DNA amplification (Espeland *et al.* 2010; Werblow *et al*. 2016). In addition, we attributed low amplification success rate to other physical factors that might affect DNA integrity. For example, partial dehydration and light and air exposure may potentially led to DNA degradation, specifically deamination of cystidine residues. Similarly, Zimmermann *et al*. (2008) claimed that DNA of 8-years old specimens could be broken to around 70 bp in lengh , specimens aged > 18 years also gave substantial fragment size of approximately 50 bp and specimens aged between 30-40 could be degraded down to oligo nucleotide (20 bp). These results indicated that primer choice is a critical factor to recover DNA from

aged museum specimens because long amplicons may fail to amplify. Obviously, collection system plays an important role in achievement of DNA recovery, thus the researchers should follow the best practice of storage condition of specimen for further molecular analysis. Based on the present results, we suggested that ethanol is favorable for insect specimen preservation. If insect pinning is required then the preservative chemical should be either paradichlorobenzene or naphthalene that not affecting PCR amplification (Espeland *et al*. 2010).

3.3 Diet analysis from bat guano using direct PCR-DGGE technique

In this section, we analyzed 240 guano samples of wrinkle-lipped free-tailed bat using the developed and validated direct PCR workflow and DGGE technique. The reason for doing this is to identify insect pest species in this bat guano for ecosystem service evaluation. These pellets were collected from two roosting caves from October 2015 to September 2016. Details of sampling method and location are shown in section 2.1-2.2. Two primer sets; ZBJ-artF/ZBJ-artR (Zeale *et al.* 2011) and Planthopper F/Planthopper R (designed in this study) were used to amplify target insect taxa. The primer ZBJ-artF/ZBJ-artR is insect universal primer which can amplify 12 insect orders and the primer 'Planthopper' was designed in this study to amplify four insect pest species which commonly found in rice field in South East Asia. The four species included brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix virescens*), and zigzag leafhopper (*Recilia dorsalis*) (Pathak and Khan 1981). The amplifiable products were then analyzed using DGGE technique. This method was used to separate mixed-insect amplifiable products obtained from bat diet. The successfully separated bands were then sequenced to allow insect species identification, and interpretation in term of prey species list, insect incidence (percentage frequency of occurrence; %FOO) and seasonal variation of insect preys consumed by wrinkle-lipped free-tailed bat.

3.3.1 Efficiency of the developed direct PCR-DGGE workflow for bat guano analysis

Two hundred and seven out of 240 guano samples (86.25%) were successfully analyzed and the prey species in the guano were identified using the developed direct PCR-DGGE workflow (Table 6). From these 207 amplifiable products, 325 fragment bands were detected on DGGE gel. Representative DGGE gels and bands from the two primer sets are shown in Figure 10. All the fragments were then subjected to sequencing, with good quality electropherograms obtained from 320 of 325 bands (98.46%). These electropherograms exhibited only minimal background noise and no multiple bases were called at the same position in the sequences (Figure 11). These good quality sequences were then aligned with reference insect species sequences in two databases: GenBank and BOLD. The results obtained from these two databases were correspondingly allowing accurate insect species identified.

Table 6 Summary of the developed workflow efficiency is shown, details in table are categorized by study process. The number of samples and success rates are given for each process.

Study process	Sample number	Successful result (%success rate)
PCR Amplification	240 pellets	207 pellets (86.25)
DGGE separation	207 amplifiable products	325 DGGE bands
Sequencing	325 DGGE bands	320 sequences (98.46)
Informative sequences	320 sequences	76 distinct OTUs
Taxon assigned OTUs using strict criteria	76 distinct OTUs	99% similarity: 42 OTUs comprising 7 orders, 25 families, 24 genera, and 26 species <99% similarity: 34 OTUs

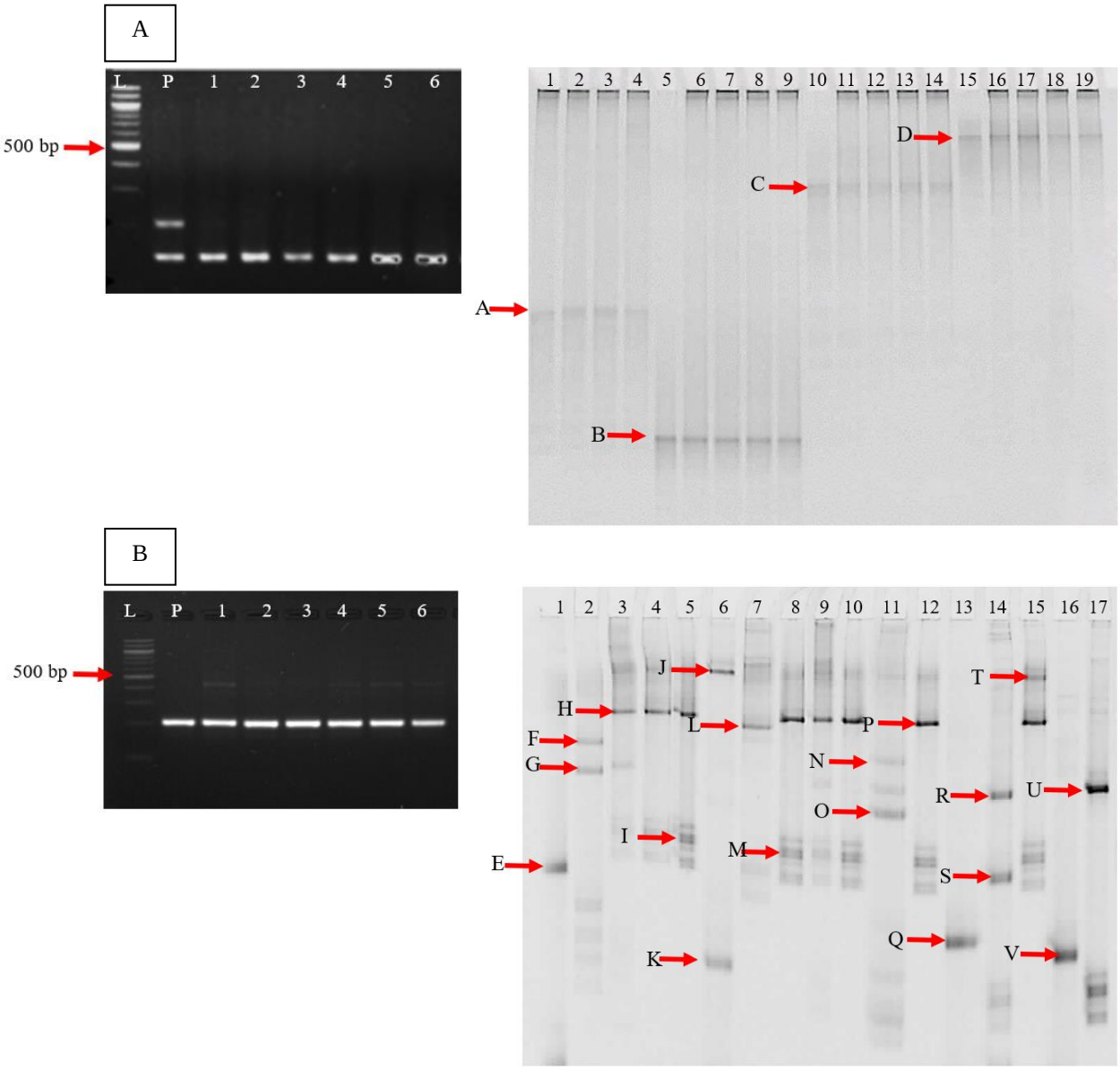

Figure 10 Representative agarose gels showing PCR products amplified using (A; left) Planthopper F/ Planthopper R and (B; left) ZBJ-artF/ZBJ-artR (Zeale *et al.* 2011). The PCR products were then further separated on DGGE gels to allow identification of prey species (A and B; right). (A) *Nilaparvata lugens*, (B) *Sogatella furcifera*, (C) *Nephotettix virescens*, (D) *Recilia dorsalis*, (E) *Chironomus javanus*, (F) *Ophionea indica*, (G) *Asota caricae*, (H) *Culex* sp., (I) *Eretes sticticus*, (J) Tephritidae sp., (K) *Sesamia inferens*, (L) *Gryllus bimaculatus*, (M) *Thaumatotibia hemitoma*, (N) *Anoplogenius microgonus*, (O) *Povilla heardi*, (P) *Culex gelidus*, (Q) *Anatrachyntis simplex*, (R) *Scirpophaga incertulas*, (S) Oecophoridae, (T) *Culex tritaeniorhynchus*, (U) *Chilo auricilius*, (V) *Blattella lituricollis*.

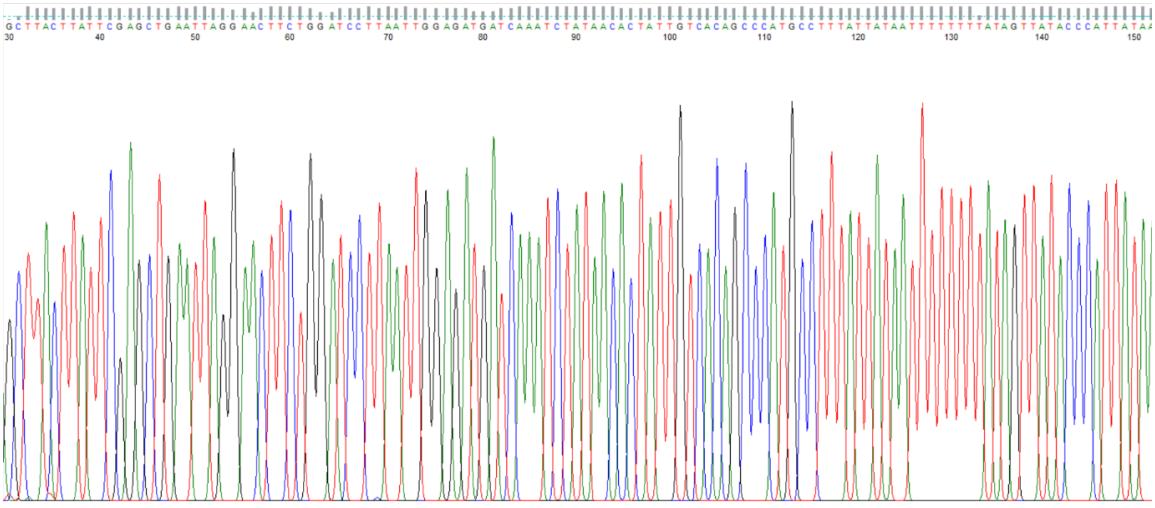

Figure 11 Representative good quality electropherogram (base position 30-153) sequenced by a single band separated from mixed insect PCR amplicons using DGGE technique. This sequence matches *Scirpophaga incertulas* in the GenBank database with 99% nucleotide similarity.

