Phylogeny of Long-Legged flies genus *Phacaspis* (Meuffels and Grootaert, 1988) in peninsular Thailand

Natcha Kaewkrajang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Zoology
Prince of Songkla University
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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(Miss Natcha Kaewkrajang)
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ชื่อวิทยานิพนธ์ วงศ์วานวิวัฒนาการของแมลงวันขายาวสกุล *Phacaspis* (Meuffels and Grootaert, 1988) ในคาบสมุทรไทย
ผู้เขียน นางสาว นัฐชา แก้วกระจ่าง
สาขาวิชา สัตววิทยา
ปีการศึกษา 2559

บทคัดย่อ

ศึกษาความสัมพันธ์เชิงวิวัฒนาการของแมลงวันขายาวสกุล *Phacaspis* ใน 6 จังหวัดตามแนวชายฝั่งทะเลอันดามันและอ่าวไทย วัตถุประสงค์ของการศึกษาเพื่อตรวจสอบวงศ์วานวิวัฒนาการของแมลงวันขายาวสกุล *Phacaspis* ในคาบสมุทรไทย โดยนำตัวอย่างของแมลงวันขายาวจากประเทศบรูไนและประเทศสิงคโปร์มาใช้เพื่อตรวจสอบความสัมพันธ์เชิงวิวัฒนาการของแมลงวันขายาวสกุล *Phacaspis* ในคาบสมุทรไทย บรูไน และสิงคโปร์ โดยเก็บตัวอย่าง *Phacaspis mitis* ทั้งหมดด้วยวิธีการวางกับดักมุ้ง เก็บด้วยมือและการเก็บด้วยสวิงบริเวณป่าชายเลน วิเคราะห์สายสัมพันธ์เชิงวิวัฒนาการระดับโมเลกุลโดยใช้ยีน *COI*, 12S rDNA, 16S rDNA และการรวมยีนทั้งสามส่วน

จากการศึกษาด้านวงศ์วานวิวัฒนาการเชิงโมเลกุลในการวิเคราะห์แบบ maximum likelihood และ Bayesian inference พบว่าผลการศึกษามีความสอดคล้องกัน โดยพบว่าแมลงวันขายาว *Phacaspis mitis* ในคาบสมุทรไทยเป็นวงศ์วานเดียวกัน (monophyletic group) และแบ่งออกเป็นสองกลุ่มอย่างชัดเจน สอดคล้องกับผลของ haplotype network พบว่า 16 haplotype patterns ของ *Phacaspis mitis* ถูกแบ่งออกเป็น 2 กลุ่มใหญ่ นอกจากนี้ผลการศึกษายังแสดงให้เห็นว่าความแตกต่างทางพันธุกรรมระหว่างประชากรมีผลมาจากระยะห่างตามชีวภูมิศาสตร์ สำหรับการศึกษาระยะเวลาการเกิดแยกกันของชนิด (divergence time) พบว่าแมลงวันขายาว *Phacaspis mitis* ในคาบสมุทรไทยมีจุดกำเนิดในยุคอิโอซีนตอนปลาย (35.5 ล้านปีก่อน) และมีความหลากหลายในช่วงยุคไพลโอ-ไพลสโตซีน (3.14-0.51 ล้านปีก่อน) โดยอิทธิพลของการเพิ่มหดของพื้นที่ป่าชายเลนมีบทบาทสำคัญต่อการแพร่กระจายของแมลงวันขายาว *Phacaspis mitis* ในคาบสมุทรไทย ณ ช่วงเวลานั้น เช่นเดียวกับสายสัมพันธ์

เชิงวิวัฒนาการของแมลงวันขายาวสกุล *Phacaspis* ในคาบสมุทรไทย บรูไน และสิงคโปร์พบว่ามีบรรพบุรุษเดียวกัน (monophyletic group) โดยผลการเชื่อมต่อกันระหว่างพื้นที่ป่าชายเลนย่อยๆ ใน Sundaland มีบทบาททำให้เกิดความแปรผันทางพันธุกรรมและแลกเปลี่ยนของยีนในแมลงวันขายาวสกุล *Phacaspis* ระหว่างประชากร

Thesis Title Phylogeny of Long-Legged flies genus *Phacaspis* (Meuffels and Grootaert, 1988) in peninsular Thailand
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Major Program Zoology
Academic Year 2016

ABSTRACT

The study of phylogenetic relationship of long-legged flies genus *Phacaspis* was focused on six coastal provinces along the Andaman and the Gulf of Thailand. The objective of this study was to determine phylogeny of long-legged flies genus *Phacaspis* in peninsular Thailand. Moreover, the specimens from Brunei and Singapore were used to resolve a phylogenetic relationship of genus *Phacaspis* in peninsular Thailand with adjacent areas. All specimens of *Phacaspis mitis* were collected using malaise trap, hand collection and net sweeping in the mangroves. The molecular phylogeny was analyzed based on *COI*, 12S rDNA, 16S rDNA and combined mitochondrial DNA genes.

The phylogenetic tree of maximum likelihood analysis and Bayesian inference were congruent. The results revealed that *P. mitis* in peninsular Thailand was monophyletic group and also can be divided into two distinct clades. According to the haplotype network, 16 haplotype patterns of *P. mitis* were separated into two major haplotype networks. Furthermore, the result also showed that level of genetic distance (F_{ST}) between populations has influenced on geographical isolation (km). Moreover, the estimating of divergence time indicated that *P. mitis* in peninsular Thailand has arisen in late Eocene (35.55 Mya) and it was diverse during Plio-Pleistocene periods (3.14-0.51 Mya). The influence of the mangrove expansion and fragmentation plays an important role to the distribution of *P. mitis* in peninsular Thailand at that time. Likewise, the phylogenetic relationship of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei was monophyletic group. The connectivity between patches of mangroves in Sundaland is suggested to be a major role to occurrence of genetic variation and gene flow in this genus.

ACKNOWLEDGEMENTS

The success of this thesis had been attributed to many people. I would like to express my sincere thanks to my major advisor, Dr. Singtoe Boonrotpong for taking me on his student and for his time, invaluable advice, persistent help, effort, kindness and constant encouragement. I also sincerely thank my co-advisor, Prof. Dr. Patrick Grootaert, who gave essential assistance and guidance throughout the study from collecting to editing the manuscript.

I would like to show my gratitude to the committee members, Asst. Prof. Dr. Taeng On Prommi and Dr. Sopark Jantarit for their valuable time, advice and kind words of encouragement.

Many special thanks go out to the graduate school of Prince of Songkla University and the Department of Biology, Faculty of Science, PSU. Furthermore, this work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (SCI540531M/2013).

Finishing work on this particular project, I would like to thank all specimens who played significant roles in this thesis. To begin with, Dr. Patamarerk Engsontia, my colleagues, my friends, especially Ms. Oratip Waranusit, Ms. Pimpanit Kongruang and Mr. Kerati Kittiwattanawong who rendered their help during the period of my work.

Finally, I also extend my greatest gratitude to my parents, my sister and my brother. They all kept me going. This thesis would not have been possible without their love, support and encouragement.

Natcha Kaewkrajang

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LIST OF ABBREVIATIONS AND SYMBOLS

bp	=	base pair (s)
BRN	=	Brunei
°C	=	degree Celsius
COI	=	cytochrome oxidase subunit I
Cytb	=	cytochrome b
DNA	=	deoxyribonucleic acid
EDTA	=	Ethylene diamine tetra-acetic acid
et al.	=	and others
etc.	=	et cetera
F primer	=	forward primer
Fig.	=	Figure
F_{ST}	=	genetic distance
KBI	=	Krabi province, Thailand
km	=	kilometer
km^2	=	square kilometer
M	=	molarity or molar concentration
mM	=	millimolar
m1+2	=	Media 1+2 (wing venation)
mt DNA	=	Mitochondrial DNA
Mya	=	million years ago
NaCl	=	Sodium chloride
NCBI	=	National center for biotechnology information

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

NRT	=	Nakhon Si Thammarat province, Thailand
P. mitis	=	*Phacaspis mitis*
PNA	=	Phang Nga province, Thailand
P. ornata	=	*Phacaspis ornata*
pmol	=	picomol/microliter
r4+5	=	Radius 4+5 (wing venation)
rDNA	=	ribosomal deoxyribonucleic acid
RNA	=	Ribonucleic acid
R primer	=	reverse primer
rpm.	=	round per minute
SDS	=	Sodium dodecyl sulfate
SGP	=	Singapore
SKA	=	Songkhla province, Thailand
SNI	=	Surat Thani province, Thailand
sp.	=	species
spp.	=	subspecies
STN	=	Satun province, Thailand
TAE	=	Tris acetate
Tris-HCL	=	Tris-(hydroxymethyl)-aminoethane hydrochloric acid
µl	=	microliter
′	=	minute of arc
″	=	second of arc

CHAPTER 1

INTRODUCTION

Rationale

Insect is the most diversity of organism in the world because they can adapt themselves for living in both aquatic and terrestrial environments. Especially, the Dolichopodidae or long-legged flies consists of more than 7,100 species in 230 genera (Yang *et al.*, 2006) which is one of the largest families from 188 families in order Diptera. Most of larvae and adults play an important role as predator in ecosystem. Moreover some species can be used as a biological indicator because they are highly sensitive to habitat change (Pollet, 2009). Furthermore, long-legged flies are found in several habitats such as damp soil, leaves, riverbanks, rock, sandy beaches and also in mangroves.

The study on marine dolichopodid flies in Thailand by Patrick Grootaert and Henk Meuffels (2001) found that 15 species in 7 genera of long-legged flies in mangroves and beaches along the Andaman Sea and the Gulf of Thailand were described. However, the discovery of new marine long-legged flies is reported an upward trend each year. The several studies of marine long-legged flies are still focused on the topic of classification and taxonomy. Some species of the taxonomic review of long-legged flies are ambiguous, especially genus *Phacaspis*. The genus *Phacaspis* is very interesting because they are true marine long-legged flies and also are able to live on the mudflats in the front of mangrove among high salinity and full sunlight exposure (*pers com*, Grootaert). Unfortunately, the study has still been scant in the topic of molecular and evolutionary genetics. The basic of these disciplines will fulfill to explain about origin of species, and investigate the evolution as well as solving problem of the classification. The first taxonomic study of genus *Phacaspis* was described in 1988 by Meuffels and Grootaert. There are several important characters for identification to the genus such as arista dorsal, shape of antennae,

clypeus shaped, bristles on coxa, wing venation and stalk hypopygium. The result can be deduced from a stalked hypopygium of genus, which is similar to subfamily Medeterinae. In contrast, the shape of the antennae and external bristle on the posterior coxae are different with subfamily Medeterinae. Then, they could not group within the other subfamilies (Robinson, 1970) which the genus *Phacaspis* was considered as incertae sedis. In 2006 the new subfamily Kowmunginae was classified in world catalog of Dolichopodidae by Yang and colleagues. It consists of two genera: *Kowmungia* and *Phacaspis*. However, it is unclear because *Kowmungia* and *Phacaspis* are different in various characters (Meuffels and Grootaert, 1988) such as shape of the antennae, stalk hypopygium formed, setae of tibiae. In addition, *Kowmungia* is apparently terrestrial whereas *Phacaspis* can be found on the mudflats in the front mangrove (*pers com,* Grootaert). At this moment, the validity of the classification in subfamily Kowmunginae is still controversial and need to be reconsidered.

Molecular analyses for classification were carried out by DNA approaches. In 2010 the phylogenetic relationships of Dolichopodidae has been studied by Lim and colleagues. The study showed that 76 Oriental species from 12 subfamilies were analyzed by six genes (12S, 16S, Cytb, COI, 18S, 28S) from both nuclear and mitochondrial DNA. Unfortunately, only one specimen of *Phacaspis mitis* from Singapore was used as a representative of the subfamily Kowmunginae to construct the phylogenetic relationship. The phylogenetic tree revealed that *Phacaspis mitis* was closely related to the genus *Thinophilus* in subfamily Hydrophorinae. Thus, the evidences of molecular and morphological analysis are conflicted to confirm the evolutionary study and classification of the genus *Phacaspis*.

Thailand is situated in Southeast Asia. There are highly diverse of mangroves ecosystem that is distributed along both coastal regions. Southern Thailand is located between transition zone of Indochinese sub-region and Sundaic sub-region. Subsequently, it is one of the important biodiversity hotspot of Southeast Asia. In 2004, department of marine and coastal resources reported that 2,758.05 km^2 is a mangroves, and 88% of mangrove ecosystem exists in southern Thailand. Previously, there are only 3 *Phacaspis* species were described around the world.