The results illustrated that the developed direct PCR-DGGE workflow is applicable to identify insect species from insect fragments in bat guano pellets. This is the first-time a combination of direct PCR-DGGE was successfully applied for this purpose. We attributed the success of this technique to several factors. Firstly, the direct PCR method used high tolerant DNA polymerase to overcome PCR inhibitor. As such, PCR was possible even in the presence of inhibitors from bat guano (Wang *et al.* 2004). Secondly, the developed protocol used PBS buffer incorporated with boiling step to dilute concentration of potential PCR inhibitors and facilitate cell lysis, which helps to prepare adequate DNA template for PCR amplification (Kitpipit *et al.* 2014). Thirdly, appropriate primers that minimized primer mismatches helped to improve amplification success rate (Varadinova *et al.* 2015; Waugh 2007). In this study, we selected two candidate primer pairs to amplify insect DNAs; one was expected to amplify DNA from at least 12 insect orders (Zeale *et al.* 2011), and another was designed to identify planthoppers common to South East Asian rice fields. This combination was used not only for maximizing amplification success, but those primers also helped to depict a realistic picture of insect preys consumed by wrinkle-lipped free-tailed bat over the study period. Another tool that contributed to the success of this study is the DGGE

system. It enabled mixed-DNA from various preys eaten by the bats to be separated, allowing correct identification from unambiguous sequences (Deagle *et al*. 2005; Lee *et al.* 2013; Martin *et al.* 2006)

3.3.2 Wrinkle-lipped free-tailed bat diet

A total 320 DGGE fragments yielded 76 operational taxonomic units (OTUs). Based on the strict threshold (99% nucleotide similarity or higher) defined in the Methodology section 2.7, 42 OTUs passed the criteria and were deemed highly informative. Based on GenBank and Barcode of Life Database, twenty-six OTUs were able to identify down to species level. While the rest of 16 discrete OTUs could be only assigned to genus or family level. Overall, the results revealed 7 insect orders from 25 families, 24 genera and 26 species as shown in Table 7. The remaining 34 OTUs that did not pass the criteria (<99% nucleotide similarity) were further investigated using an "eye-test" strategy, in which the top 20 matches were screened using the following points: (1) the matches were from insect and (2) those insects belong to genera that can be found in Thailand. All 34 OTUs passed this "eye-test", and as such a likely explanation of why the percent nucleotide similarity is lower is the limitation in the currently available taxonomic delineations of the databases (Floyd *et al.* 2009; Jinbo *et al.* 2011; Wilson *et al.* 2017).

Diptera was the most abundant insect order found in wrinkle-lipped free-tail bat diet, in which its percentage frequency of occurrence was 32.8%. The second highest proportion was from Hemiptera (27.2%) followed by smaller proportion from Lepidoptera (24.1), and Coleoptera (10.3%), respectively. While the other insect taxa were found in minority proportion, in which, comprised Orthoptera (2.8%), Blattodea (1.6%), and Ephemeroptera (1.3%) respectively. The five most frequently found prey species were *Nilaparvata lugens* (16.3%), *Culex gelidus* (5.6%), *Culex tritaeniorhynchus* (5.0%), *Eretes sticticus* (4.4%), and *Scirpophaga incertulas* (4.1%).

Table 7. Percentage frequency of occurrence (%FOO) of insect preys in fecal samples of *C. plicatus* collected from October 2015 to September 2016 at Khao Wongkot Cave and Khao Chakan Cave in Central Thailand is shown. Details in table include list of taxa which could be identified using the developed direct PCR-DGGE workflow, number of pellets containing given taxa, and % FOO. The frequency of occurrence is a proportion between number pellets containing given taxa divided by the total occurrences of all taxa. %FOO per order is the sum of all %FOO in the respective order.

Taxon assignment			Number of pellets containing given taxa	Percentage frequency of occurrence (%FOO)	Total %FOO
Order	**Family**	**species**			
Blattodea	Ectobiidae	*Blattella lituricollis*	5	1.6	**1.6**
Coleoptera	Carabidae	*Anoplogenius microgonus*	2	0.6	**10.3**
	Carabidae	*Ophionea indica*	10	3.1	
	Carabidae	Unknown species	7	2.2	
	Dytiscidae	*Eretes sticticus*	14	4.4	
Diptera	Agromyzidae	Unknown species	17	5.3	**32.8**
	Chironomidae	*Chironomus javanus*	4	1.3	
	Chironomidae	Unknown species	4	1.3	
	Culicidae	*Culex gelidus*	18	5.6	
	Culicidae	*Culex tritaeniorhynchus*	16	5.0	
	Culicidae	*Culex vishnui*	9	2.8	
	Culicidae	*Culex sp*	8	2.5	
	Culicidae	Unknown species	21	6.6	
	Limoniidae	Unknown species	2	0.6	
	Musidae	Unknown species	2	0.6	
	Tephritidae	*Ensina sonchi*	2	0.6	
	Tephritidae	Unknown species	2	0.6	
Ephemeroptera	Polymitarcyidae	*Povilla heardi*	4	1.3	**1.3**
Hemiptera	Cicadellidae	*Nephotettix virescens*	11	3.4	**27.2**
	Cicadellidae	*Recilia dorsalis*	7	2.2	
	Delphacidae	*Nilaparvata lugens*	52	16.3	
	Delphacidae	*Sogatella furcifera*	9	2.8	
	Delphacidae	*Toya propinqua*	8	2.5	
Lepidoptera	Cosmopterigidae	*Anatrachyntis simplex*	3	0.9	**24.1**
	Cossidae	Unknown species	6	1.9	
	Crambidae	*Chilo auricilius*	6	1.9	
	Crambidae	*Scirpophaga incertulas*	13	4.1	
	Depressariidae	Unknown species	3	0.9	
	Erebidae	*Asota caricae*	4	1.3	
	Erebidae	Unknown species	3	0.9	
	Geometridae	*Taxeotis perlinearia*	4	1.3	
	Geometridae	Unknown species	6	1.9	
	Noctuidae	*Sesamia inferens*	5	1.6	
	Oecophoridae	Unknown species	2	0.6	
	Pyralidae	*Anassodes mesozonalis*	3	0.9	
	Pyralidae	*Meyrickiella homosema*	8	2.5	
	Pyralidae	Unknown species	3	0.9	
	Tortricidae	*Thaumatotibia hemitoma*	2	0.6	
	Tortricidae	Unknown species	2	0.6	
	Uraniidae	*Schidax squamaria*	2	0.6	
	Xyloryctidae	Unknown species	2	0.6	
Orthoptera	Gryllidae	*Gryllus bimaculatus*	9	2.8	**2.8**

The result demonstrated that over the study period of one year, Diptera played a key role as the main food source of wrinkle-lipped free-tailed bats, which is probably due to opportunistic feeding. Their abundance in the area might be due to several water reservoirs located within flying distance of the study site's roosting habitat. These reservoirs could serve as propagation resources for various Dipteran insects. Dipteran insects (e.g. mosquitoes and midges) normally swarm in great numbers over body of water where they are born at twilight or near vegetation landscape, and are likely to be dispersed by wind (Becker *et al*. 2010), making them available for consumption by the open-space foraging bats (Kunz and Fenton 2003).

The Hemipteran and Lepidopteran insects also make up the majority proportion of the diet of wrinkle-lipped free-tailed bats. This incidence could be based on specific wing morphology and echolocation behavior, they are apparently an aerial-hawking type which typically fed on high-flying insects (Norberg and Rayner 1987). These specific characteristics shape them with evolutionary arms race to hunt insects in open space at high altitude where they met insect preys, e.g., planthoppers and moths that are known to migrate at night in that height (Chapman *et al.* 2010, 2011; Riley *et al*. 1991). The Brazilian free-tailed bat (*Tadarida brasiliensis*), a similar species to *C. plicatus*, has also been found to feed on migratory moths which are major pest in the southern United States (Altringham 2011; Cleveland *et al*. 2006; Krauel *et al*. 2014; Lee and McCracken 2005; McCracken *et al*. 2007). This could be an evidence demonstrating that these bats mainly feed on insect preys which have migrated to high altitude.

Coleopteran insects were also detected using the developed workflow. *Anoplogenius microgonus* and *Ophionea indica* (ground beetle) are common species living in rice paddy field; they are also natural enemies of some rice pest insects, e.g., planthoppers and leafhoppers (Maisarah *et al*. 2014; Pathak and Khan 1981). The appearance of these insects in guano of wrinkle-lipped free-tailed bats also confirmed that these aerial predators have foraged in open space above rice fields surrounding their roosting cave. This agrees with previous study that found wrinkle-lipped free-tailed bats foraged around farmland area (Utthammachai 2009) and acted as potentially biological control agents that help to regulate insect pest population (Leelapaibul *et al*.

2005; Srilopan *et al*. 2018). Also, aerial insectivorous bats in this genus normally forage at high altitude in open- space vegetation, which suits searching for wind-borne nocturnal migratory insects, as such co-existence of these predators and preys in term of time and space was then resulted in ecosystem service which wrinkle-lipped free-tailed bat help to suppress population of brown planthoppers prevalent around rice fields.

Most of the findings in this study regarding prey species agree with previous studies performed with other techniques. The fact that Diptera played a key role agrees with data given by stable isotope analysis, which found Dipteran insects were estimated as high as 50-55% in wet and dry season, respectively (Ruadreo and Voigt 2019). The second highest proportion, Hemipteran insects, were also found as one of the dominant prey groups of wrinkle-lipped free-tailed bats using microscopic analysis of fecal matter (Srilopan *et al.* 2018). The third and fourth major groups, Lepidopteran and Coleopteran insects, found in present study correspond to Leelapaibul *et al*. (2005) findings, which stated that Homoptera (28.4%), Lepidoptera (20.8), Hemiptera (16.4), and Coleoptera (14.4%) were the four major taxa that contributed around 80% of diet of wrinkle-lipped free-tailed bats living in Central Thailand. A slight contradiction of this study with conventional microscopic method is seen regarding the percent frequency of occurrence, especially with Dipteran insects. Using the same study site over the same study period with this study, Srilopan *et al.* (2018) reported that Coleopteran insect made up the majority of this bat's diet. Ephemeroptera and Blattodea were absent using microscopic approach while Odonata, and Hymenoptera were absent using direct PCR-DGGE. We attribute this difference to the limitation and bias of the two methods. Several prey taxa that could not be detected using the developed technique may be due to the poor efficiency of the candidate primers for those taxa. Although these primers were designed originally to detect a broad range of insect taxa, their limited usefulness for only some insect orders have been reported (Wray *et al.* 2018). On the other hand, conventional microscopic analysis might miss digested soft-bodied insect parts. Rigid, hard-bodied Coleopteran insects were more easily observed using microscope because their body contains 44% chitinous

tissue (Lease and Wolf 2010). It is important to note that this is the first time that the diet of *C. plicatus* was revealed genetically down to species level.

3.3.3 Seasonal variation in wrinkle-lipped free-tailed bat diet

To study seasonal variation in wrinkle-lipped free-tailed bat diet, the percentage frequencies of occurrence of the prey species were plotted temporally (month-by-month) over the course of the year (12 months). A total of 26 successfully identified OTUs which were assigned down to species level was included. Rice growing season status (active and inactive) was explored as a factor as it was intrinsically linked to the abundance of certain insect species. The season was categorized as active rice-growing season (October-November, 2015 and August-September, 2016) and inactive rice-growing season (December, 2015 to July, 2016) according to Geo-Informatics and Space Technology Development Agency (GISTDA) data (Srilopan *et al*. 2018).