Phacaspis petiolata was reported in Prachuap Khiri Khan province (Meuffels and Grootaert, 1988). In 2001, Patrick Grootaert and Henk Meuffels described new species *Phacaspis mitis* from Krabi, Trang and Satun provinces. Both species are discovered in Thailand. However, *Phacaspis ornata* was not found in Thailand.

In this study, the phylogeny of long-legged flies genus *Phacaspis* Meuffels & Grootaert, 1988 in peninsular Thailand was studied in 8 coastal provinces from the Gulf of Thailand and the Andaman Sea (Chumphon, Surat Thani, Nakhon Si Thammarat, Songkhla, Pattani, Phang Nga, Krabi and Satun). The phylogenetic tree was constructed by mitochondrial DNA (12S rDNA, 16S rDNA, Cytochrome oxidase subunit I and the combined mitochondrial genes) using maximum likelihood analysis and Bayesian inference. This study investigates the question of monophyletic group of long-legged flies among genus *Phacaspis* in peninsular Thailand. Phylogenetic relationship of genus *Phacaspis* in peninsular Thailand and adjacent areas (Singapore and Brunei) are grouped together in phylogenetic tree.

Key questions

1. Are there any long-legged flies *Phacaspis mitis* in peninsular Thailand monophyletic group?
2. What is the phylogenetic relationship of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei?

Hypotheses

1. Long-legged flies *Phacaspis mitis* in peninsular Thailand is a monophyletic group.
2. Phylogenetic relationship of long-legged flies in genus *Phacaspis* from peninsular Thailand, Singapore and Brunei was monophyly.

Objectives

1. To determine phylogeny of long-legged flies *Phacaspis mitis* in peninsular Thailand.
2. To resolve a phylogenetic relationship of long-legged flies of genus *Phacaspis* in peninsular Thailand with Singapore and Brunei.

Literature reviews

1. Biology of long-legged flies

Long-legged flies are a very small insect (1.8-10 mm of body length). They have metallic green or coppery body color. Male genitalia or hypopygium is an important characteristic for identify between male and female. The development of long-legged flies is complete metamorphosis (Fig. 1). An adult and larvae of long-legged flies play major role as predator in ecosystem. Some species can be used as bioindicators because they are highly sensitive within habitat change (Pollet, 2009). An adult of long-legged flies can occupy in sandy beach, leaves, rock, trunk and mangroves while larvae lived in moist-soil where is next to swamp, stream etc. These habitats can be found the soft body invertebrate such as culicid larvae, chironomid and ceratopogonid. Consequently, it was used to breeding site and foraging site (Grootaert and Meuffels, 2005). In general, long-legged flies were found in 3 habitats such as terrestrial, freshwater and marine habitats. It indicated that long-legged flies can be adapted in several environments and also the adaptation process brings to the evolution. Especially, true marine long-legged flies are focused on evolutionary study because they can be adapted and occupied in the habitat tolerance, including high salinity and full sunlight exposure. Moreover, the origin of true marine long-legged flies was hypothesized that it would be the freshwater long-legged ancestor (*pers com*, Grootaert).

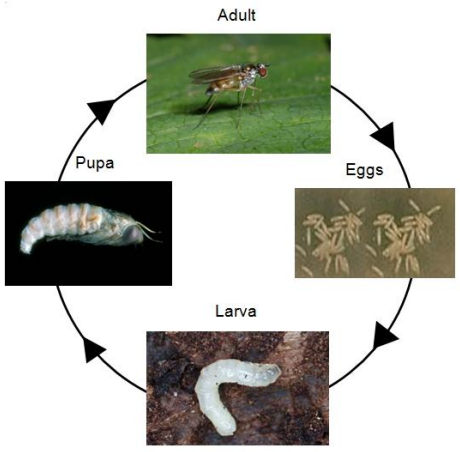

Figure1. Life cycle of long-legged flies.
(modified from http://ohioline.osu.edu)

2. Taxonomy and classification of long-legged flies genus *Phacaspis*

Long-legged flies were classified in Order Diptera, Superfamily Empidoidae and Family Dolichopodidae. They consist of 230 genera and over 7,100 species within 17 subfamilies (Yang *et al*., 2006). In general, the identification to genus level uses mainly on external characters in male such as shape of antennae, antennal segment, wing venation, setae on fore and hind leg etc. Especially, the male genitalia are a necessary character for decision.

There are several important characters of genus *Phacaspis* for identification: arista dorsal, antennal on first dorsal segment absent, third antennal segment is triangular shape, clypeus lentiform, acrostichal bristles absent, postocular hair present, tarsal segments on fore leg are longer than wide as well as they have apical part of m1+2 nearly straight and parallel to r4+5 on the wing (Grootaert and Meuffels, 2001). Genus *Phacaspis* was described in 1988 by Henk Meuffels and Patrick Grootaert. This genus consists of 3 species following:

Order Diptera

Suborder Brachycera

Infraorder Asilomorpha

Superfamily Empidoidea

Family Dolichopodidae

Subfamily Kowmunginae

Genus *Kowmungia* (Bickel, 1987)

Kowmungia angustifrons (Bickel, 1987)

Kowmungia crassitarsus (Bickel, 1987)

Kowmungia flaviseta (Bickel, 1987)

Kowmungia nigrifemorata (Bickel, 1987)

Genus *Phacaspis* (Meuffels and Grootaert, 1988)

Phacaspis ornata (Meuffels and Grootaert, 1988)

Phacaspis petiolata (Meuffels and Grootaert, 1988)

Phacaspis mitis (Grootaert and Meuffels, 2001)

3. Previous studies of long-legged flies genus *Phacaspis*

Genus *Phacaspis* consists of 3 species; they are *Phacaspis petiolata* (Meuffels and Grootaert, 1988), *Phacaspis ornata* (Meuffels and Grootaert, 1988) and *Phacaspis mitis* (Grootaert and Meuffels, 2001).

The first study, external characteristics of new genus *Phacaspis* were considered for classification (Meuffels and Grootaert, 1988). The result showed that stalk hypopygium of *Phacaspis* was similar to genus *Medeterinae* but the shape of antennae and the external bristle on the posterior coxae were different. In addition, *Phacaspis* was also showed the similar characters to the other genera. It was reasonable to infer that the position of genus *Phacaspis* in family Dolichopodidae was uncertain (*incertae sedis*).

However, genus *Phacaspis* was classified to a new subfamily Kowmunginae with genus *Kowmungia* in 2006 by Yang and colleagues. It was unclear because some characters of both genera were quite different (Meuffels and Grootaert, 1988) (Table 1). Apart from external characteristic, genus *Phacaspis* can be found on mud flats in the front of mangroves whereas genus *Kowmungia* lived in terrestrial habitat indeed (*pers com*, Grootaert). According to these reason, the classification of genus *Phacaspis* was still controversial.

In 2010, the subfamilies relationships in Dolichopodidae were unveiled by Lim and colleagues. The study comprises seventy-six species from 12 subfamilies including 1 specimen of *Phacaspis mitis* in new subfamily Kowmunginae. They were analyzed using two nuclear genes (18S, 28S) and four mitochondrial genes (12S, 16S, Cytb, COI). The result showed that *Phacaspis mitis* cannot be placed within any subfamilies and there is no necessary that creating the Kowmunginae.

Genus *Phacaspis* distributed in several countries in Southeast Asia such as Singapore, Brunei, Malaysia as well as Thailand. In Thailand, long-legged flies have been published at least five papers so far but there is only study about genus *Phacaspis*. In 1988, *Phacaspis petiolata* was reported in Prachuap Khiri Khan Province by Henk Meuffels and Patrick Grootaert. In 2001, Patrick Grootaert and Henk Meuffels described new species *Phacaspis mitis* in Thailand. They were found in Krabi province, Trang province and Satun province.

In contrast, *Phacaspis ornata* was absence in Thailand. However, the previous study of *Phacaspis* focused on taxonomy but there is no study in terms of molecular genetics in Thailand.

Table1. The different characteristics of genus *Phacaspis* and *Kowmungia*. (Meuffels and Grootaert, 1988)

Characteristics	Genus *Phacaspis* Grootaert and Meuffels, 1988	Genus *Kowmungia* Bickel, 1987
Clypeus	Clypeus is lentiform.	There is frontoclypeal suture.
Arista	Arista dorsal	Arista apical
Eyes with facets	Only species (*P. ornata*)	All species
Acrostichals	Absence	Presence
Dorsocentral segments	3-4 segments	6 segments
There is external bristle on coxa III	Absence	Presence
Type of setae on tibia	Weak	Strong
The development of anal lobe on wing.	Presence	Absence
The positions of segments were formed to stalk hypopygium.	7^{th} or 6^{th} + 7^{th} segment were fused together.	7^{th} segment

*Genus *Phacaspis* excludes *P. mitis*

4. Mangrove forest

Mangroves are plant community that was found mainly in fringing sheltered tropical shore (Lugo & Snedaker, 1974). Mangroves were covered by small shrubs and tall trees. *Rhizophora* spp. is the dominant tree. Most of plants in mangroves have respiratory root or prop root, branch and there are an ability to grow in saline environment and tidal areas. Mangroves play an important role in ecosystem such as transferring energy and nutrients from the land to marine. In addition, mangroves are nesting site, habitat and food source of small fish, crabs, shrimps, mollusk, insects, reptile, amphibian and some bird species (Hogarth, 2007). Mangrove distributes in American, west coast of Africa, east coast of Africa, Asia, Australia and Oceania regions (Woodroffe and Grindrod, 1991). According to the international tropical timber organization (2010), the majority of mangroves about 51,049 km^2 are found in South East Asia within total 150,000 km^2. However, the current distribution of mangroves can be explained by the chronology of mangroves evolution and the movement of continents. The fossil evidence of oldest mangrove pollen is the fruit of the palm *Nypa*. The Nypa pollen has been found in eastern Brazil, North and West Africa and South East Asia during the early Palaeocene. Consequently, the mangroves in South East Asia were originated in early Palaeocene and it also the origin of mangroves in the world (Hogarth, 2007).

Thailand is located in South East Asia between 15 °00′ North latitude and 100°00′ East longitude. There are several landscapes such as plateau, mountains and hills, river plains including coastal plain. The coastal provinces in Thailand were covered by 2,758.05 km^2 mangroves such as 96.51 km^2 in central part, 227.49 km^2 in eastern part and 2,434.05 km^2 in southern part. Almost mangroves can be found in Phang Nga, Satun and Krabi provinces respectively (Department of marine and coastal resources, 2004). There are 23 coastal provinces in Thailand which is covered by mangrove as following:

Bangkok	Samut Prakan	Petchaburi
Samut Songkhram	Trat	Rayong
Chanthaburi	Samut Sakhon	Chon Buri
Chachoengsao	Chumphon	Surat Thani

Nakhon Si Thammarat	Phatthalung	Songkhla
Pattani	Ranong	Phangnga
Phuket	Krabi	Trang
Satun	Prachuap Khiri Khan	

5. Molecular phylogenetic and mitochondrial genes

Phylogeny is the relationship of organism which has descended from common ancestor. Morphological, physiological and molecular analyses were used for inferring the relationship and they were represented by phylogenetic tree (Dowell, 2008). Especially, the molecular information (DNA, RNA ,Proteins) was recognized as an evidence of evolution more than other evidence because they are suitable and easy to analysis in term of quantitatively and statistics (Suárez, 2008). Molecular phylogenetic used both of molecular techniques and statistical analysis for explanation the evolution relationship of organisms (Dowell, 2008).

In the beginning, the phylogenetic tree was constructed by morphological characteristics until DNA sequencing analysis has been revealed in 1977 by Sanger and colleages. After that, molecular genetics have been getting attention. The most study of molecular phylogeny was investigated by mitochondrial DNA and nuclear DNA but in this study will be focused on mitochondrial DNA (mtDNA). There are several disciplined for using mitochondrial genes to exploring the relationship between lineages. First, mitochondrial genes lack of introns. Second, mitochondrial genes are maternal inherited and there is not recombination. Third, mitochondrial genes have widely usable primers and it is available to amplification. Fourth, the rates of evolution in mitochondrial genes are faster than nuclear genes such as mitochondrial genes of insects have evolutionary rate more than nuclear genes about 2-9 times (DeSalle *et al*., 1987; Moriyama and Powell, 1997; Monteiro and Pierce, 2001; Lin and Danforth, 2004). For these reason mitochondrial genes can be shown the evolution in small scales such as species levels, genera levels.