As expected, frequency of occurrence of insect prey species statistically related to rice growing season ($X^2 = 40.89$, *d.f.* = 25, $P < 0.05$). The most abundance insect prey found were brown planthoppers, with the probability that this species were found in bat diet during active rice growing season higher than non-farming season was 98% (Figure 12). The study result agreed with Srilopan *et al.* (2018) which found that the number of brown planthopper (*Nilaparvata lugens*) in bat guano directly correlated to rice-growing season. The brown planthopper and other sucking insects, e.g., zigzag leafhopper (*Recilia dorsalis*), white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix virescens*) and Delphacid planthopper (*Toya propinqua*), can feed on rice plants. As such, they were abundant during the rice-growing months. Especially the planthoppers (Delphacidae; Hemiptera), both nymph and mature insects can feed on rice stem, establish, and propagate using leaf-sheet of rice plants as substrates to lay their eggs. In each cultivated season, around 2-3 generations of planthopper can be produced, continuation growing rice all the year for a few decades in the study area therefore probably serve a favorable resource for this insect group (Matteson 2000 ; Heinrichs 1994; Heong and Hardy 2009). However, in the year that study was conducted, drought hindered year-round farming, and rice was only grown

during October-November 2015 and August-September 2016. As a result, these sucking insects were found in low frequency due to lacking of food and propagation resource, which they were also detected fewer in bat guano during inactive rice-growing months.

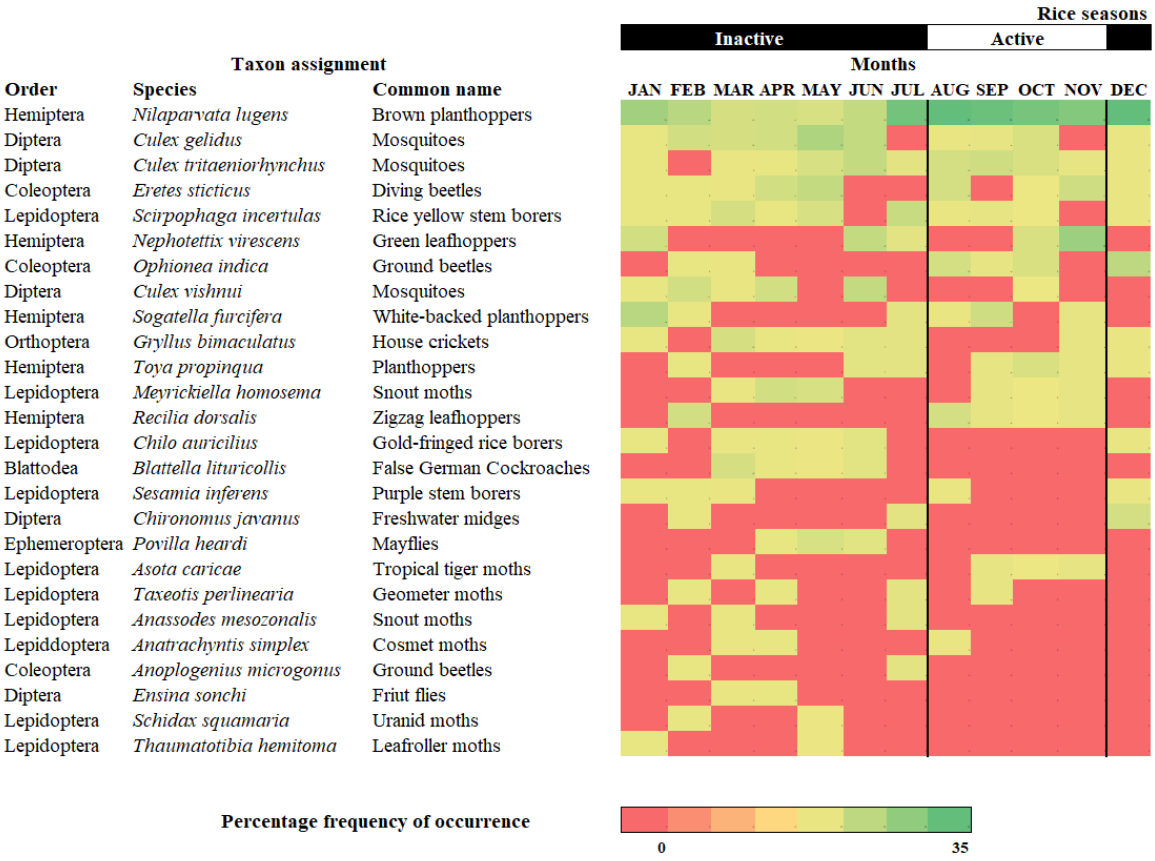

Figure 12 Heat map of the percentage frequency of occurrence for each prey species plotted monthly for 12 months. A color scale indicates the frequency of occurrence, with red being lowest (not found in that month) to green being highest (35 % FOO).

These bats then switched to other species, such as moths, beetles, and mosquitoes, during inactive rice-growing months. Both mayflies (*Povilla heardi*) and mosquitoes have life stages that are heavily reliant on water bodies (Abu Hassan *et al*. 2010; Becker *et al*. 2010; Sartori and Brittain 2015). In fact, Dipteran insects trapped around body of water contributed about half of all Dipteran biomass in all habitats, especially during hot-dry season during which the biomass of Dipteran can be as high as 90% (Suksai and Bumrungsri 2019). Figure 12 shows that these aquatic insects (mosquitoes, mayflies, midges) were consumed mainly during the hot-dry season. This indicates that *C. plicatus* may shift their foraging range from agricultural landscape to be closer to water resources, a finding that agrees with Suksai and Bumrungsri (2019). These months also correspond to the critical reproductive stages of *C. plicatus*. Typically, *C. plicatus* gives birth during March to May (Furey *et al*. 2018; Hillman 1999; Leelapaibul *et al*. 2005). Adequate water is necessary for milk production (Adams and Hayes 2008). The bats therefore need to fly frequently to water bodies where they could also forage on the insect that swarm in those areas during dusk and dawn. Previous studies showed that during lactation period, insectivorous bats may adjust their foraging style in several ways, e.g., increase foraging time (Barclay 1989), reduce home range size, and include more feeding bouts (Henry *et al*. 2002).

Other insect species that feed on non-rice plants were also detected in bat guano. These insect species feed on several host plants that belong to the same family with rice (Poacea) or they are oligophage. For example, purple stem borer (*S. inferens*) is highly damaging to corn sorghum; gold-fringed rice borer (*C. auricilia*) and Delphacid planthopper (*T. propinque*) are a potential pest of sugarcane. The false German cockroach (*B. lituricollis*) and cricket (*G. bimaculatus*) use leave litter both in open rice field and sugar cane plantation to be their shelter. As corn, sorghum, sugarcane plantations make up roughly half of the land areas of the study sites, it was not surprising to find these species as part of the bat's diet. However, it was apparent from Figure 12 that *C. plicatus* prefers brown planthoppers likely due to the coincidence of the planthoppers' migratory height and the bats' flying pattern (Nguyen *et al.* 2019).

The result demonstrated that prey preference of *C. plicatus* probably depends on both intrinsic and extrinsic factors, namely reproductive status, closeness to water bodies, and the availability of preys around their roosting cave in each season. In other words, they are opportunistic feeder who prey on the abundant insect species available in the area. Interestingly, certain insects that are strictly cave-dwellers were not detected in bat guano. The presence of these species in bat guano suggests that *C. plicatus* use hunting strategy that suits open-space foraging and do not primarily feed on cave-dwelling insects. With their long-narrow wings, the bats' movement are unfit for hunting activity in the small crevices of their roosting cave.

3.3.4 Rice pest consumed and conservation implication

Eight species detected in bat guano have been recognized as insect pest prevalent around rice fields (Pathak and Khan 1981). These are brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix virescens*), zigzag leaf hopper (*Recilia dorsalis*), Delphacid planthopper (*Toya propinqua*), gold-fringed rice stemborer (*Chilo auricilius*), Asiatic pink stem borer (*Sesamia inferens*), and rice yellow stem borer (*Scirpophaga incertulas*). For planthoppers, these pests were suspected in reducing rice yield through transmission of pathogenic viruses and cannot be controlled using intensive chemical since there are various biotypes resistant to pesticides (Heong *et al.* 1994, 2015; Sogawa 2015). Being an opportunistic feeder, *C. plicatus* can be a key species that functioned in regulating these pest as an effective biological control agent during the months that these pests are most abundant. In addition to rice pest, *C. plicatus* also consumed some *Culex* sp., the Japanese encephalitis (JE) virus carrier (Abu Hassan *et al*. 2010; Centers for Disease Control and Prevention 2019). These results agreed with previous studies that found *C. plicatus.* provides ecological service by regulating insect pests (Leelapaibul *et al*. 2005; Srilopan *et al*. 2018) and help to suppress pathogen insects (Wray *et al*. 2018).

The present study deconvoluted diet of *C. plicatus* genetically for the first time. These prey species have never reported as diet of these bats before except for brown planthoppers (*N. lugens*), a potential pest of rice which is a staple food for Thai people (Leelapaibul *et al*. 2005; Srilopan *et al*. 2018). In addition to brown planthoppers, a few pest species that is recognized as insect pests of various crops includes sugarcane, corn, and sorghum, were also found in their diet corresponding to land utility around the study site. The manifest prey species list obviously illustrated their ecosystem service in contributing to food security throughout this region since they play an important role as a biological pest control agent. Moreover, the result confirmed several mosquito species were consumed over the study period, this provides a basis for ecosystem service assessment in another aspect which has never been expected in the colonial cave bats. Although *C. plicatus* now is categorized in 'Least Concern' species (IUCN red list), chemical intensification in agriculture, habitat loss, climate change and other anthropogenic disturbance (e.g. wind turbine, hunting for meat, limestone extractive industry etc.) can possible cause population decline in this vulnerable species (Furey *et al*. 2010; Hughes 2017). Conservation plans should be therefore established considering both cave and farmland management, in which helps to reserve their shelter, propagation site, and to provide reliable food source within their foraging range. The results from this study could be used to encourage farmers to organize sustainable farmland system, especially agricultural landscape adjacent to the roosting cave since the favorable farmland can be attractive to potential predators particularly the generalist feeders who can help to suppress various insect pest species (Gurr *et al*. 2012; Naylor and Ehrlich 1997; Puig-montserrat *et al*. 2015).

CHAPTER 4

Conclusion

This research successfully developed and validated the direct PCR protocol to achieve high amplification success rate for a wide range of insect taxonomic group. Also, the developed method could be applied to analyze various sample types; oven-dried, ethanol-preserved, museum, cooked insect and bat guano samples using one universal method. Species identification based on insect morphology confirmed that barcoding results obtained using the developed protocol can be reliable. The workflow had high sensitivity that requires only 1x1 mm^2 insect tissue for starting material, this could be beneficial in applying to degraded specimens which encounter in ecology studies.

The developed direct PCR protocol could be employed together with the Denaturing Gradient Gel Electrophoresis (DGGE) called direct PCR-DGGE technique. This was the first time direct PCR-DGGE was successfully used to analyzed wrinkle-lipped free-tailed bat diet (*C. plicatus*) from guano samples. Diet of the bat was revealed genetically down to species level resulting in, more complete picture of ecosystem service. *C. plicatus* was found to play important roles not only in insect pests regulating over agricultural landscape adjacent to their roosting cave but also controlling disease transmitting insect.

Based on our finding, further studies can be conducted to explore foraging behaviors of *C. plicatus* in other colonies or other bat species, and surrounding farmland type influence on different ecosystem service and prey species preference. Management plans need to be established properly to protect their habitat and prevent population decline, which may help to improve productivity and profitability of the agriculture industry.