Moreover, mitochondrial genes have been used for insect molecular phylogeny and phylogeography studies (Avise, 1987, 1994, 2000; Caterino *et al*., 2000; Harrison, 1989; Simmons and Weller, 2001; Simon *et al*., 1994). Especially, cytochrome oxidase I gene because *COI* gene can be shown a great signal of phylogenetic and universal primers of *COI* are very strong (Hebert *et al*., 2003). Apart from *COI* gene, 12S rDNA and 16S rDNA genes have been used in insect phylogenetic study as well. The *COI* gene, 12S rDNA gene and 16S rDNA gene are key molecular marker to investigate the evolution studies and phylogeny (Germann *et al*., 2011). The molecular phylogeny and evolutionary studies are an important role to explain the origin and evolution of organisms. Nowadays the studies of long-legged flies are focused on survey, taxonomy and classification. However, the taxonomic studies are still not clear to explain about the evolutionary problem in some species. Unfortunately, genus *Phacaspis* has not study in term of evolutionary history. Consequently, the molecular phylogenetic study of *Phacaspis* was examined.

CHAPTER 2

MATERIALS AND METHODS

1. Study sites

The study sites were sampled from mangrove areas. Coastal provinces across the Gulf of Thailand and the Andaman Sea were chosen. It comprised of Chumphon, Surat Thani, Nakhon Si Thammarat, Songkhla, Pattani, Phang Nga, Krabi and Satun provinces (Fig. 2).

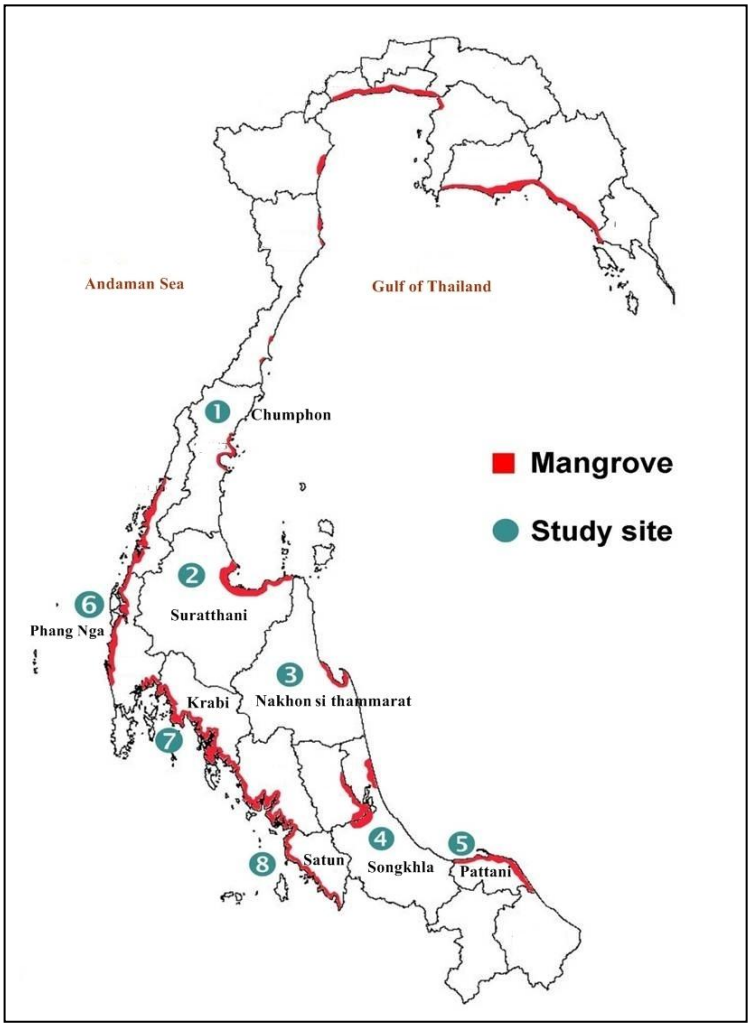

Figure2. Collecting sites along peninsular Thailand.
(modified from Aksornkoae, 2002)

1. Chumphon province is located on Isthmus of Kra between Latitudinal 10 degrees 29′N and Longitudinal 99 degrees 11′E. The average annual rainfall is 4,100 mm. Mangroves was found in district of Pathio, Mueang Chumphon, Sawi, Thung-Tako, Lang Suan and Lamae (152.126064 km^2). The dominant tree is family Rhizophoraceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

2. Surat Thani province is located at Latitudinal 9 degrees 7′N and Longitudinal 99 degrees 21′E. The average annual rain fall is 1,710 mm. Mangroves was found in district of Kanchanadit, Koh Samui, Chaiya, Donsak, Tha Chang, Phunphin and Mueang Surat Thani (74.51872 km^2). Family Rhizophoraceae and Acanthaceae are dominant trees. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

3. Nakhon Si Thammarat province is located at Latitudinal 8-10 degrees N and Longitudinal 99 degrees 15′ to 100 degrees 5′E. The average annual rainfall is 2,429.04 mm. Mangroves was found in district of Khanom, Pak- Phanang, Mueang Nakhon Si Thammarat and Tha Sala (246.339968 km^2). However, more than a half of area was changed to agriculture, shrimp farm and port. The dominant trees are family Rhizophoraceae and Acanthaceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

4. Songkhla province is located at Latitudinal 6 degrees 17′ to 7 degrees 56′N and Longitudinal 100 degrees 1′ to 101 degress 6′E. The average annual rainfall is 1,750.9 mm. Mangroves was found in district of Mueang Songkhla, Chana, Hatyai, Khuan Niang, Bang Klam, Krasae Sin, Sathing Phra, Singhanakhon and Thepha (86.663216 km^2). The dominant trees are family Combretaceae, Rhizophoraceae, Arecaceae, Lythraceae and Cycadaceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

5. Pattani province is located at Latitudinal 6 degrees 52′ to 6 degrees 87′N and Longitudinal 101 degrees 14′ to 101 degrees 24′E. The average annual rainfall is 1,750.9 mm. Mangroves was found in district of Mueang Pattani, Yaring, Panare, Sai Buri and Mai Kaen (66.129504 km^2). Family Rhizophoraceae, Arecaceae and Euphorbiaceae are dominant trees. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

6. Phang Nga province is located at Latitudinal 8 degrees 27′52.3″N and Longitudinal 98 degrees 32′E. The average annual rainfall is 3,638.3 mm. Mangroves was found in district of Khura Buri, Takua Pa, Thai Mueang, Takua Thung, Mueang Phang Nga, Thap Put and Koh Yao (529.988016 km^2). The dominant tree is family Acanthaceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

7. Krabi province is located at Latitudinal 7 degrees 22′ to 8 degrees 41′N and Longitudinal 8 degrees 21′ to 99 degrees 19′E. The average annual rainfall is 169.4 mm. Mangroves was found in district of Ao- Luek, Mueang Krabi, Khlong Thom, Nuea Khlong and Koh Lanta (349.097184 km^2). The dominant trees are family Rhizophoraceae and Meliaceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

8. Satun province is located at Latitudinal 6 degrees 4′ to 7 degrees 2′N and Longitudinal 99 degrees 5′ to 100 degrees 3′E. The average annual rainfall is 2,215.4 mm. Mangroves was found in district of Thung Wa, La-ngu, Tha Phae and Mueang- Satun (516.329216 km^2). The dominant tree is family Rhizophoraceae. (Office of Mangrove Conservation, Department of marine and coastal resources, 2012).

2. Insect sampling

The specimens were collected in eight provinces from February to July 2015. The three techniques were used such as the malaise traps, the hand collecting and the net sweeping. The non-disturbance area in mangroves, including sunlight exposure on the ground and quite open space is the suitable area. Malaise traps were set up in 3 plots at mangroves in each sampling site and then the trap were collected consecutively for 1 week in each province (Fig. 3). Long-legged flies were preserved in a bottle with 70% ethanol.

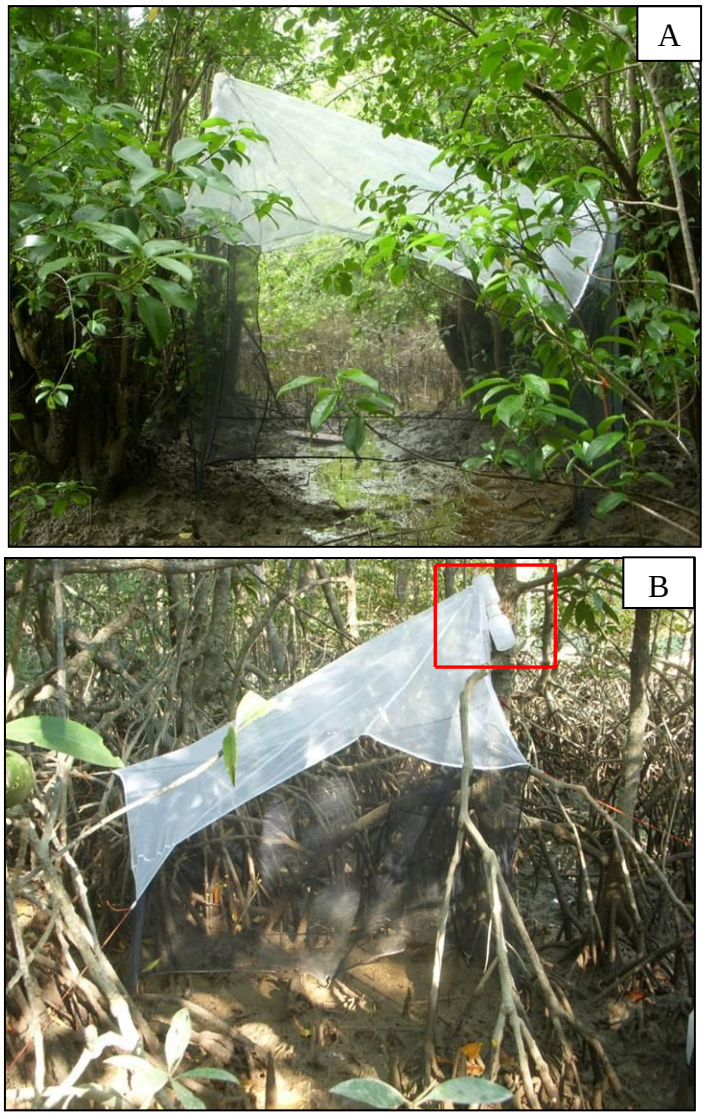

Figure 3. (A) Locality of malaise traps setting up in mangrove.
(B) A bottle contains 70% ethanol.

In addition, specimen was collected by hand collection using a plastic bottle and net sweeping on the ground or above the vegetation (Fig. 4). The specimen was collected in the semi-diurnal (two high waters and two low waters each day) in the sampling site of Andaman sea and also diurnal (one tidal cycle per day) of Gulf of Thailand. The suitable habitat of *Phacaspis mitis* consists of the highly sunlight exposure area and intertidal mudflats. Ethyl acetate was used to preserve the specimen. It was left in 1 week for collecting specimens in each province. All samples were preserved in 70% ethanol alcohol and keep freezing at - 4° C for the identification and molecular analyses.

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Figure 4. (A) Hand collecting method and (B) net sweeping method.

3. Identification

The wet specimens were identified to genus and species level using identification papers of Henk Meuffels and Patrick Grootaert (1988, 2001) including http://evolution.science.nus.edu.sg/. The important external characters were used to identification such as shape of antennae, wing venation and the structure of male genitalia. Each specimen was taken a photo under stereo microscope using compact digital camera Fuji X-A1 before molecular analyses. Moreover, the specimen photo was edited by Adobe Photoshop CS5.

4. DNA extraction

Total DNA was extracted from the whole body of male specimen using manual method. The specimen was pooled in 1.5 µl added 100 µl of lysis buffer and grind tissue by micro pestle then added 2 µl of proteinase K and incubated at 60° C for 24 hours. After that the specimen was added 7µl of 8 M Potassium acetate and stored at -20° C for 30 minutes. The mixture was centrifuged at 13,400 rpm for 15 minutes and transfer supernatant into a new micro centrifuge tube added 100 µl of 95% ethanol alcohol, and then centrifuge at 13,400 rpm for 15 minutes. Next step, it supernatant was discarded and added 100 µl of 70% ethanol alcohol, after that centrifuged at 13,400 rpm for 15 minutes. Then, waste was discarded and DNA was desiccated for 2-3 hours. Finally, the DNA was diluted in 50 µl of TAE buffer and store at - 20° C for deep freezing.

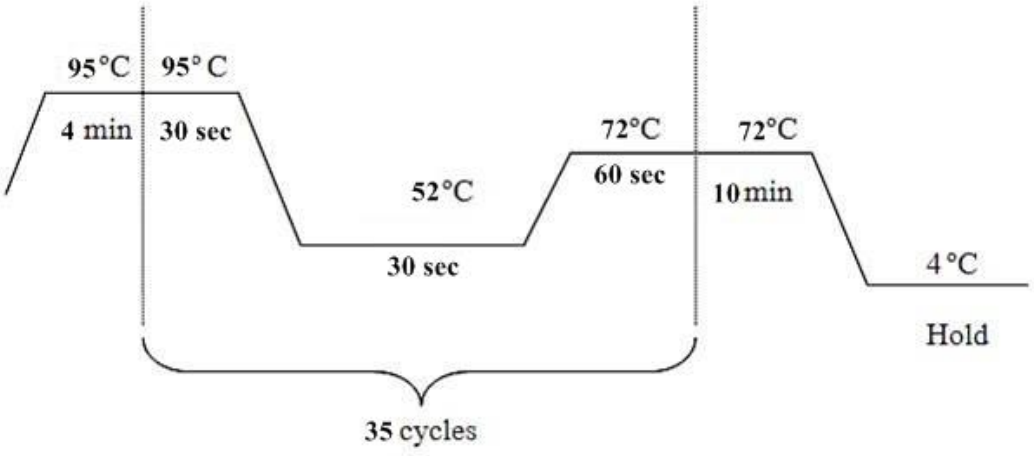
5. DNA amplification

Amplification of 12S rDNA was performed using two primers; SR-J-14233(F) and SR-N-14588 I; targeting a 355 base pair (bp). Meanwhile, 16S rDNA was performed using two primers; LR-J-12887 (F) and LR-N-13398 I; targeting a 511 bp. The universal primers of LCO1490 (F), HCO2198 I; targeting a 710 bp was amplified for cytochrome oxidase subunit I gene (Table2).

The Polymerase Chain Reaction condition

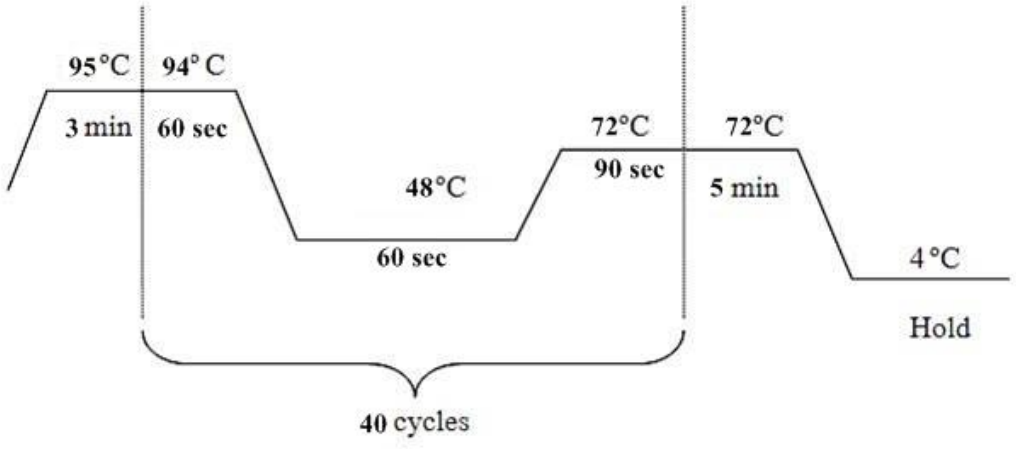
Total volume of PCR product was 50 µl. It contains 25 µl Dream*Taq* Green PCR Master Mix, 1µl of 10 pmol Forward primer, 1µl of 10 pmol Reverse primer, 5 µl DNA template and added up water or nuclease-free to total volume of 50 µl.

The PCR reactions for 12S rDNA and 16S rDNA (Germann *et al*., 2011) were run using the following program:



Initial denaturation	1 cycle	4 minutes at 95°C
Amplification	35 cycles	30 seconds at 95°C
		30 seconds at 52°C
		60 seconds at 72°C
Final extension	1 cycle	10 minutes at 72°C

The PCR reactions for universal primer mt*COI* (Folmer *et al*., 1994) were run using the following program:



Initial denaturation	1 cycle	3 minutes at 95°C
Amplification	40 cycles	60 seconds at 94°C
		60 seconds at 48°C
		90 seconds at 72°C
Final extension	1 cycle	5 minutes at 72°C

PCR products were kept deep freezing for -20°C.

6. Gel electrophoresis

PCR products were carried out on 1.5% agarose gels in 0.5X TAE buffer at 100 voltages for 30 minutes. Before loading the samples, 5 µl of PCR products were mixed together with 1 µl of maestro safe loading dye. Gel electrophoresis was visualized under UV light using Gel Documentation. After that, PCR product was sequenced at First BASE Laboratories in Malaysia.

7. Sequencing alignment and data analyses

The individuals of *Phacasspis mitis* in Thailand including *Phacaspis mitis, Phacaspis ornata* from Brunei and Singapore were examined using 12S rDNA, 16S rDNA, Cytochrome oxidase subunit I gene. Moreover, genus *Thinophilus and Nanothinophilus* were outgroups. The lists of specimens were summarized in Table 3.

Totally, 69 sequences of *Phacaspis mitis* and 8 sequences of *Phacaspis ornata* were verified using BLAST search in GenBank. In addition, the sequence of *P. mitis* from Singapore was retrieved from NCBI GenBank (*FJ808401*). The alignment and editing of all sequences were performed by program BioEdit version 7.2 (Hall, 1999). Uncorrected pairwise distance and nucleotide composition of gene were calculated by MEGA6 (Tamura *et al*., 2013). Nucleotide diversity (*Pi*), haplotype diversity (*Hd*) and polymorphic sites were analyzed by DNAsp program version 5.10.01 (Librado and Rozas, 2009) (Table 4). A statistical parsimony network was constructed phylogenetic tree using TCS1.21 program (Clement *et al*., 2000). R program version 3.3.2 was used to calculate the correlation between genetic distances (F_{ST}) and geographical distances (km) using Mantel Test for Analysis of Molecular Variance (AMOVA).

8. Phylogenetic analyses

The aligned of *COI* sequences, 12S rDNA sequences, 16S rDNA sequences (appendix 2, 3 and 4, respectively) and combined sequences were subjected to maximum likelihood (ML) and neighbor-joining (NJ) analyses using MEGA6. Maximum likelihood analysis was carried out with a heuristic search option; mental test, general time-reversible (GTR) model, gamma distribution (G) and complete deletion of gap/missing data treatment. The supporting at each node was assessed based on bootstrap resampling with 10,000 replicates. Neighbor-joining analysis was performed for estimating divergence times by uncorrected pairwise distance. Because there is no fossil record for *Phacaspis*, the divergence time value about 37.2-33.9 Mya of genus *Thinophilus* was used to calibrate the divergent time of this genus (Pollet *et al.*, 2004).

According to Bayesian inference, those of aligned sequences were converted from plain text file to Nexus format by http://sing.ei.uvigo.es/ALTER/. The completed sequencing was analyzed by Bayesian inference method using MrBayes program version 3.2.6 (Ronquist *et al*., 2012). Bayesian analysis was implemented by Markov Chain Monte Carlo simulations (MCMC) of 3×10^6 generations and it was sampled tree in each 100 generations. Rate variation among sites using gamma models, or gamma+ invariant sites models (Swofford *et al.*, 1996).The confident value based on standard deviation of split frequencies was less than 0.05 or 0.01. The first of 25% generation was discarded as burn-in. Bayesian phylogram was illustrated by Figtree version 1.4.2

Table 2. Lists of primers used to amplify DNA template.

Primer names	Strand	Sequences	Sizes of regions (bp)	References
LCO1490	Forward	5′-GGTCAACAAATCATAAAGATATTGG-3′	710	Folmer *et al*.,1994
HCO2198	Reverse	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′		
SR-J-14233	Major	5′-AAGAGCGACGGGCGATGTGT-3′	355	Germann *et al*.,2011
SR-N-14588	Minor	5′-AAACTAGGATTAGATACCCTATTAT-3′		
LR-J-12887	Major	5′-CCGGTTTGAACTCAGATCATGT-3′	511	Germann *et al*.,2011
LR-N-13398	Minor	5′-CGCCTGTTTAACAAAAACAT-3′		

Table 3. List of *Phacaspis* species, abbreviation (Abbrev.) and number of individuals analyzing. (Surat Thani (SNI); Satun (STN); Phangnga (PNA); Krabi (KBI); Songkhla (SKA); Nakhon Si Thammarat (NRT); Brunei (BRN) and Singapore (SGP))**.**

Species	Abbrev.	Location	Number of individuals		
			COI	12S rDNA	16S rDNA
Phacaspis mitis	SNI.1, 2, 3, 4	Surat Thani	4	4	4
P. mitis	NRT.1, 2	Nakhon Si-Thammarat	2	2	2
P. mitis	SKA.1,2,3,4,5	Songkhla	5	5	5
P. mitis	PNA.1,2	Phang Nga	2	2	2
P. mitis	KBI.1,2,3,4	Krabi	4	4	4
P. mitis	STN.1,2,3,4	Satun	4	4	4
P. mitis	BRN.1	Brunei	1	1	1
P. ornata	SGP.2,4	Singapore	2	2	2
P. mitis	FJ808401	Singapore	1	0	0
P. mitis	AB42406110	Singapore	1	0	0
P. mitis	AB42406116	Singapore	1	0	0
P. ornata	AB42406139	Singapore	1	0	0
P. ornata	AB42406145	Singapore	1	0	0
Nanothinophilus hoplites	-	Satun	1	1	1
Thinophilus sp.	-	Songkhla	1	1	1

CHAPTER 3

RESULTS

1. Species composition

A total of 229 individuals of long-legged flies, belonging to 4 genera 6 species were sampled in peninsular Thailand, Brunei and Singapore (Table 4). In peninsular Thailand *Phacaspis mitis* was found in 6 provinces such as Surat Thani (30 individuals), Nakhon Si Thammarat (3 individuals), Songkhla (7 individuals), Phang Nga (12 individuals), Krabi (39 individuals) and Satun (55 individuals). However, *Phacaspis mitis* was not found in Chumphon and Pattani provinces. Moreover, *Phacaspis mitis* was collected by Patrick Grootaert from Brunei (3 individuals) and Singapore (2 individuals). In addition, *Phacaspis ornata*, *Ornamenta* sp. were collected from Singapore (8 individuals) and Brunei (7 individuals), respectively. Apart from genus *Phacaspis*, genus *Nanothinophilus* and *Thinophilus* were also found in peninsular Thailand. *Nanothinophilus hoplites* (40 individuals) in Surat Thani and Satun, *Thinophilus chaetolosus* (7 individuals) in Nakhon Si Thammarat and Surat Thani, *Thinophilus* sp. (16 individuals) in Songkhla were collected and were used as an outgroup.

Table 4. List of specimens, localities and number of individuals.

Species	Number of individual		Localities	Coordinate
	Male	Female		
Phacaspis mitis	13	15	Lam-Pho, Chaiya Distrct, Surat Thani, Thailand	9°22'33.6"N 99°16'00.3"E
	2	0	Ban-Nuea-Num, Chaiya District, Surat Thani, Thailand	9°23'34.0"N 99°15'24.0"E
	2	1	Bang Gong Khong, Nakhon Si Thammarat, Thailand	8°24'09.4"N100°11’29.9"E
	7	0	Nathub, Chana District, Songkhla, Thailand	7°01'20.1"N 100°42'59.4"E
	4	8	Bang Dong, Takua Pa District, Phang Nga, Thailand	8°55'46.5"N 98°23'22.0"E
	11	23	Saithai, Mueang-Krabi District, Krabi, Thailand	8°03'23.5"N 98°53'38.2"E
	5	0	Bang-Li-Ki, Koh Lanta District, Krabi, Thailand	7°44'25.2"N 99°02'52.3"E
	27	15	Ban Ba Kan To Thit, La-ngu District, Satun, Thailand	6°47'29.8"N 99°48'53.5"E
	2	3	Pak-Bara, La-ngu District, Satun, Thailand	6°50'30.4"N 99°46'32.9"E
	3	5	Mueang-Satun District, Satun, Thailand	6°36'59.5"N 99°57'23.9"E
	2	1	Labu, Brunei	4°51'22.49"N 115°07'04.84"E
	2	0	Semakau, Singapore	1°12'00.25.2"N 103°34.56"E

Table 4 (Continued). List of specimens, localities and number of individuals.