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APPENDICES

APPENDIX A

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RESOURCE ARTICLE

WILEY MOLECULAR ECOLOGY RESOURCES

A new cost-effective and fast direct PCR protocol for insects based on PBS buffer

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Abstract

Insect DNA barcoding is a species identification technique used in biodiversity assessment and ecological studies. However, DNA extraction can result in the loss of up to 70% of DNA. Recent research has reported that direct PCR can overcome this issue. However, the success rates could still be improved, and tissues used for direct PCR could not be reused for further genetic studies. Here, we developed a direct PCR workflow that incorporates a 2-min sample preparation in PBS-buffer step for fast and effective universal insect species identification. The developed protocol achieved 100% success rates for amplification in six orders: Mantodea, Phasmatodea, Neuroptera, Odonata, Blattodea and Orthoptera. High and moderate success rates were obtained for five other species: Lepidoptera (97.3%), Coleoptera (93.8%), Diptera (90.5%), Hemiptera (81.8%) and Hymenoptera (75.0%). High-quality sequencing data were also obtained from these amplifiable products, allowing confidence in species identification. The method was sensitive down to 1/4th of a 1-mm fragment of leg or body and its success rates with oven-dried, ethanol-preserved, food, bat guano and museum specimens were 100%, 98.6%, 90.0%, 84.0% and 30.0%, respectively. In addition, the pre-PCR solution (PBS with insect tissues) could be used for further DNA extraction if needed. The workflow will be beneficial in the fields of insect taxonomy and ecological studies due to its low cost, simplicity and applicability to highly degraded specimens.

KEYWORDS
biodiversity assessment, *COI*, direct PCR, DNA barcodes, DNA extraction, insecta

1 | INTRODUCTION

DNA barcoding is used to identify species based on a short DNA sequence from the cytochrome oxidase subunit I (*COI*) gene (Hebert, Cywinska, Ball, & deWaard, 2003; Hebert & Gregory, 2005; Hebert, Ratnasingham, & deWaard, 2003). Compared to conventional morphological identification, it is a cost-effective, quicker and more robust method. It has been successfully employed in a wide range of metazoans (Waugh, 2007), including insects (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Janzen et al., 2005; Kumar, Srinivasan, & Jambulingam, 2012). This method is particularly useful when morphology is difficult to observe, or limited sample is found. For insects, species identification using DNA barcoding also provides an easy system to assess biodiversity (Hajibabaei et al., 2006; Hebert, Cywinska et al., 2003; Hebert, Ratnasingham et al., 2003; Janzen et al., 2005), reveal cryptic species (Hebert et al., 2004) and monitor invasive alien species which are harmful to both agricultural and medical aspects (Armstrong & Ball, 2005; Kumar, Rajavel, Natarajan, & Jambulingam, 2007; Kumar et al., 2012). However, conventional DNA barcoding requires DNA extraction prior to DNA amplification,

which leads to additional costs, time and loss of DNA through the inefficient DNA extraction process (Asghar, Malik, Anwar, Javed, & Raza, 2015; Dittrich-Schröder, Wingfield, Klein, & Slippers, 2012; Kranzfelder, Ekrem, & Stur, 2016).

Direct PCR is a technique that amplifies DNA directly from biological samples without prior DNA extraction. It reduces time and cost and obviates complex process and toxic chemicals (Mercier, Gaucher, Feugeas, & Mazurier, 1990; Panaccio, Georgesz, Hollywell, & Lew, 1993; Wong et al., 2014). The method is achieved using genetically modified DNA polymerases, which have higher tolerance to inhibitors, and proprietary additives, such as PCR enhancers, to the reaction buffer (Śpibida, Krawczyk, Olszewski, & Kur, 2017). Direct PCR was first introduced to the entomology community about two decades ago (Grevelding, Kampkötter, Hollmann, Schäfer, & Kunz, 1996). It has been successfully used to identify some insects mainly from order Diptera (e.g. fruit flies, nonbiting midges and mosquitoes) and Lepidoptera (e.g. fall armyworm) (Grevelding et al., 1996; Loto, Romero, Baigori, & Pera, 2013; Werblow et al., 2016; Wong et al., 2014). Direct PCR also shows promising results in amplifying DNA from ethanol-preserved samples (Loto et al., 2013; Shokralla, Singer, & Hajibabaei, 2010). However, the numbers of samples in these studies were limited and many taxonomic groups still failed to amplify, especially hard-bodied insects such as Coleoptera and Odonata (Wong et al., 2014). Beyond their use in routine identification, direct PCR has the potential to be used for ecological investigations, e.g. direct PCR to amplify DNA in faecal samples (Kitpipit, Chotigeat, Linacre, & Thanakiatkrai, 2014). More recently, direct PCR has been used with next-generation sequencing (NGS) to metabarcode hundreds and thousands of samples at a very low cost per sample (less than 1 USD per barcode) (Baloğlu, Clews, & Meier, 2018; Meier, Wong, Srivathsan, & Foo, 2016; Wang, Srivathsan, Foo, Yamane, & Meier, 2018). However, the success rates of these NGS-based studies can still be improved (e.g. 60%–80% in Baloğlu et al. (2018) and 82% in Wang et al. (2018)), and some potentially problematic insect sample types have not been assessed. A direct PCR assay or a simple and effective pre-PCR step that can be universally applied across most insect taxa, sample types, and detection techniques will greatly benefit conservation, epidemiological, agricultural studies.

Therefore, in this study, we aimed to develop an improved direct PCR protocol for DNA barcoding that can be used for species identification in a broad range of insect taxonomic groups. We also aimed to demonstrate the applicability of the developed workflow to museum samples, food samples and even to bat diet analysis. First, we examined whether the developed workflow can be used

TABLE 1 Amplification and sequencing success rates obtained from the developed workflow categorized by insect order. List of the sample types, sample size and age of the samples used in this study is shown

		Success rate (%)				
Sample types[a]	Taxa	1'Amplification	2'Amplification	Sequencing	Sample size	Age (years)
Fresh specimens	Mantodea	100	-	100	4	0
	Phasmatodea	100	-	100	8	0
	Neuroptera	100	-	100	5	0
	Odonata	100	-	92.3	13	0
	Blattodea	100	-	100	15	0
	Orthoptera	100	-	90.0	10	0
	Lepidoptera	92.1	97.3	97.3	38	0
	Coleoptera	75.0	93.8	87.5	16	0
	Diptera	85.7	90.5	85.7	21	0
	Hemiptera	81.8	81.8	77.3	22	0
	Hymenoptera	75.0	75.0	62.5	8	0
Ethanol-preserved specimens	Various taxa	98.6	-	-	143	3–5
Oven-dried specimens	Various taxa	100	-	-	30	3[b]
Museum specimens	Lepidoptera	30.0	-	30.0	10	3–39[c]
Bat faecal specimens	Unknown	84.0	-	60.0	25	0
Food specimens	Lepidoptera	90.0	-	80.0	10	0

[a]The fresh specimens (*N* = 160) were used for optimization and to show that the workflow could be used for insect barcoding. The nonfresh specimens (*N* = 218) were used to validate the workflow. [b]Indicates that samples were three years old, but they were freshly dried by hot air oven for this study. [c]Indicates that samples were dried immediately after collection and kept in a dried state for 3 to 39 years in the museum. A hyphen (-) indicates that the experiment was not conducted. Amplification success rate was calculated by dividing the number of samples successfully amplified by the total number of samples. The second amplification success rate was calculated by summing the samples that were amplified in the first amplification and the samples that were additionally amplified in the second amplification. Only samples successfully amplified were submitted for sequencing. Sequencing efficiency was determined by dividing the number of usable sequence (with high-quality sequence) by the total number of samples in the corresponding taxon. As such, sequencing success rates do not exceed their corresponding amplification success rates.

for species identification purpose in various taxa of insects; a full validation of the developed workflow was performed to determine sensitivity and reproducibility of the workflow. Second, the workflow was tested in various insect sample types typically encountered in entomological studies, including oven-dried specimens, ethanol-preserved specimens, museum specimens, bat guano specimens, and food specimens, to investigate a range of applicability. We also compared cost and analytical time of the developed workflow and other competitive commercial kits. The developed workflow has the potential to overcome the limitation of conventional insect species identification method and could be applied to a wide range of sample types and detection techniques. Also, this workflow helps to preserve insect tissues and further DNA extraction is possible from the processed fragments.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 378 insect samples, spanning 11 orders, 36 families and 94 species, were tested in this study. Table 1 shows samples categorized by types: fresh, dried, museum, ethanol-preserved, bat guano and food samples. Further details are shown in Supporting Information Table S1. Of the 378 samples, 160 fresh insect specimens were used to optimize and test the performance of the workflow, and the remaining 218 nonfresh samples (dried, museum, ethanol-preserved, bat guano and food samples) were used to validate the workflow.

The 160 fresh samples were collected from two sources: collected at the forestry area of Prince of Songkla University, Thailand, by hand, swipe net, light trapping, fruit trapping and pitfall trapping, and donated by the National Biological Control Research Center and His Majesty the King Insects Park, Kasetsart University, Thailand (Supporting Information Table S1). Specimens were identified to various taxonomic levels before further processing using morphological features following experts' use of classification keys (Kononenko & Pinratana, 2005; Pinratana & Černy, 2009; Triplehorn, Borror, Triplehorn, & Johnson, 2005). All samples were separately kept in sterile plastic bags and stored at −20°C until further analysis. At the end of the study, the samples were labelled and deposited according to the suggestion of the Princess Maha Chakri Sirindhorn Natural History Museum, Thailand.

To test the efficiency of workflow on stored samples and ecological samples, four sample types were used (total $N = 218$), including dried specimens ($N = 40$), ethanol-preserved specimens ($N = 143$), pellets of bat droppings ($N = 25$) and food specimens ($N = 10$) (see Supporting Information Table S1 for details). Forty dried specimens were collected from two sources. First, thirty insects were dried from pinned specimens by drying in a hot air oven at 50°C for 2 weeks. Second, ten dried specimens (age between 3 and 39 years) were provided by the Princess Maha Chakri Sirindhorn Natural History Museum. For ethanol-preserved specimens, 143 wholly preserved specimens (age between 3 and 5 years) were donated by Bats, Small Mammals and Birds Research Unit, Department of Biology, Faculty of Science, Prince of Songkla University. These specimens were preserved individually in a 1.5 ml microcentrifuge tube (or 50 ml vial for large insect) containing 70% ethanol and were preserved immediately after sampling in the field. Twenty-five pellets of insectivorous bat guanos were also donated by the same group. In addition, 10 food samples collected from street food markets were included to test in this study.

2.2 | Sample preparation

Fresh samples and dried insect samples were prepared by putting a few pieces of approximately 1×1 mm^2 leg (for large insect) or whole body (for small insect) in a 1.5 ml microcentrifuge tube. Twenty micro litres of 1X phosphate buffer saline (PBS) was added to the tube before briefly mixing at room temperature and incubating at 98°C for 2 min. The supernatant, called pre-PCR solution, was then added directly to a PCR mastermix instead of purified DNA.