Species	Number of individual		Localities	Coordinate
	Male	Female		
Phacaspis ornata	4	4	Semakau, Singapore	1°12'00.25.2"N 103°34.56"E
Ornamenta sp.	6	1	Tutong1, Brunei	4°47'11.94"N 114°37'33.61"E
Nanothinophilus hoplites	4	1	Tha Chang District, Surat Thani, Thailand	9°18'36.0"N 99°11'16.8"E
	10	10	Mueang-Satun District, Satun, Thailand	6°36'59.5"N 99°57'23.9"E
	10	5	Ban Ba Kan To Thit, La-ngu District, Satun, Thailand	6°47'29.8"N 99°48'53.5"E
Thinophilus chaetolosus	2	2	Bang Gong Khong, Nakhon Si Thammarat, Thailand	8°24'09.4"N 100°11’29.9"E
	1	2	Tha Chang District, Surat Thani, Thailand	9°18'36.0"N 99°11'16.8"E
Thinophilus sp.	11	5	Ban-Huo-kao, Songkhla, Thailand	7°12'03.6"N 100°34’36.8"E
Total	**128**	**101**		

2. Nucleotide composition

The nucleotide composition of *Phacaspis mitis* in each mitochondrial gene was analyzed from 21 sequences in peninsular Thailand. The cytochrome oxidase subunit I gene, 600 bp consists of Adenine (A) (28.70 %), Cytosine (C) (18.50 %), Guanine (G) (16.50 %), Thymine (T) (36.30 %). 12S rDNA of 250 base pairs consists of A (38.00 %), C (15.00 %), G (10.20 %) and T (36.90 %). For 410 base pairs of 16S rDNA contains A (38.60 %), C (15.60 %), G (8.70 %) and T (37.00 %).

In addition, the nucleotide composition of *Phacaspis mitis* in each province was analyzed based on 3 mitochondrial genes (Table 5). According to the nucleotide composition in each province, nucleotide composition in Surat Thani province of cytochrome oxidase subunit I gene comprised A (29.15 %), C (18.05 %), G (16.20 %), T (36.60 %). 12S rDNA consists of A (37.10 %), C (14.30 %), G (10.50 %) and T (38.10 %). 16S rDNA consists of A (39.00 %), C (15.60 %), G (8.60 %) and T (36.80%). Nucleotide composition in Nakhon Si Thammarat province of cytochrome oxidase subunit I gene consists of A (27.75 %), C (19.30 %), G (17.10 %), T (35.80 %). 12S rDNA consists of A (40.00 %), C (16.30 %), G (9.40 %) and T (34.30 %). 16S rDNA consists of A (37.80 %), C (15.80 %), G (8.90 %) and T (37.50 %). The nucleotide composition in Songkhla province of cytochrome oxidase subunit I gene consists of A (27.80 %), C (19.30 %), G (17.20 %), T (35.70 %). 12S rDNA consists of A (40.00 %), C (16.30 %), G (9.40 %) and T (34.30 %). 16S rDNA consists of A (37.80 %), C (15.80 %), G (8.90 %) and T (37.50 %). Nucleotide composition in Phang Nga province of cytochrome oxidase subunit I gene consists of A (29.10 %), C (18.25 %), G (16.25 %), T (36.40 %). 12S rDNA consists of A (36.80 %), C (14.30 %), G (10.65 %) and T (38.25 %). 16S rDNA consists of A (39.00 %), C (15.30 %), G (8.60 %) and T (37.00 %). The nucleotide composition in Krabi province of cytochrome oxidase subunit I gene comprised A (29.10 %), C (18.18 %), G (16.22 %), T (36.50 %). 12S rDNA consists of A (36.77 %), C (14.28 %), G (10.65 %) and T (38.30 %). 16S rDNA consists of A (39.07 %), C (15.60%), G (8.50 %) and T (36.80 %).

The nucleotide composition in Satun province of cytochrome oxidase subunit I gene consists of A (29.10 %), C (18.20 %), G (16.20 %), T (36.50 %). 12S rDNA consists of A (37.00 %), C (14.30 %), G (10.60 %) and T (38.10 %). 16S rDNA consists of A (39.08 %), C (15.65 %), G (8.55 %) and T (36.72 %)

Table 5. The average of percentages of nucleotide composition in 3 genes based on each province.

Gene / Province	Cytochrome oxidase I (Universal)				12S rDNA				16S rDNA			
	A	**C**	**G**	**T**	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
Surat Thani	29.15	18.05	16.20	36.60	37.10	14.30	10.50	38.10	39.00	15.60	8.60	36.80
Nakhon Si Thammarat	27.75	19.30	17.10	35.80	40.00	16.30	9.40	34.30	37.80	15.80	8.90	37.50
Songkhla	27.80	19.30	17.20	35.70	40.00	16.30	9.40	34.30	37.80	15.80	8.90	37.50
Phang Nga	29.10	18.25	16.25	36.40	36.80	14.30	10.65	38.25	39.00	15.30	8.60	37.00
Krabi	29.10	18.18	16.22	36.50	36.77	14.28	10.65	38.30	39.07	15.60	8.50	36.80
Satun	29.10	18.20	16.20	36.50	37.00	14.30	10.60	38.10	39.08	15.65	8.55	36.72

3. Nucleotide diversity and genetic variation

The result of polymorphic site analysis from 21 sequences using 3 mitochondrial genes (Table 6) showed that the invariable sites (monomorphic) of cytochrome oxidase subunit I gene was the highest value (502 sites). The value of 16S rDNA (375 sites) is higher than 12S rDNA (215 sites). Cytochrome oxidase subunit I gene contained 4 singleton variable sites, 94 sites of parsimony informative and 90 sites of parsimony informative (two variants). For 16S rDNA, it was revealed 2 singleton variable sites, 28 sites of parsimony informative and 27 sites of parsimony informative (two variants). Moreover, 12S rDNA contained 29 sites of parsimony informative and 29 sites of parsimony informative (two variants) but the singleton variable sites were not found.

In addition, 8 parameters were analyzed for measuring the DNA polymorphism among 3 mitochondrial genes (Table 6). The result showed that cytochrome oxidase subunit I gene was found 98 polymorphic sites, 102 of mutations number. There are 16 haplotype patterns, with haplotype diversity of 0.948, variance of haplotype diversity of 0.00157, and standard deviation of haplotype diversity (0.040). The nucleotide diversity of *COI* was 0.07321 and theta (per site) from *Eta* was 0.04725. The result of 12S rDNA showed that the number of polymorphic sites, total numbers of mutations, number of haplotype, haplotype diversity, variance of haplotype diversity and standard deviation of haplotype diversity were 29, 29, 4, 0.633, 0.00553 and 0.074, respectively. For 12S rDNA, 0.05578 of nucleotide diversity and 0.03304 of theta (per site) from *Eta* were measured. In addition, the result of 16S rDNA showed that the number of polymorphic sites, total numbers of mutations, number of haplotype, haplotype diversity, variance of haplotype diversity, standard deviation of haplotype diversity, nucleotide diversity and theta (per site) from *Eta* were 30, 31, 7, 0.762, 0.00427, 0.065, 0.03185 and 0.02128, respectively.

Moreover, total number of InDels events analysis (Table 6) showed that the polymorphism of 12S rDNA gene was a result of evolutionary process (insertion or deletion) and the polymorphism in cytochrome oxidase subunit I gene and 16S rDNA was not found from this study. Tajima's *D* method was used to

execute neutrality test (Table 6). Although the result revealed that the Tajima's value of cytochrome oxidase subunit I gene was significantly different ($p<0.05$), 12S rDNA was highly significant difference ($p<0.01$), 16S rDNA was not significantly different.

Table 6. Variability estimates in the mitochondrial genes.

	Cytochrome oxidase I (Universal)	12S rDNA	16S rDNA
(a) Total base pair	600	250	410
(b) Polymorphic Sites			
Invariable (monomorphic) sites	502	215	375
Singleton variable sites	4	0	2
Parsimony informative sites	94	29	28
Parsimony informative sites (two variants)	90	29	27
(c) DNA Polymorphism			
Number of polymorphic sites, *S*	98	29	30
Total number of mutations, *Eta*	102	29	31
Number of Haplotypes, *h*	16	4	7
Haplotype (gene) diversity, H_d	0.948	0.633	0.762
Variance of Haplotype diversity	0.00157	0.00553	0.00427
Standard Deviation of Haplotype diversity	0.040	0.074	0.065
Nucleotide diversity, *Pi*	0.07321	0.05578	0.03185
Theta (per site) from Eta	0.04725	0.03304	0.02128

Table 6 (Continued). Variability estimates in the mitochondrial genes.

	Cytochrome oxidase I (Universal)	12S rDNA	16S rDNA
(d) InDels (Insertion-Deletion) Polymorphism			
Total number of InDels events analyses, I	0	3	0
(e) Tajima's Test , D			
** $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$* ns = not-significant	2.23643*	2.66331**	1.93172 ns

4. Distance analysis

The degree of nucleotide substitution was estimated among 6 provinces in peninsular Thailand by uncorrected pairwise distance analysis based on 3 mitochondrial genes (Table 7). The result in cytochrome oxidase subunit I gene showed the range of genetic distance value between Songkhla and Surat Thani were 0.180-0.185. The genetic distance of Satun and Surat Thani were ranged between 0.007 to 0.012, whereas Satun and Songkhla were 0.185-0.188. Krabi and Surat Thani were 0.005-0.021, whereas Krabi and Songkhla were 0.185-0.191, Krabi and Satun were 0.000-0.019. The genetic distance of Phang Nga and other provinces were ranged between Phang Nga and Surat Thani (0.012-0.021), Phang Nga and Songkhla (0.185), Phang Nga and Satun (0.012-0.017) and Phang Nga and Krabi (0.007-0.017). The distance analysis of Nakhon Si Thammarat with Surat Thani, Songkhla, Satun, Krabi and Phang Nga were contained by 0.178-0.183, 0.002-0.003, 0.183-0.185, 0.183-0.185, and 0.183, respectively. Of this result the highest difference value in cytochrome oxidase I gene, the highest value was found between Krabi and Songkhla (0.191) and the lowest difference value was found between Krabi and Satun (0.000).

In addition the result of uncorrected pairwise distance in 12S rDNA showed that the range of genetic distance value between Songkhla and Surat Thani were 0.137-0.143 (Table 8). Satun and Surat Thani were 0.000-0.004, whereas Satun and Songkhla were 0.137-0.143. The genetic distance of Krabi and Surat Thani were ranged between 0.000-0.004, Krabi and Songkhla were 0.143, whereas Krabi and Satun were 0.000-0.004. The distance analysis of Phang Nga with Surat Thani, Songkhla, Satun and Krabi were contained by 0.000-0.004, 0.143, 0.000-0.004 and 0.000, respectively. The genetic distance of Nakhon Si Thammarat and other provinces were ranged between Nakhon Si Thammarat and Surat Thani (0.137-0.143), Nakhon Si Thammarat and Songkhla (0.000), Nakhon Si Thammarat and Satun (0.137-0.143), Nakhon Si Thammarat and Krabi (0.143) and Nakhon Si Thammarat and Phang Nga (0.143). Moreover, this result revealed that the genetic distance between Songkhla and Surat Thani, Satun, Krabi, Phang Nga, including Nakhon Si Thammarat and Surat Thani, Satun, Krabi, Phang Nga had highest

different value (0.143). Meanwhile, the lowest value (0.000) was found between Surat Thani and Satun, Krabi, Phang Nga. Moreover, the lowest value was also found between Satun and Krabi, Phang Nga as well as Phang Nga and Krabi, Nakhon Si Thammarat and Songkhla.

The result of uncorrected pairwise distance in 16S rDNA gene showed the range of distance value between Songkhla and Surat Thani were 0.071-0.077 (Table 9). The genetic distance of Satun and Surat Thani were ranged between 0.000-0.008, Satun and Songkhla were 0.071-0.074. The distance value between Krabi and other provinces were ranged between Krabi and Surat Thani (0.000-0.008), Krabi and Songkhla (0.071-0.077) as well as Krabi and Satun (0.000-0.002). The genetic distance between Phang Nga and Surat Thani were 0.002-0.008, Phang Nga and Songkhla were 0.074, Phang Nga and Satun were 0.002-0.005, Phang Nga and Krabi were 0.002-0.005. The distance analysis of Nakhon Si Thammarat with Surat Thani, Songkhla, Satun, Krabi and Phang Nga were contained by 0.071-0.077, 0.000, 0.071-0.074, 0.071-0.077 and 0.074, respectively. The highest difference value in 16S rDNA was found between Songkhla and Surat Thani, Krabi and Songkhla, Nakhon Si Thammarat and Surat Thani as well as Nakhon Si Thammarat and Krabi (0.077). The lowest differences value was found between Satun and Surat Thani, Krabi and Surat Thani, Krabi and Satun as well as Nakhon Si Thammarat and Songkhla (0.000).

Table 7. Uncorrected pairwise distance of *Phacaspis mitis* in cytochrome oxidase subunit I gene.

	SNI.1	SNI.2	SNI.3	SNI.4	STN.1	STN.2	STN.3	STN.4	PNA.1	PNA.2	KBI.1	KBI.2	KBI.3	KBI.4	SKA.1	SKA.2	SKA.3	SKA.4	SKA.5	NRT.1	NRT.2
SNI.1																					
SNI.2	0.002																				
SNI.3	0.003	0.005																			
SNI.4	0.005	0.007	0.005																		
STN.1	0.007	0.008	0.007	0.012																	
STN.2	0.008	0.010	0.008	0.010	0.002																
STN.3	0.008	0.010	0.008	0.010	0.005	0.003															
STN.4	0.007	0.008	0.007	0.008	0.003	0.002	0.002														
PNA.1	0.015	0.017	0.015	0.021	0.015	0.017	0.017	0.015													
PNA.2	0.012	0.014	0.012	0.017	0.012	0.014	0.014	0.012	0.003												
KBI.1	0.005	0.007	0.005	0.010	0.002	0.003	0.003	0.002	0.014	0.010											
KBI.2	0.019	0.021	0.019	0.021	0.019	0.017	0.017	0.015	0.010	0.007	0.017										
KBI.3	0.007	0.008	0.007	0.008	0.003	0.002	0.002	0.000	0.015	0.012	0.002	0.015									
KBI.4	0.008	0.010	0.008	0.010	0.005	0.003	0.003	0.002	0.017	0.014	0.003	0.017	0.002								
SKA.1	0.180	0.183	0.185	0.183	0.185	0.188	0.188	0.188	0.185	0.185	0.185	0.185	0.188	0.191							
SKA.2	0.180	0.183	0.185	0.183	0.185	0.188	0.188	0.188	0.185	0.185	0.185	0.185	0.188	0.191	0.000						
SKA.3	0.180	0.183	0.185	0.183	0.185	0.188	0.188	0.188	0.185	0.185	0.185	0.185	0.188	0.191	0.000	0.000					
SKA.4	0.180	0.183	0.185	0.183	0.185	0.188	0.188	0.188	0.185	0.185	0.185	0.185	0.188	0.191	0.000	0.000	0.000				
SKA.5	0.180	0.183	0.185	0.183	0.185	0.188	0.188	0.188	0.185	0.185	0.185	0.185	0.188	0.191	0.000	0.000	0.000	0.000			
NRT.1	0.178	0.180	0.183	0.180	0.183	0.185	0.185	0.185	0.183	0.183	0.183	0.183	0.185	0.188	0.002	0.002	0.002	0.002	0.002		
NRT.2	0.178	0.180	0.183	0.180	0.183	0.185	0.185	0.185	0.183	0.183	0.183	0.183	0.185	0.188	0.003	0.003	0.003	0.003	0.003	0.002	

The lowest value (0.000)

The highest value (0.191)

Table 8. Uncorrected pairwise distance of *Phacaspis mitis* in 12S rDNA gene.

	SNI.1	SNI.2	SNI.3	SNI.4	STN.1	STN.2	STN.3	STN.4	PNA.1	PNA.2	KBI.1	KBI.2	KBI.3	KBI.4	SKA.1	SKA.2	SKA.3	SKA.4	SKA.5	NRT.1	NRT.2
SNI.1																					
SNI.2	0.004																				
SNI.3	0.000	0.004																			
SNI.4	0.004	0.008	0.004																		
STN.1	0.000	0.004	0.000	0.004																	
STN.2	0.004	0.008	0.004	0.000	0.004																
STN.3	0.000	0.004	0.000	0.004	0.000	0.004															
STN.4	0.000	0.004	0.000	0.004	0.000	0.004	0.000														
PNA.1	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000													
PNA.2	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000	0.000												
KBI.1	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000	0.000	0.000											
KBI.2	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000	0.000	0.000	0.000										
KBI.3	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000									
KBI.4	0.000	0.004	0.000	0.004	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000								
SKA.1	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143							
SKA.2	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000						
SKA.3	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.000					
SKA.4	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.000	0.000				
SKA.5	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.000	0.000	0.000			
NRT.1	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.000	0.000	0.000	0.000		
NRT.2	0.143	0.137	0.143	0.137	0.143	0.137	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.000	0.000	0.000	0.000	0.000	0.000	

The lowest value (0.000)

The highest value (0.143)

Table 9. Uncorrected pairwise distance of *Phacaspis mitis* in 16S rDNA gene.

	SNI.1	SNI.2	SNI.3	SNI.4	STN.1	STN.2	STN.3	STN.4	PNA.1	PNA.2	KBI.1	KBI.2	KBI.3	KBI.4	SKA.1	SKA.2	SKA.3	SKA.4	SKA.5	NRT.1	NRT.2
SNI.1																					
SNI.2	0.005																				
SNI.3	0.005	0.000																			
SNI.4	0.005	0.000	0.000																		
STN.1	0.005	0.000	0.000	0.000																	
STN.2	0.005	0.000	0.000	0.000	0.000																
STN.3	0.008	0.002	0.002	0.002	0.002	0.002															
STN.4	0.002	0.002	0.002	0.002	0.002	0.002	0.005														
PNA.1	0.008	0.002	0.002	0.002	0.002	0.002	0.005	0.005													
PNA.2	0.008	0.002	0.002	0.002	0.002	0.002	0.005	0.005	0.000												
KBI.1	0.005	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002											
KBI.2	0.008	0.008	0.008	0.008	0.008	0.008	0.010	0.005	0.005	0.005	0.008										
KBI.3	0.005	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.000	0.008									
KBI.4	0.005	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.000	0.008	0.000								
SKA.1	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071							
SKA.2	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000						
SKA.3	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000	0.000					
SKA.4	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000	0.000	0.000				
SKA.5	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000	0.000	0.000	0.000			
NRT.1	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000	0.000	0.000	0.000	0.000		
NRT.2	0.077	0.071	0.071	0.071	0.071	0.071	0.074	0.074	0.074	0.074	0.071	0.077	0.071	0.071	0.000	0.000	0.000	0.000	0.000	0.000	

The lowest value (0.000)

The highest value (0.077)

5. Haplotype networks of *Phacaspis mitis* in peninsular Thailand

The aligned sequences of cytochrome oxidase subunit I gene, 610 bp were analyzed by DNAsp program based on 21 sequences of *Phacaspis mitis* in peninsular Thailand. The result showed 16 distinct haplotype patterns (Table10). Haplotype pattern 1, 2, 9 and 10 comprises of sequence of Surat Thani. Haplotype pattern 4, 5 and 11 were found in Satun. Haplotype pattern 6, 7and 13 comprises of sequence of Krabi. Haplotype pattern 8 and 14 were aligned sequence of Phang Nga. Haplotype pattern 15 and 16 consists of sequence of Nakhon Si Thammarat. Interestingly, haplotype pattern 3 and 12 showed that haplotype pattern 3 was included only 5 sequences from Songkhla. Sequences of Satun and Krabi were combined together in same pattern (haplotype pattern 12).

A statistical parsimony network was analyzed by TCS program. Although the result revealed 16 haplotype patterns, they were divided into 2 haplotype networks (Fig. 5, 6). Haplotype network A contained 13 distinct haplotype patterns (Fig. 5A). The origin of this group might be found in Satun (ST.4) and then, it was dispersed to Satun (ST.1, ST.2 and ST.3) and Krabi (KB.1 and KB.4). After that Krabi (KB.1) can be divided into 2 sub-patterns. The first sub-pattern (1^{st} sub-pattern) showed it comprises Phang Nga (PG.1 and PG.2) and Krabi (KB.2). Meanwhile, the second-pattern (2^{nd} sub-pattern) showed that there were only found the sequences of Surattani (S.1, S.2, S.3 and S.4). Conversely, haplotype network B contained 3 distinct haplotype patterns (Fig. 5B). The result showed Nakhon Si-Thammarat (NK.1) might be the origin of all sequence of this group and Songkhla (SK.1) was separated from Nakhon Si Thammarat (NK.1).

Table 10. Haplotype variation among *Phacaspis mitis* in peninsular Thailand.

Haplotype	Sequences	10 20 30 40 50 * * * * *
Haplotype 1	SNI.1	AAACCTATATTAATTTTCCCAATTGTATTCACTTTATAATATATTATTAT
Haplotype 2	SNI.2	.....
Haplotype 3	SKA.1,2,3,4,5	TTTATAGATACCTAACCTT.TTCCTCGCCTTTA.CTA.TCTATC.TA.CA
Haplotype 4	STN.1	T.....A.....G.C.....
Haplotype 5	STN.2	T.....A.....G....G.C.....
Haplotype 6	KBI.1	T.....A.....C.....
Haplotype 7	KBI.2	....T.....T.....A.....C...G.....
Haplotype 8	PNA.1	.....T....A.....C.....
Haplotype 9	SNI.3	.....C..
Haplotype 10	SNI.4	.....G.....C..
Haplotype 11	STN.3	TG.....A.....G.....C.....
Haplotype 12	STN.4 , KBI.3	T.....A.....G.....C.....
Haplotype 13	KBI.4	T.....A.....G.....C.....
Haplotype 14	PNA.2	.....A.....C.....
Haplotype 15	NRT.1	TTTATAGATACCTAACCTT.TTCCTCGCCTTTA.CTA.TCTATC.TA.CA
Haplotype 16	NRT.2	TTTATAGATGCCTAACCTT.TTCCTCGCCTTTA.CTA.TCTATC.TA.CA

Table 10 (Continued). Haplotype variation among *Phacaspis mitis* in peninsular Thailand.

Haplotype	Sequences	60 70 80 90
		* * * *
Haplotype 1	SNI.1	TACTTACCTTAAACTTTTATAAATTATTTTCTTTAATACTATTACTAAT
Haplotype 2	SNI.2	...C.....
Haplotype 3	SKA.1,2,3,4,5	CGT.C.TA.CTGTTCAACTCTT.AATAAACTAAATT.TTATCGCT.TGG
Haplotype 4	STN.1	.....C...
Haplotype 5	STN.2	.....C...
Haplotype 6	KBI.1	.....C...
Haplotype 7	KBI.2	..T..G.....G.....C.....C.....C...
Haplotype 8	PNA.1	..T.....G.....G.....C.....C.....C...
Haplotype 9	SNI.3	.....C...
Haplotype 10	SNI.4	.....C.....
Haplotype 11	STN.3	.....C...
Haplotype 12	STN.4 , KBI.3	.....C...
Haplotype 13	KBI.4	.....C.....C...
Haplotype 14	PNA.2	..T.....G.....C.....C.....C...
Haplotype 15	NRT.1	CGT.C.TA.CTGTTCAACTCTT.AATAAACTAAATT.TTATCGCT.TG.
Haplotype 16	NRT.2	CGT.C.TA.CTGTTCAACTCTT.AATAAACTAAATT.TTATCGCT.TG.