For ethanol-preserved specimens, the samples were dip-rinsed in sterile distilled water, briefly shaken using vortex mixer, wiped dry with a filter paper and prepared as same as fresh specimens (i.e. dissected, added to 20 µl PBS, mixed and incubated for 2 min).

For bat guano samples, a single pellet was ground in a 1.5 ml sterile tube into fine powder using sterile plastic pestle. The powder was then mixed with 1,000 µl of PBS and briefly centrifuged. Twenty micro litres of clear supernatant was transferred to a new tube, incubated for 2 min and used as pre-PCR solution.

For food samples, insect tissue was dissected to 1×1 mm^2 in cross section surface and prepared like the fresh specimens (i.e. dissected, added to 20 µl PBS, mixed and incubated for 2 min).

2.3 | Direct PCR amplification

PCR amplification was carried out using the Phire® Hot Start II DNA polymerase kit (Thermo Fisher Scientific, USA). PCRs were prepared in total volume of 20 µl comprising 1X PCR buffer, 0.2 mM dNTPs, 1.0 unit Phire® Hot Start II DNA polymerase, 1 µl of pre-PCR solution, sterile distilled water and primers shown in Table 2. PCR was performed using the T100™ Bio-Rad thermal cycler (Bio-Rad, USA) using the PCR conditions listed in Table 2. In case of failure to amplify initially, a second amplification was performed by using a freshly prepared pre-PCR solution made from a different starting tissue of the same specimen.

2.4 | PCR product detection and purification

PCR products were checked using agarose gel electrophoresis. To do this, 2% agarose gel stained with ethidium bromide was prepared and loaded with 20 µl PCR products. The system was run in 1X Tris-Borate-EDTA (TBE buffer) at 120 V for 30 min then visualized under Bio-Rad Gel Doc™ EZ system (Bio-Rad, USA). Successfully amplified products were purified using illustra™ ExoProStar™ (GE Healthcare Life Sciences, USA). In case of presence of nonspecific DNA bands, expected PCR products were cut from the gel and

TABLE 2 List of the seven primer pairs incorporating the optimized protocol in this study including primer name, sequence, expected amplicon size (bp), target taxa, PCR conditions and references. For sequences used to design primer PlanthopperF/R, please see Supplementary Table 2

Primer name	Sequence (5′ to 3′)	Size (bp)	Target species	Thermal steps		References
				Typical PCR	Touchdown PCR	
UEA7	TACAGTTGGAATAGACGTTGATAC	700	Diptera	Initial denature: 94°C, 5 min		Lunt, Zhang, Szymura, and Hewltt (1996)
UEA10	TCCAATGCACTAATCTGCCATATTA			Denaturation: 94°C, 40 s		
				Annealing 55°C, 60 s		
				Extension: 72°C, 40 s		
				Final extension 72°C, 2 min		
				Number of cycles: 35		
tRWF1	AAACTAATARCCTTCAAAG	700	Orthoptera	Initial denature: 95°C, 2 min	Denaturation: 94°C, 40 s	Park, Suh, Oh, and Hebert (2010)
LepR	TAAACTTCTGGATGTCCAAAAAATCA		Mantodea	Denaturation: 94°C, 40 s	Annealing 51°C, 40 s	
			Phasmatodea	Annealing 45°C, 40 s	Extension: 72°C, 70 s	
				Extension: 70°C, 70 s	Final extension 72°C, 2 min	
				Number of cycles: 5	Number of cycles: 40	
LepF	ATTCAACCAACCAATCATAAAGATATTGG	658	Universal primer	Initial denature: 94°C, 1 min	Denaturation: 94°C, 30 s	Hebert et al. (2004)
LepR	TAAACTTCTGGATGTCCAAAAAATCA		for insect taxa	Denaturation: 94°C, 30 s	Annealing 55°C, 40 s	
				Annealing 45°C, 40 s	Extension: 72°C, 60 s	
				Extension: 72°C, 60 s	Final extension 72°C, 2 min	
				Number of cycles: 5	Number of cycles: 35	
CI-J-1632	TGATCAAATTTATAAT	570	Coleoptera	Initial denature: 95°C, 3 min		Simon et al. (1994)
CI-N-2191	GGTAAAATTAAAATATAAACTTC			Denaturation: 95°C, 30 s		
				Annealing 45°C, 60 s		
				Extension: 72°C, 60 s		
				Final extension 72°C, 2 min		
				Number of cycles: 35		
ShortF	CAATTTCCAAATCCNCCAAT	220	Coleoptera	Initial denature: 98°C, 30 s		Gilbert, Moore, Melchior, and Worobey (2007)
ShortR	GGTCAACAAATCATAAAGATATTGGAA			Denaturation: 98°C, 5 s		
				Annealing 50°C, 5 s		
				Extension: 72°C, 10 s		
				Final extension 72°C, 1 min		
				Number of cycles: 35		

(Continues)

TABLE 2 (Continued)

Primer name	Sequence (5′ to 3′)	Size (bp)	Target species	Thermal steps		References
				Typical PCR	Touchdown PCR	
ZBJ-ArtF	AGATATTGGAACWTTATATTTTATTTTTGG	157	Universal primer	Initial denature: 94°C, 3 min	Denaturation: 94°C, 30 s	Zeale, Butlin, Barker, Lees, and Jones (2011)
ZBJ-ArtR	WACTAATCAATTWCCAAATCCTCC		for insect taxa	Denaturation: 94°C, 30 s	Annealing 53°C, 30 s	
PlanthopperF	TTAATAATTGGTGCACCAGATATAG	145	Hemiptera	Annealing 61°C, 30 s	Extension: 72°C, 30 s	Self-developed primer
PlanthopperR	AWAGGGGGGGATAAAYDGTTC			Extension: 72°C, 30 s	Final extension 72°C, 2 min	
				Number of cycles: 16	Number of cycles: 24	

purified using QIAquick® Gel Extraction Kit (Qiagen, Germany). Purified PCR products were then quantified using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific) and kept at −20°C until further analysis.

2.5 | Sequencing and species identification

Purified PCR products were sequenced at First BASE Laboratories SDN BHD, Malaysia. Ambiguous bases were checked and corrected using the software FINCH TV Version 1.4.0 (Geospiza Inc, USA). To identify species, good quality sequences were then matched with known reference sequences in the NCBI database using the program BLASTN. A species was called if there was a match with 98%–100% nucleic acid similarity score (Clare, Barber, Sweeney, Hebert, & Fenton, 2011).

2.6 | Validation of the developed direct PCR workflow

2.6.1 | Reproducibility test

The reproducibility test was conducted for two purposes. First, to determine the applicability and robustness of the developed protocol to samples collected from different body parts, sources or sizes. Due to possible variations in DNA availability of insects from different sources and in different stages, sizes or body parts, we wanted to demonstrate that the optimized tissue amounts were robust to these variations. Therefore, 40% (63/160 fresh samples) of specimens used for the optimization experiment above were reprepared as pre-PCR solution using a new piece of insect tissue. These samples spanned 13 species from eight orders (see Supporting Information Table S1). Second, we wanted to investigate how long the DNA in the pre-PCR solution (PBS buffer) lasts, 31 pre-PCR solutions kept at −20°C for 3 months to three years were subjected to amplification and detection, using the same protocols detailed above. These samples constituted 24 species from six orders (see Supporting Information Table S3).

2.6.2 | Sensitivity test

The sensitivity test was performed to determine the optimal and minimum amount of insect tissue that could be analysed by the developed protocol. The legs of large-bodied insects or the body of small insects were dissected. Varying number of 1-mm^2 fragments (8, 6, 4, 2, 1, ½, ¼ and ⅛th pieces) was tested. The amplification success rates and PCR product band intensities were evaluated. Ten replicates were performed for each number of fragments.

2.6.3 | Applicability to various ecological sample types

Five sample types (oven-dried, ethanol-preserved, museum, bat guano and food specimens) were used to test the applicability and robustness of the developed protocol to commonly found

	Amplification success rate (%) for each sample amount (1-mm^2 pieces)							
Insect size	8	6	4	2	1	½	1/4th	1/8th
Large	100	100	100	80	50	20	10	0
Small	–	–	–	80	100	30	10	10

TABLE 3 Sensitivity test for the developed protocol for large-bodied and small-bodied insects. Different insect specimens were used for each amount (N = 10). Eight to 1/8th pieces of 1-mm^2 were used to prepare pre-PCR solutions. A successful amplification means a visible PCR product band was observed

ecological sample types. A modified protocol was used for bat guano specimens, as follows: if a PCR product were not seen on the agarose gel, nested-PCR was then performed with the PCR product from the first round of amplification as the DNA template.

2.7 | Recovery of genomic DNA from PBS-treated tissue

In order to determine whether the PBS-treated tissue could be DNA-extracted and the DNA could still be used for further genetic studies, we performed DNA extraction from 15 randomly selected PBS-treated samples. These 15 samples (pre-PCR solution) had been prepared from fresh insect tissues (N = 5), ethanol-preserved specimens (N = 5) and oven-dried specimens (N = 5), all of which had been successfully amplified using the developed direct PCR workflow (Section 2.2 to 2.4) in the earlier phase of the study. We used the QIAamp® DNA Mini Kit (Qiagen) with minor modification: incubation time was extended from 3 hr to overnight. The extracted DNA was then used as DNA template for amplification with the LEP primers. All reagents and PCR conditions were prepared as described in Section 2.3.

3 | RESULTS

3.1 | Optimization of the developed direct PCR workflow

3.1.1 | Amplification and sequencing from fresh specimens

The developed workflow was optimized and used to analyse 160 fresh insect specimens. Table 1 shows the amplification and sequencing success rates (usable sequence) for these samples. In summary, 150 out of 160 fresh samples were successfully amplified (93.8%), all 150 were successfully sequenced (93.8%), and 144 sequences passed filtering (90%). The result showed that the first pass amplification success rates were between 75% and 100%, depending on the insect order. Samples that did not yield any visible PCR product in the first amplification (1st amplification) were re-amplified using a newly made pre-PCR solution (2nd amplification). The result showed improved efficiency in three insect orders included Lepidoptera, Diptera and Coleoptera (Table 1). The six samples (6/150 PCR products) that did not pass filtering were due to the presence of multiple peaks in the electropherograms, which was probably due to carry-over contamination during sample collection. These six samples were prepared from legs and as such were highly unlikely to be cross-reactivity of primers with gut bacteria, and the samples prepared from whole bodies never exhibited multiple peaks in our study.

3.1.2 | OTU delimitation

One hundred forty-four good quality sequences were queried against reference sequences available on GenBank for insect species identification. BLAST queries showed that all 144 sequences could be matched with the nucleotide similarity of 85%–100% (Supporting Information Table S4). Results were further compared with prior identification based on morphological features. The BLAST matches for all 144 sequences matched the expected taxon identification (Supporting Information Table S4). This indicates that the developed protocol could be used as a tool to successfully amplify DNA for barcoding in insect species. Sequences with lower similarity percentages (<97%) in GenBank were also queried against the Barcode of Life Database (BOLD) and resulted in "no match" against BOLD.

3.2 | Reproducibility test

A total of 63 samples randomly chosen from the pool of fresh samples were reprepared as pre-PCR solution and retested with the developed workflow to demonstrate its robustness and accuracy in insect species identification. The results showed 100% amplification success rate and identification accuracy (Supporting Information Table S3) in all the samples reprepared. For reproducible performance of 31 pre-PCR solutions stored for one to three years, all 31 samples (100%) were successfully amplified and detected on agarose gel electrophoresis.