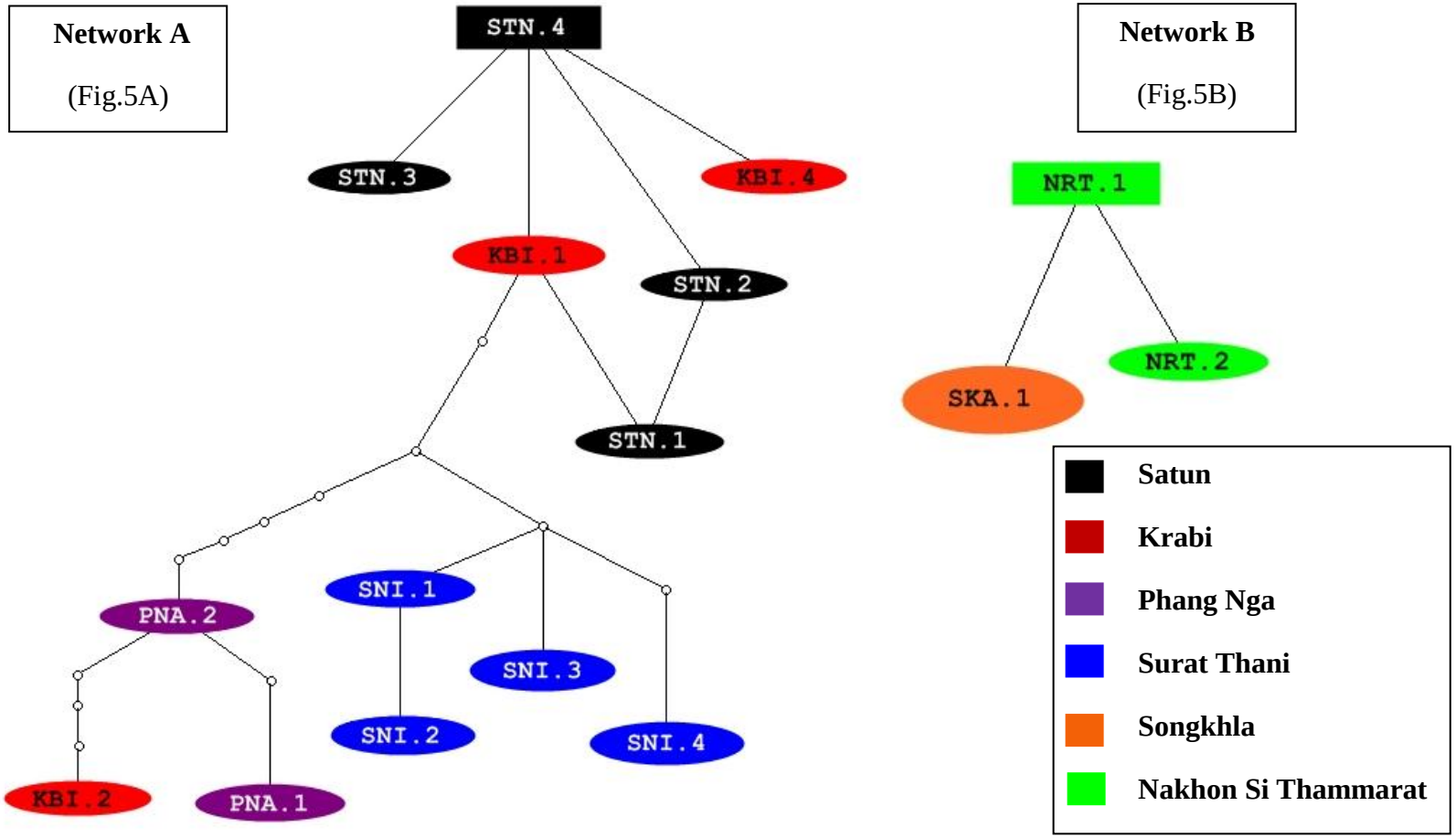


Figure 5. Haplotype networks of *Phacaspis mitis* in peninsular Thailand.
5A) haplotype network in 1st sub-pattern, 5B) haplotype network in 2st sub-pattern.

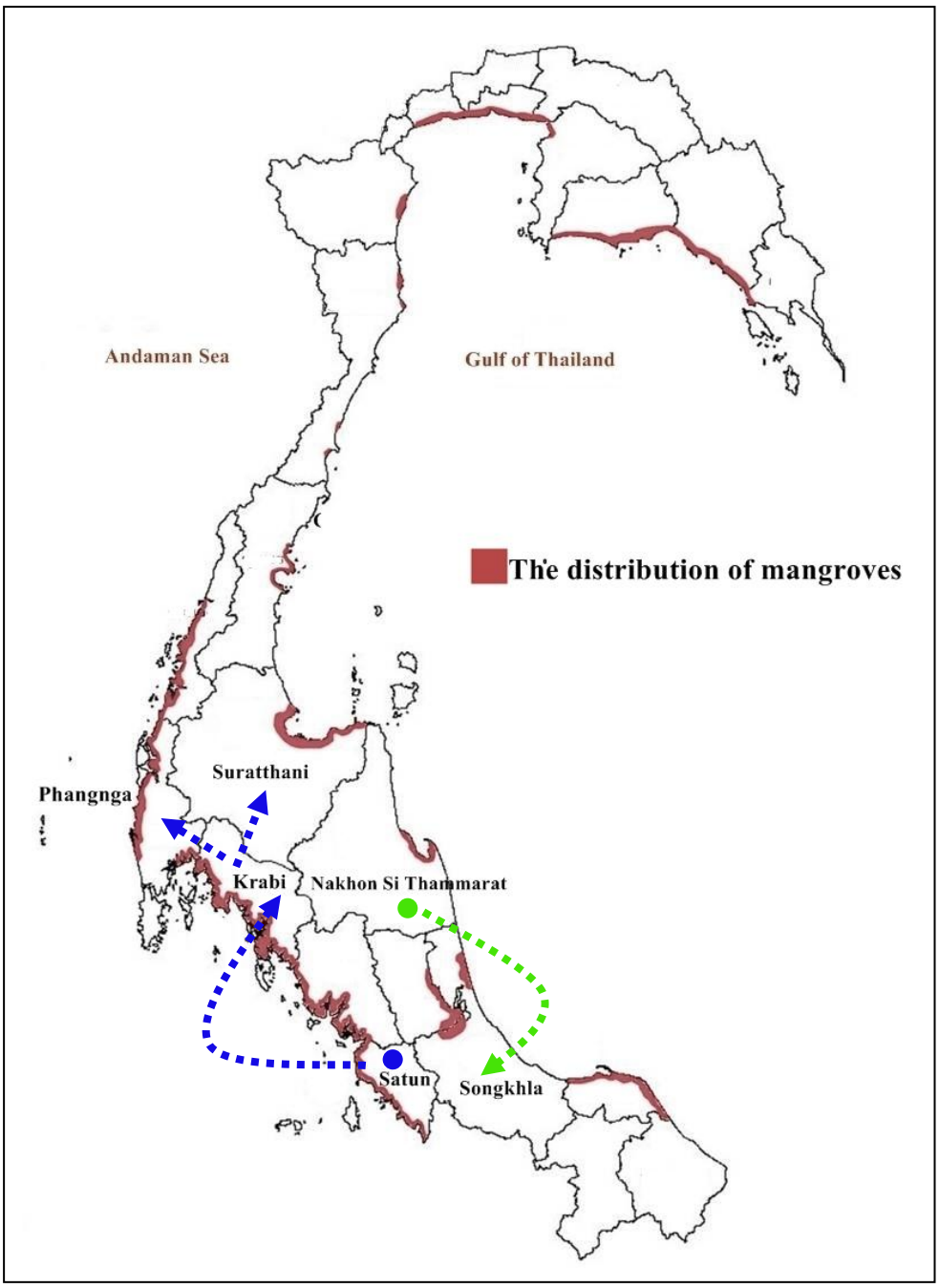


Figure 6. The distribution patterns of *Phacaspis mitis* in peninsular Thailand. (modified from Aksornkoae, 2002).

6. Phylogenetic tree of *Phacaspis mitis* in peninsular Thailand

6.1. Phylogenetic analysis based on maximum likelihood

According to the research question 1, 21 specimens were described as taxon, represented from 6 provinces. The 2 specimens (*Thinophilus* sp., *Nanothinophilus hoplites*) were treated as outgroup. The sequence of cytochrome oxidase I gene was carried out by universal primer. The cytochrome oxidase subunit I gene contained 600 bp, including analyzed gaps (Fig. 7). The result showed that the phylogenetic tree was monophyletic group. It can be divided into 2 main clades. Clade A consists of Krabi, Satun, Surat Thani and Phang Nga provinces. The bootstrap value was strongly supported with 99%. Within, clade A, it was separated into 3 subclades: A1, A2 and A3. Subclade A1 consists of Krabi and Satun. *P. mitis* from Surat Thani was separated in subclade A2. Subclade A3 contained *P .mitis* from Phang Nga and Krabi. Whereas, clade B consists of Songkhla and Nakhon Si Thammarat provinces, which has strongly bootstrap support (100 %).

The phylogenetic tree of maximum likelihood analysis based on 12S rDNA gene of 250 bp including analyzed gaps (Fig. 8). By this analysis, the result showed that *Phacaspis mitis* in peninsular Thailand was monophyletic group. It can be divided into 2 distinct clades. Clade A was composed of *P. mitis* from Krabi, Satun, Surat Thani and Phang Nga provinces, which it was strongly supported in 99 % of bootstrap value. However, clade A was still separated into 3 subclades: A1, A2 and A3. Subclade A1 was the specimens from Krabi, Satun, Surat Thani and Phang Nga that it was grouped together in this subclade. Subclade A2 consists of *P. mitis* of Satun, Phang Nga and Surat Thani. Eventually, subclade A3 was only found the *P. mitis* in Surat Thani province. Meanwhile, clade B comprises the *P. mitis* in two provinces of Songkhla and Nakhon Si Thammarat. Bootstrap value was highest in 99 % of bootstrap analysis.

With regard to the 16S rDNA gene, the sequences based on 410 bp including gaps were analyzed (Fig. 9). The result revealed that the presence of monophyletic tree of *Phacaspis mitis* in peninsular Thailand was separated into 2 main clades. There are 4 provinces; Krabi, Satun, Surat Thani and Phang Nga

provinces were grouped together in clade A. Moreover, clade A consists of 3 subclades: A1, A2 and A3. Subclade A1 was only found the *P. mitis* in Krabi province. *P. mitis* from Phang Nga was the subclade A2. Subclade A3 consists of *P. mitis* from Krabi, Satun and Surat Thani. However *P. mitis* from Satun of clade A was related to Clade B with lower bootstrap support. Clade B was composed of specimens of Nakhon Si Thammarat and Songkhla provinces, which has 100 % bootstrap support.

In the combined gene of 1,260 bp from 12S rDNA, 16S rDNA and cytochrome oxidase subunit I genes were analyzed (Fig. 10). The result showed that the monophyletic tree of combined gene can be divided into 2 main clades: cladeA and cladeB. Krabi, Satun, Surat Thani and Phang Nga provinces were grouped together in clade A which had 99 % of bootstrap support. However, *Phacaspis mitis* of clade A was separated into 3 subclades: subclade A1, A2 and A3. *P. mitis* of subclade A1 contained Satun and Krabi that was grouped together. *P. mitis* of subclade A2 was only found in Surat Thani province. *P. mitis* from Krabi and Phang Nga was included into subclade A3. The rest specimens of Songkhla and Nakhon Si Thammarat were grouped together in clade B and this clade had 100 % of bootstrap value. However, *P. mitis* of Songkhla and Nakhon Si Thammarat were still separated into subclade.

The phylogenetic tree of maximum likelihood analysis based on 3 mitochondrial genes and combined genes were congruent. The results showed that *Phacaspis mitis* from 6 provinces in peninsular Thailand was monophyletic group and it divided into 2 distinct clades. Clade A consists of Krabi, Satun, Surat Thani and Phang Nga provinces. Meanwhile, Songkhla and Nakhon Si Thammarat provinces were grouped together in clade B.

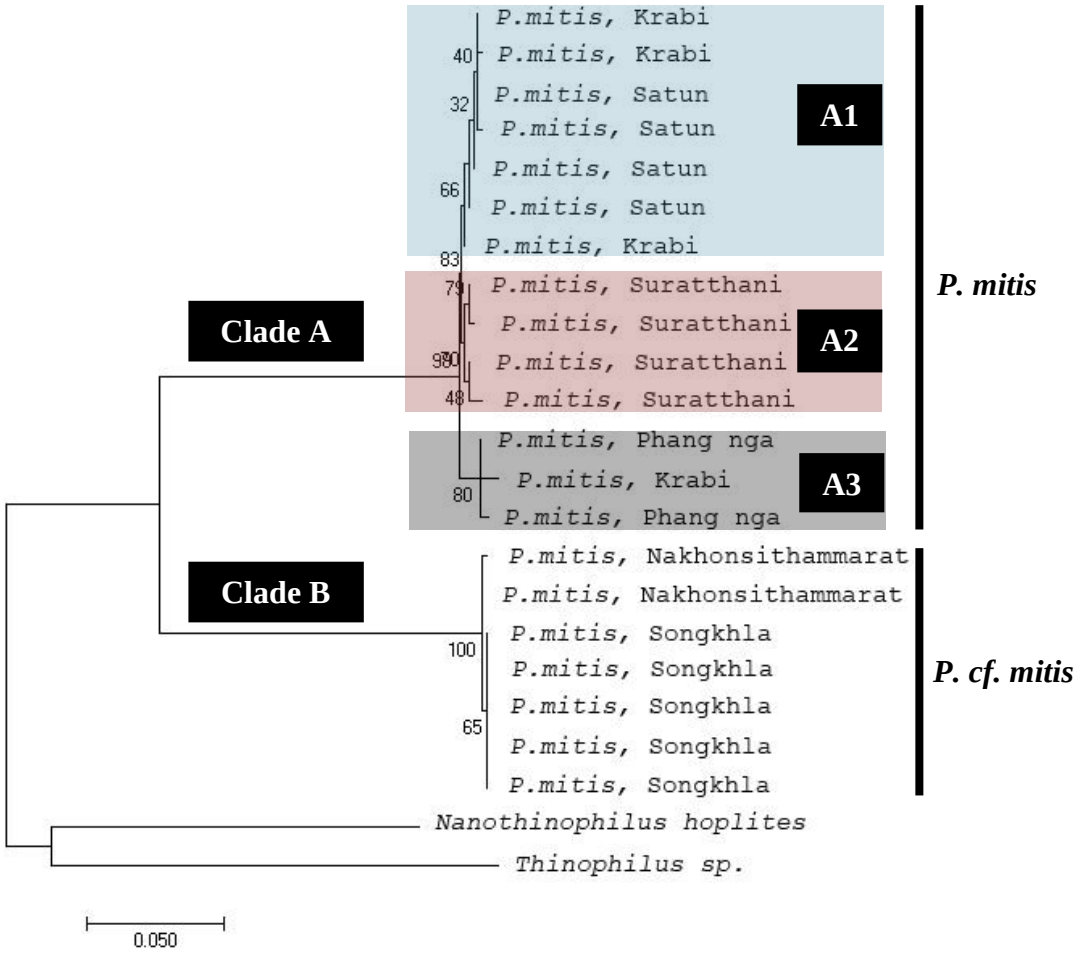


Figure 7. Maximum likelihood tree obtained from *COI* gene of *Phacaspis mitis* in peninsular Thailand.