3.3 | Sensitivity test

The optimal sample amounts for the developed protocol were determined (ten replicates were performed for each sample amount). For large-bodied insects, four to eight 1-mm^2 pieces dissected from the leg consistently yielded 100% success rates (Table 3). For small-bodied insects, only one 1-mm^2 piece dissected from the body yielded 100% success rate (Table 3). The minimum amounts that generated detectable PCR products at least once in ten amplifications were ¼ of a piece and ⅛th of a piece for large- and small-bodied insects, respectively.

3.4 | Applicability to various ecological sample types and cooked samples

In order to determine applicability of the developed workflow, five types of insect specimens commonly found in entomological studies (total *N* = 218), including oven-dried samples (*N* = 30), ethanol-preserved samples (*N* = 143), museum samples (*N* = 10), bat guano samples (*N* = 25) and food samples (*N* = 10) were tested. Ethanol-preserved and oven-dried samples were prepared from fresh samples to mimic the standard practices of preservation method for further molecular analysis whereas museum samples, guano samples and food samples were included to determine whether the developed protocol can be used to amplify the naturally degraded samples.

The results showed that the amplification success rates from oven-dried samples, ethanol-preserved samples, food samples, bat guano and museum samples was as high as 100% (*N* = 30), 98.6% (*N* = 143), 90% (*N* = 10), 84% (*N* = 25), 30% (*N* = 10), respectively. Only three museum specimens were successfully amplified, and all of these samples gave good quality electropherograms equating 30% success rate in both amplification and sequencing (Supporting Information Table S5) For food samples, nine PCR products were successfully amplified and eight high-quality sequences (80%) were obtained. These barcodes allowed the identification of unknown cooked insect via BLAST (Supporting Information Table S5).

For bat guano samples, only 60% of the samples could be sequenced and identified. The rest showed multiple peaks at the same base position in the electropherogram, indicating presence of DNA from multiple species. The successfully amplified bat guano samples were found to contain DNAs from *Lispe* sp. and Hemiptera sp.

3.5 | Recovery of DNA from PBS-treated tissue

Fifteen samples of PBS-treated tissue were DNA-extracted using a commercial extraction kit and subjected to conventional DNA amplification procedure. The DNA extracts from all 15 samples (100%) showed the expected size of PCR products (650 bp), demonstrating that the remaining insect tissues in PBS can still serve as starting material for further genetic studies.

4 | DISCUSSION

In this study, we optimized an economical, rapid and highly efficient direct PCR workflow that can be applied to a wide range of insect taxa, as well as demonstrated its effective for ecological samples. Overall, these results demonstrate that the developed workflow is applicable for various types of insect specimens and, therefore, has the potential to be used for assessment of biodiversity, for ecological monitoring, and for taxonomic study. We attributed the high success rates to many factors. First, this direct method incorporated a dilution step using PBS, which helped to dilute the potential PCR inhibitors in the samples and maintain the correct pH of the reaction (Kitpipit et al., 2014). Second, the heating step aided cell lysis which released DNA for the PCR reaction and denatured proteins that could degrade DNA or affect PCR (Grevelding et al., 1996; Loto et al., 2013). Third, the high-fidelity DNA polymerase used in this study was more tolerant of PCR inhibitors. This modified enzyme allowed amplification of a wide range of insect species with varying degrees of inhibitors (Wang et al., 2004). Fourth, we designed and selected additional primer pairs based on extensive sequence alignments. These primers minimized primer-template mismatches (Varadinova et al., 2015; Waugh, 2007), which increased success rates for some taxa that could not be amplified in previous reports.

Although Phire Hot Start II is a modified polymerase, its cost is comparable to standard *Taq* polymerases (Supporting Information Table S6), which makes it suitable for high throughput applications. The superiority of Phire Hot Start II for direct PCR has been demonstrated by Kitpipit et al. (2014). Supporting Information Table S7 compares the advantages and disadvantages of commercial direct PCR kits and the developed workflow using six criteria, which are cost per reaction, handling time during sample preparation, insect sample tested, amounts of tissue require, reproducible performance and harmful chemical used. As shown in the Supporting Information Table S7, our proposed workflow is not the fastest to prepare, but it is cost-effective (i.e. only PBS is required), can be used for a wide range of insect taxa, does not use toxic chemicals, and the prepared pre-PCR solution can be reused for direct PCR up to three years. The remaining tissues could also be subjected to DNA extraction for further genetic analyses that might not be amenable to direct PCR. Further studies could be conducted to confirm the DNA quality of tissues stored at varying time intervals to determine whether these pre-PCR solutions could be used for very long storage times (e.g. up to 10 years). If high-quality DNA could still be extracted from these tissues, this method would prove beneficial for rare and vulnerable specimens.

Bat guano was included in our validation of the workflow in order to determine whether the sample preparation steps (pre-PCR solution of PBS) would be sufficient to obtain barcodes in a difficult ecological sample. We were able to obtain barcodes of both hard- and soft-bodied insect preys available in the guano, but almost half of the samples resulted in mixed DNA barcodes from many species. This was expected as bats feed on many insect species available near their cave (Leelapaibul, Bumrungsri, & Pattanawiboon, 2005). The ability of NGS to do metabarcoding, that is sequencing and identifying many species in environmental samples in which DNA are mixed, is ideal for such situations. In order to truly reap the time and cost benefits of NGS, thousands of samples are required. However, (a) access to instrument (i.e. NGS-ready lab set-up cost of at least 100,000 USD) (El-Metwally, Ouda, & Helmy, 2014; Helmy, Awad, & Mosa, 2016), (b) the large sample pool required to drive down the cost per barcode (i.e. 1,000 to 10,000 samples), and (c) specialist availability (e.g. bioinformaticians and highly trained technicians) are still obstacles that need to be overcome for small- to medium-scale projects in developing countries (Helmy et al., 2016). In this study, we still relied on Sanger sequencing, which is readily available

in most university laboratories at reasonable prices (5–15 USD per specimen) when dealing with a relatively smaller scale study (e.g. 100–200 specimens). Potentially, the pre-PCR step of incubating in PBS buffer could be used to prepare samples for NGS in large-scale studies. This would maximize the opportunity to retry samples that may have failed due to the lower success rates of NGS; to retry samples in order to see if the first PCR/sequencing run has missed certain species that are present in lower amounts; and to extract DNA from the remaining tissues in PBS for further genetic studies of valuable specimens.

The commonly used HCO-LCO primers for insect barcoding were initially tried in our preliminary tests. This primer set failed to amplify many specimens, which could be due to only three species of insects were included during primer design (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), resulting in primer-template mismatches when applied to a broad range of orders. As such, we decided to use LEP primers, which were developed by (Hebert & Gregory, 2005) as the main primer pairs due to high amplification efficiency in a wide range of taxa based on preliminary studies. Although these primer pairs were developed to amplify mainly insects in order Lepidoptera, we successfully used them to amplify butterflies, moths, cockroaches, ants, dragonflies, green lacewings, mosquitoes and some beetles. Moreover, there are around 700,000 sequences archived in the Barcode of Life Data System (BOLD) using LEP primers, compared to only 25,000 for LCO primer. Thus, the LEP primers can be used as the main universal primers for insects. Unfortunately, GenBank does not provide statistics for primers used and, therefore, a comparison could not be made. Even with GenBank's and BOLD's current sizes, only about half of the sequences obtained in our study was identified down to the species level. Efforts to metabarcode from ten and hundred thousands of insect using high throughput platforms such as NGS would be one way address the incompleteness of these databases (Jinbo, Kato, & Ito, 2011). Other primers from the literature were also employed to increase amplification success for difficult taxa (i.e. in cases which universal primers had shown low success rates). Our customized primers were developed specifically to amplify trace DNA in bat guano samples for ecological assessment purpose (i.e. for Hemiptera). The customized primers amplified a subsection of the standard barcoding region. Our results showed that the developed workflow could be used with a wide range of primers under different conditions.

The taxonomic orders which produced high success rates agreed with previous studies (Armstrong & Ball, 2005; Loto et al., 2013). This study was the first time Phasmatodea and thick exoskeleton taxa (e.g. Coleoptera and Odonata) were successfully amplified with direct PCR. Prior to this study, these thick exoskeleton taxa had been deemed unsuitable for direct PCR (Wong et al., 2014). The slightly lower success rates seen with Hymenoptera, Hemiptera, Diptera and Coleoptera could be due to the presence of various PCR inhibitors in these insects, such as melanin in their compound eyes, haemocyanin, and formic acid. Melanin binds with DNA templates and hinders the activity of DNA polymerase (Boncristiani, Li, Evans, Pettis, & Chen, 2011; Opel, Chung, & McCord, 2010). Haemocyanin, due to its structural similarity to haemoglobin, may act as a chelating agent to prevent enzymatic functions. Glandular legs also contain other secretions which could inhibit PCR (Billen, 2009; Wong et al., 2014). Similarly, light-coloured exoskeletons contain phenolic compound (e.g. arterenone, dopamine and noradrenaline) in their integuments (Kramer et al., 2001). These compounds are precursors in many metabolic pathways and may act as PCR inhibitors by chelating metal ions (Schrader, Schielke, Ellerbroek, & Johne, 2012). Diptera is a highly diverse taxa, and thus, it is difficult to find a truly universal primer (Waugh, 2007). For Coleoptera, the primers we selected from previous studies (CI-J-1632/ CI-N-2191 and ShortF/ShortR) were responsible for the lower success rates. Even with conventional PCR using a purified DNA template, no PCR product was obtained using universal primers (Castalanelli et al., 2010).

In sensitivity test, the optimal sample amounts for the developed protocol were determined. The results demonstrated that the developed protocol has similar sensitivity to the conventional extraction method, which requires at least a single leg for large insects (Foottit, Maw, & Hebert, 2014; Hebert et al., 2004; Oba, Ohira, Murase, Moriyama, & Kumazawa, 2015) and multiple legs or whole bodies for smaller insects (Gutiérrez, Vivero, Vélez, Porter, & Uribe, 2014; Hebert & Gregory, 2005). This makes it highly useful for cases where the samples are degraded (i.e. ecological specimens such as bat guano) and cases where morphological characteristics are obscure. Also, it provides an option to perform DNA barcoding without deconstruction of whole voucher specimens in museums.

For museum specimens, low amplification success rate was obtained even when well-optimized protocol was used. We extracted DNA from the actual specimens using a standard extraction kit (QIAamp DNA mini kit, QIAGEN) to test whether the low success rate was associated with the use of the developed protocol. The result indicated that although standard protocol was used, amplification success rate still remained low (only 50% success rate) and low PCR product yields (i.e. faint bands or no band observed on agarose gel) were observed in specimens aged over six years old. We attributed this to several possible reasons. First, the specimens were pinned and dried to study external morphology; as such, the soft tissue inside exoskeleton was dried and decomposed by time without chemical preservative treatment (Dick, Bridge, Wheeler, & Desalle, 1993; Sutrisno, 2012), resulting in limited intact DNA source. Second, dichlorvos, a preservative chemical used to prevent collection pest, may negatively affect DNA amplification (Espeland et al., 2010; Werblow et al., 2016) and third, other physical factors might affect DNA integrity. For example, partial dehydration and exposure to air and light may potentially lead to DNA degradation, particularly deamination of cystidine residues (Mitchell, Willerslev, & Hansen, 2005). Similarly, Zimmermann et al. (2008) claimed that DNA of 8-years old specimens could be fragmented to around 70 bp in length, specimens aged >18 years also gave abundant fragments of 50 bp in size, and specimens aged between 30–40 years could be degraded down to oligonucleotide sizes (20 bp). These results indicated that primer choice is a critical factor to recover DNA from aged museum specimens because long amplicons may not survive. Conservation and

biodiversity researchers should follow the best practice of storage condition of specimen for further molecular study. Based on the present result, we recommend that ethanol is favourable for insect specimen preservation. If insect pinning is required then the preservative chemical should be either paradichlorobenzene or naphthalene that do not affect PCR amplification (Espeland et al., 2010).

5 | CONCLUSION

Our study demonstrated that for many insect taxa, high amplification success rates could be achieved through direct PCR amplification using the pre-PCR dilution protocol. In addition to the reduced analysis time and cost of direct PCR, we showed that the pre-PCR dilution method allowed various insect sample types, for example oven-dried, ethanol-preserved, museum and even bat guano samples, to be prepared using one universal method. The comparison between morphology and molecular-based method confirmed that the developed assay should be reliable for routine identification. In combination with previously published and newly designed primers, the workflow had high sensitivity and could be applied to dried samples, ethanol-preserved samples and food samples with high success rates. The whole workflow could be beneficial in the fields of insect taxonomy and ecological studies due to its low cost, simplicity and applicability to degraded specimens. Lastly, the pre-PCR dilution part of the workflow may be combined with other amplification or detection techniques, such as denaturing gel gradient electrophoresis and next-generation sequencing, to deconvolute mixed DNA samples and drive down metabarcoding costs even further.

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AUTHOR CONTRIBUTIONS

(a) The study detailed in the manuscript is part of master thesis of K.T. (b) The experiments were designed by T.K., W.C. and S.B. (c) K.T. conducted insect sample collection and molecular laboratory while bat guano collection was conducted by S.B. (d) T.K., P.T. and W.C. contributed laboratory supplies and analytical tools. (e) K.T., T.K. and P.T. analysed data and wrote and revised the manuscript.

DATA ACCESSIBILITY

DNA sequences were assigned Accession nos: MH686444–503 and MK033641–738.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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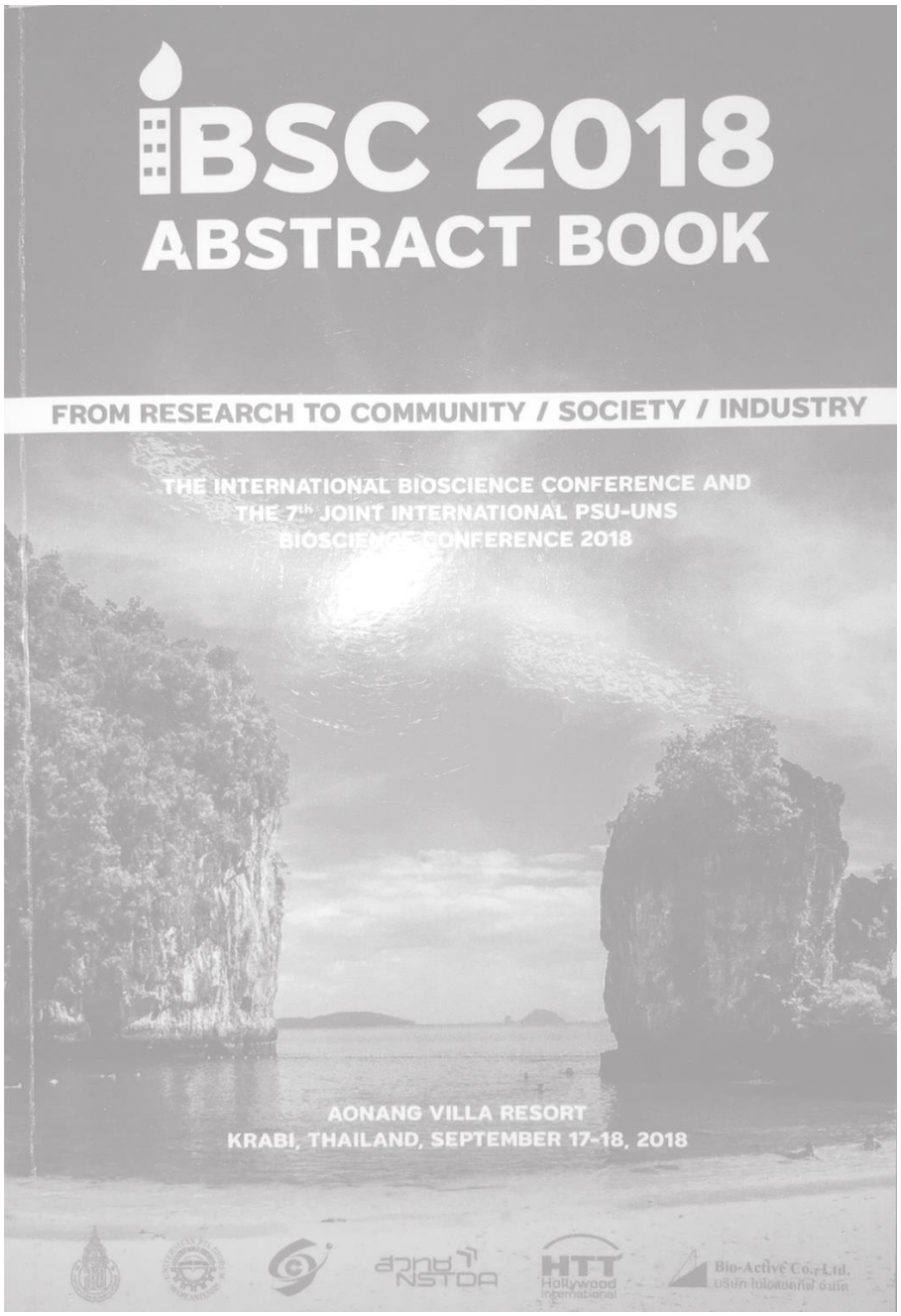
APPENDIX B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Track A

Biodiversity, Environment and Physiology of Living Organisms

POA

POA-100

A preliminary diet analysis of wrinkle-lipped free-tailed bat *Chaerephon plicatus* (Buchannan, 1800) using direct PCR-DGGE technique

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Abstract. *Chaerephon plicatus* has been reported as a pest control agent which plays an important role for rice pest suppression. Diet analysis is used to reveal this ecology service. However, fecal examination using microscopic method have never provided reliable prey list due to degradation of remains. In this preliminary study, we identified insect preys in bat guano using a direct PCR-DGGE technique which incorporates a short pre-PCR preparation step prior to PCR amplification. PCR products amplified from multiple species were then separated by DGGE system. Forty five of 55 bat guano pellets collected monthly for six months from bat caves surrounded by rice fields were successfully amplified (81.8% amplification success rate). Eighty one bands were separated from PCR products; sequencing confirmed that these bands comprised 20 operational taxonomic units (OTU) of insects and could be assigned to 7 orders, 13 families, 17 genera and 16 species. The results showed that *C. plicatus* diet composition depends on rice-growing season. Potential rice pest species, e.g. brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix virescens*), zigzag leafhopper (*Recilia dorsalis*) and stem rice borer (*Scirpophaga incertulas*), were consumed by *C. plicatus*, indicating its function as pest suppressing agent. We propose the first prey species list of *C. plicatus* which allows further understanding of predator-prey interaction and illustrates this insectivorous bat's foraging behaviour. These findings also provide basic data which could further benefit conservation and sustainable management of bat caves.

Keywords: diet analysis, PCR-DGGE, insectivorous bat, ecology service, *Chaerephon plicatus*

1. Introduction

Insectivorous bats routinely regulate insect pest in agricultural landscape worldwide (Kunz *et al.*, 2011). Particularly, molosid bats that usually form large aggregated colonies have significantly contributed to human well-being due to the ecological service they provide. For example, Brazilian free-tailed bats (*Tadarida brasiliensis*) feed on moths preventing cotton yield losses in the southern US which is valued at 741,000 USD annually (Cleveland *et al.*, 2006). In Thailand, similar biological control is provided by wrinkle-lipped free-tailed bats (*Chaerephon plicatus*), which consume white-backed planthoppers in rice fields around their roosting cave (Leelapaibul *et al.*, 2005). This rice yield protection is valued at 1.2 million USD each year (Wanger *et al.*, 2014). Moreover, large quantities of bat guano produced in their habitat are harvested for use as fertilizers by local communities (Furey *et al.*, 2018).

Diet analysis of bats is done using both visual observation and DNA-based approaches. However, direct observation of feeding is sophisticated, whereas microscopic examination of stomach contents or guano required expertise and intensive labor. Also, because of thorough mastication, soft-bodied preys were often missed (Whitaker *et al.*, 2009; Wray *et al.*, 2018). Techniques based on DNA barcoding have been used to overcome these difficulties. Either specific PCR primers or universal primers can be used. With specific primers, one or a few targeted prey species can be detected. For universal primers,

cloning to isolate mixed-prey amplicons followed by sequencing individual clone is used to identify species (Zeale *et al.*,2011). However, this approach is labor-intensive and expensive. Currently, next generation sequencing (NGS) is becoming a powerful tool in diet studies, as it reduces costs and allows faster processing when used to analyze large batches of samples, but bioinformatics pipelines needs to be developed and interpretation can be difficult (Pompanon *et al.*, 2012). The high cost of establishing and maintaining an NGS facility and the lack experts hinder NGS applicability, especially for developing countries (Helmy *et al.*, 2016). Therefore, other alternative approaches for diet analysis should be developed

Recently, direct PCR – DNA amplification without DNA extraction – has been shown to have high efficiency for mammalian species identification from highly degraded samples such as feces (Kitpipit *et al.*, 2014). It is rapid, economic and more sensitive than conventional PCR because no DNA is lost during the extraction steps. Improved DNA polymerases and PCR buffer help to increase the amplification efficiency in the presence of inhibitors. Direct PCR has been successfully used to identify some insects, mainly from the order Diptera (e.g. fruit flies, non-biting midges, and mosquitoes) and Lepidoptera (e.g. fall armyworm), both of which are present in large proportions of bat diet. Denaturing gradient gel electrophoresis (DGGE) is another option successfully used for dietary study. This electrophoretic system allowed mixed PCR products from various preys eaten by a predator to migrate through a denaturant-integrated polyacrylamide matrix. PCR products of each prey is then separated based on different GC content or melting temperature (Tm). DGGE has been applied for dietary analysis of prey community in gut of krill, squid, sea lion, and leopard cat (Lee *et al.*, 2013). However, the combination of direct PCR and DGGE has never been used to study insect preys in bat feces.

To improve our understanding in ecology service of insectivorous bats, we aimed to analyse the diet of *C. plicatus* . Firstly, we examined whether direct PCR-DGGE could be used to study bat diets. Secondly, we investigated the number of bat guano pellets which given sufficient information to illustrate feeding behavior. Lastly, we compared the insect order revealed using microscopic method and the developed technique. This study should elucidate insectivorous bats' contribution to pest suppression, which should encourage future conservation plans.

2. Materials and Methods

2.1. Study areas

POA

Chaerephon plicatus fecal samples were contributed by the project "Ecosystem services of *C. plicatus* to rice fields in Thailand". In this study we focused on the Khao Wong kot Cave in Lopburi province (15°01'06.04"N, 100°32'42.81"E), a cave in central Thailand which is home to a large colony of *C. plicatus*. The cave was mainly surrounded by rice fields (70% of land used within 20 km radius). Fecal samples were collected every month from January until June 2016 by 30 plastic baskets set underneath roosting position. For each collected position, pellets were kept in 1.5 ml tube with silica gel absorption in field before transfer to storage at -20°C for long-term storage. A single pellet in each collection tube was randomly chosen for further processes.

2.2. Sample preparation and direct PCR amplification

A total of 55 bat fecal samples, based on 5-9 pellets randomly selected each month, were included in this study. Single pellets were ground in a 1.5 mL sterile tube into fine powder using sterile plastic pestle. The powder was then mixed with 1000 µL of PBS and briefly centrifuged. Twenty µL of clear supernatant was transferred to a new tube and heated at 98°C for 2 min and used as pre-PCR solution. For all samples, PCR amplification was carried out separately using two candidate mitochondrial COI primer pairs: 157 bp product from universal primers for insect (ZBJ-ArtF1c AGATATTGGAACWTTA TATTTTATTTTTGG and ZBJ-ArtR2c WACTAATCAATTWCCAAATCCTCC) and 145 bp product from planthoppers-specific primers (Forward TTAATAATTGGTGCACCAGATATAG and Reverse AWAGGGGGGGATAAAYDGTTC). PCRs were prepared in total volume of 20 µL comprising 1X

PCR buffer, 0.2 mM dNTPs, 1.0 unit Phire® Hot Start II DNA polymerase, 1 µL of pre-PCR solution, 0.5 uM of each primer and sterile distilled water. PCR was performed using a T100™ Bio-Rad thermal cycler (Bio-Rad, California, USA) using the PCR conditions of Zeale *et al.*, 2011.

2.3. PCR product detection and DGGE performance

PCR products were visualized using 2% agarose gel stained with ethidium bromide. The system was run in 1X Tris-Borate-EDTA (TBE buffer) at 100 V for 30 min then photographed using Bio-Rad Gel Doc™ EZ system (Bio-Rad, USA). 1:10 dilution of successfully amplified products were used as DNA templates in the second PCR amplification for DGGE analysis. In this step, PCR reagents and conditions remained the same as those used in section 2.2 except the forward primer. GC-clamped forward primers (GGGGCGGGGCGGGGCGGGGCGGGGGGGCAGATATTGGAAC WTTATATT TTATTTTTGG and CCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC GCCGCCGCTTAATAATTGGTGCACCAGATATAG) were used instead of ZBJ-ArtF1c and Forward planthopper-specific primer, respectively. The PCR products amplified using the universal primers (GC-clamped ZBJ-ArtF1c and non-clamped ZBJ-ArtR2c) were then loaded onto 20-25% denaturant gradient (5.6 M urea and 30% deionized formamide v/v) in 10% polyacrylamide whereas 20-50% denaturant gradient (7 M urea and 40% deionized formamide v/v) in 10% polyacrylamide was prepared for PCR products amplified using the GC-clamped Forward planthopper-specific primer and non-clamped Reverse planthopper-specific primer. The DGGE separation was performed using OmniPAGE VS20WAVE-DGGE (Cleaver Scientific, Warwickshire, United Kingdom) at a constant voltage of 50 V and temperature of 55°C for 18 h. After electrophoresis, the gels were stained in 0.5 mg/ml ethidium bromide solution for 15 min, soaked to de-stain for 30 minute , visualized and photographed using UVIdoc HD2 (UVITEC, Cambridge, United Kingdom). The separated bands were excised from the gel, and incubated in sterile distilled water for an hour to extracted PCR product from gel. Diffused PCR product were removed GC-clamp by re-amplification using reagents and conditions according to section 2.2. Amplified PCR products were checked on agarose gel and purified using QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Purified PCR products were then quantified using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA) before sequencing.

2.4. Sequencing and species identification

Purified PCR products were sequenced at First Base Laboratories SDN BHD, Malaysia. Ambiguous bases were checked and corrected using the software FINCH TV Version 1.4.0 (Geospiza Inc, Washington, USA). To identify species, good quality sequences were matched with known reference sequences in BOLD system. A species was called if it was matched to a reference sequence with ≥98 similarity score. When multiple species share the highest matching score, identification was downgraded to highest common taxonomic level (Aizpurua *et al.*, 2017).

3. Results and Discussion

3.1. Efficiency of the developed direct PCR-DGGE workflow for bat diet analysis

Forty five out of 55 fecal samples (81.8%) were successfully amplified using the developed workflow. Eighty one bands were detected after separation of PCR products using DGGE system. Sequencing results confirmed that these bands comprised 20 insect OTUs. Data analysis assigned them to 7 orders, 13 families, 17 genera and 16 species. Figure 1 shows bat diets based on frequency of occurrence in ordinal level (shown as mean±SE), which included Diptera (42.2±2.4%), Homoptera (15.9±4.4%), Lepidoptera (22.5±3.4%), Coleoptera (5.0±2.7%), Orthoptera (6.1±2.2%), Ephemeroptera (5.0±2.5%) and Blatodea (3.2±2.2%). At the family level, we observed a combination of Culicidae and Agromyeidae within Diptera; Dephacidae and Cicadellidae within Homoptera; Crambidae, Pyralidae,

Cossidae, Erebidae and Depressariidae within Lepidoptera. For the other minor proportions, only one family was assigned in each order, which were Carabidae, Gryllidae, Polymitarcyidae and Ectobiidae within order Coleoptera, Orthoptera, Epheomeroptera and Blatodea, respectively. This is the first time the prey species of *C. plicatus* has been definitely confirmed, and the result demonstrated that *C. plicatus* consumed rice pests e.g. brown planthopper (*Nilaparvata lugens)*, white-backed planthopper (*Sogatella furcifera)*, green leafhopper (*Nephotettix virescens)* and zigzag leafhopper (*Recilia dorsalis)*. These insects are categorized as pest by Pathak and Khan (1994); they cause reduced rice yield through transmission of pathogenic viruses. We also found that *C. plicatus* consumed stem rice borer (*Scirpophaga incertulas)*, a pest that prevents rice growth. These preliminary results confirmed that *C. plicatus* provides ecological service by regulating insect pests.

We attributed the success of workflow to two factors. Firstly, the direct PCR method used high tolerant DNA polymerase to overcome PCR inhibitor in combination with appropriate primers that minimized mismatches. The heating step also helped to lyze cell. Secondly, the DGGE system enabled mixed-DNA from various preys eaten by bat to be separated. This technique has been used to study stomach content in many wildlife (e.g. sea lion, monkey and leopard cat) (Lee *et al.*, 2013).

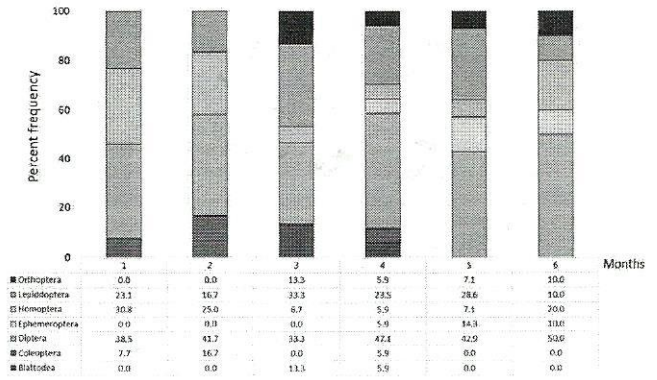

Fig. 1. Percent frequency of insect in *C. plicatus* diet between January and June 2016) categorized by insect orders are shown. Percent frequency was calculated monthly using number of occurrences of interested taxon divided by total occurrences for all taxa then multiplied by 100.

3.2. OTUs accumulation curve

To investigate the number of bat guano pellets suitable for illustrating feeding behaviour of *C. plicatus*, accumulated number of OTUs obtained from different numbers of bat guano pellets were plotted, six replications were conducted to obtain consistent results (Figure 2). The results show that accumulated number of OTUs dramatically increased when 1 to 3 bat guano pellets were tested. The accumulated number of OTUs remained consistent after subsequent addition of 1 bat guano pellet. The results indicated that around 4 bat guano pellets were sufficient to represent diet of *C. plicatus* in each month.

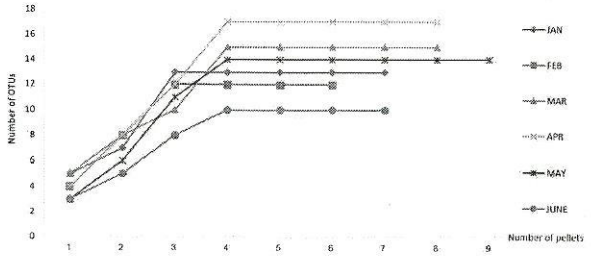

Fig 2. OTUs accumulation curve in each month In comparison, studying bat diet using microscopic method, five pellets of bat guano were required to illustrate diet of an individual (Whitaker *et al.*, 2009).

3.3. Percent frequency in ordinal level obtained from microscopic method VS developed workflow

Conventional microscopy-based method was used by Srilopan *et al.* (2018) to analyze *C. plicatus* fecal samples collected at the same study site. Comparison of percent frequency using conventional method and our DNA-based workflow showed agreement in terms of the four major insect orders observed, including Coleoptera (22.9±0.8%), Homoptera (19.8±1.5%), Diptera (17±0.7%) and Lepidopter (14.1±1.1%). However, discrepancy in quantity was observed between the results obtained by conventional method and molecular method. Also, some taxa were not observed: Ephemeroptera and Blattodea were absent using microscopic approach while Hemiptera, Odonata and Hymenoptera were absent using direct PCR-DGGE. The results indicated that using DNA barcoding as a tool to study diet is advantageous for identifying less-chitinous insects. Several prey taxa not detected using the developed workflow could be due to the poor efficiency of candidate primers for those taxa. Although these primers were designed originally to detect a broad range of insect taxa, its limited usefulness for only some insect orders have been reported (Wray *et al.*, 2018). Further work should therefore consider suitable primers or a mixed of primers in order to provide a more complete, accurate picture of preys.

4. Conclusions

Our preliminary study demonstrated that bat diet analysis can be achieved using direct PCR-DGGE workflow, which could be further applied to study other insectivorous predators. This is the first time that the diet of *C. plicatus* was revealed down to the species level. The results clearly showed the ecology service of this bat colony as regulator of rice pests around their roosting cave. Further study for whole year bat pellet should be conducted to accurately depicture complete diet on annual basis. Conservation management plan should be drafted to protect their habitat and prevent population decline.

5. Acknowledgements

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6. Conflict of interest

none

7. References

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Thongjued, K., Chotigeat, W., Bumrungsri, S., and Kitpipit, T. 2018. A preliminary diet analysis of wrinkle-lipped free-tailed bat (Chaerephon plicatus, Buchannan, 1800) using direct PCR-DGGE technique. Proceedings of the International Bioscience Conference and the 7th Joint International PSU-UNS Bioscience, 114-119.