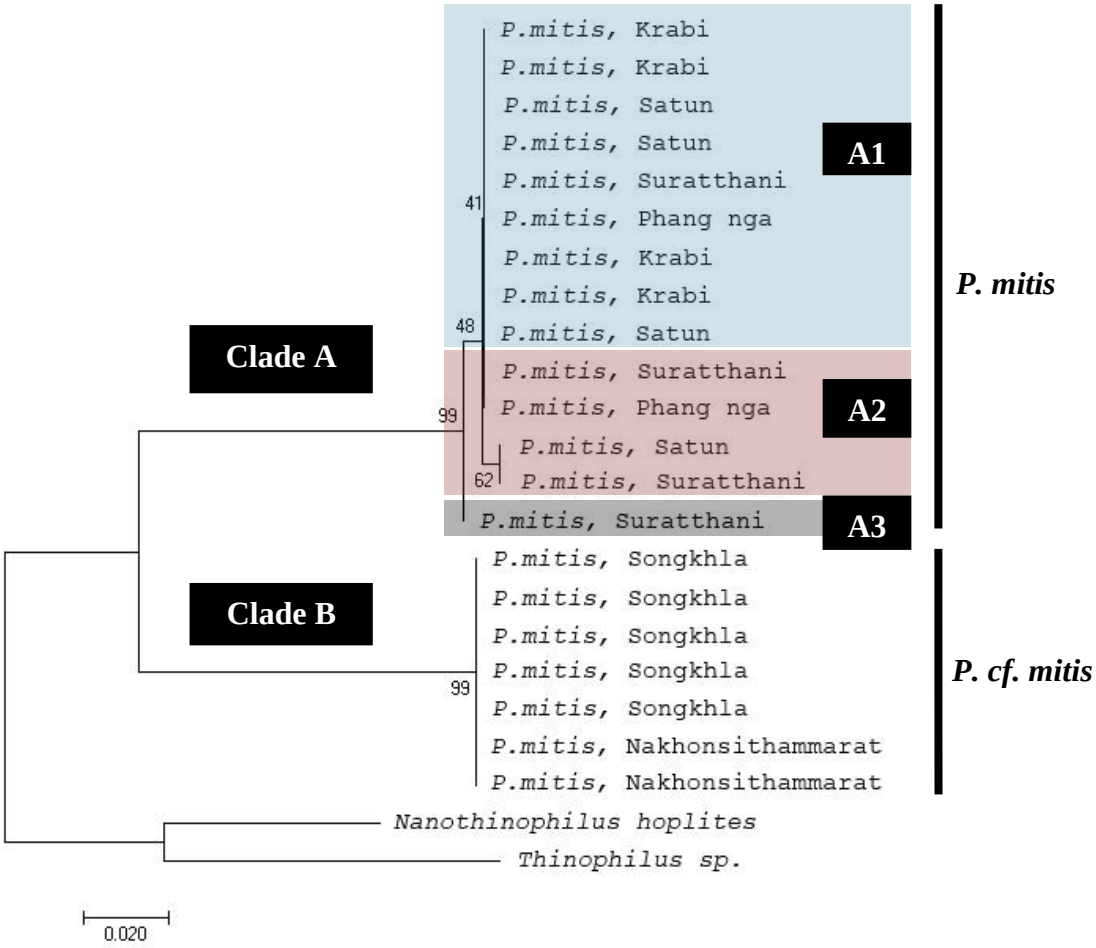


Figure 8. Maximum likelihood tree obtained from 12S rDNA gene of *Phacaspis mitis* in peninsular Thailand.

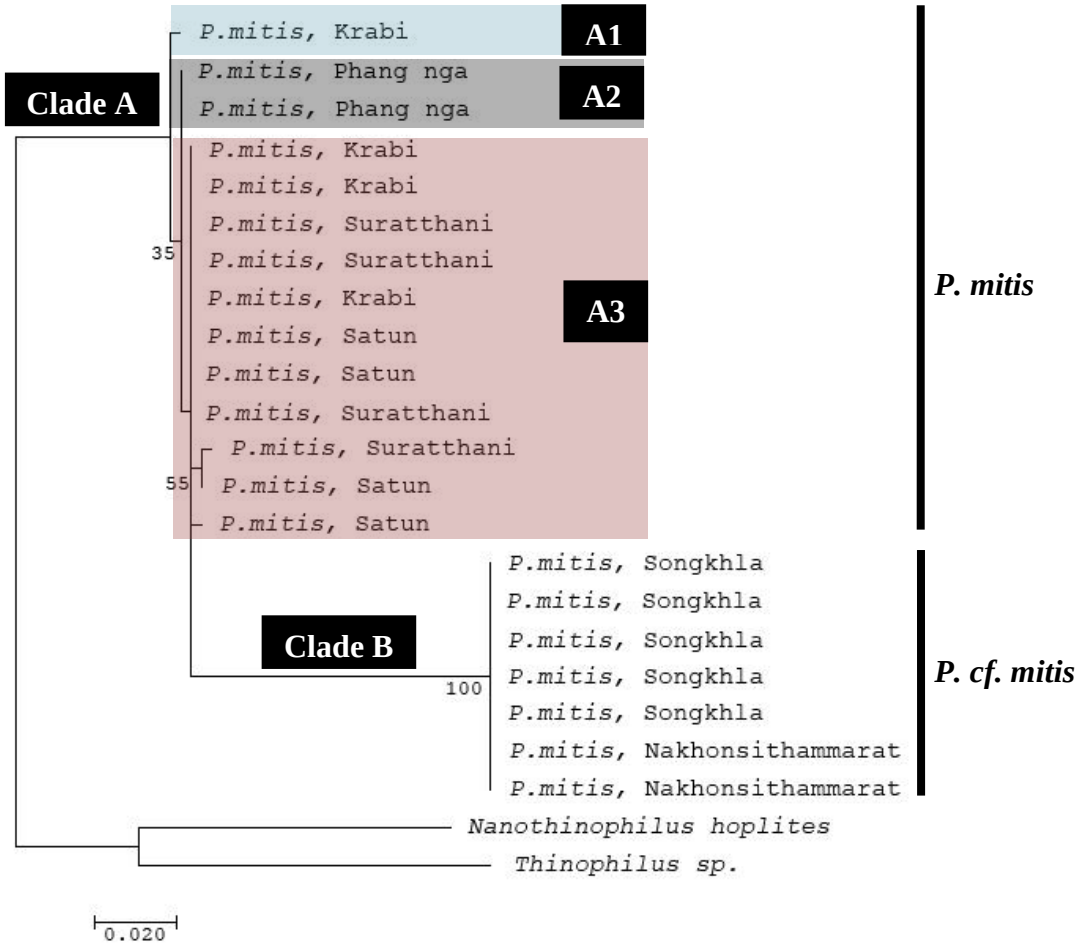


Figure 9. Maximum likelihood tree obtained from 16S rDNA gene of *Phacaspis mitis* in peninsular Thailand.

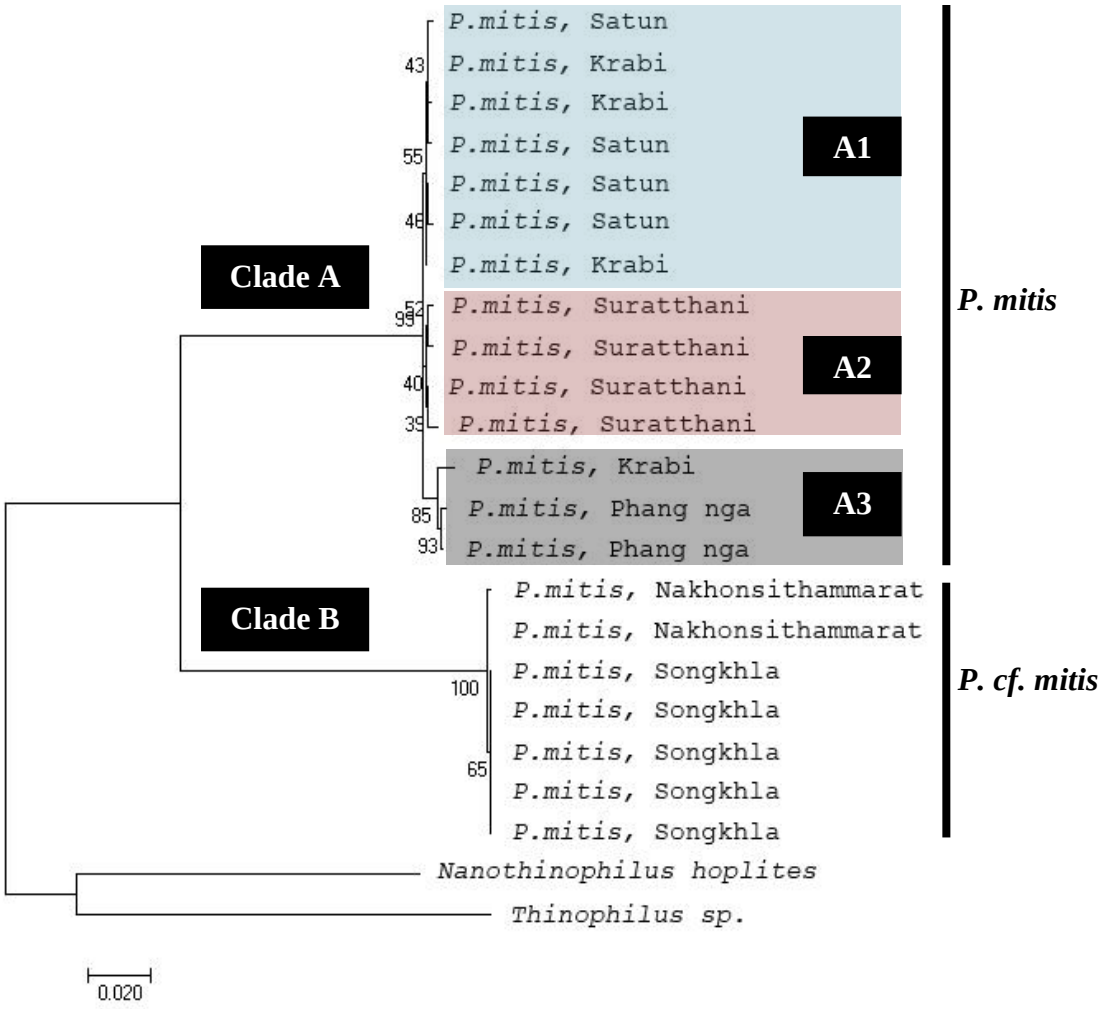


Figure 10. Maximum likelihood tree obtained from combined genes of *Phacaspis mitis* in peninsular Thailand.

6.2. Phylogenetic analysis based on Bayesian inference

Apart from maximum likelihood analysis, 21 sequences of *Phacaspis mitis* in peninsular Thailand were constructed the phylogenetic tree by Bayesian inference based on 3 mitochondrial genes. Universal primer was used to analysis for cytochrome oxidase subunit I gene. The result revealed the monophyletic tree and it was separated into 3 subclades: A1, A2 and A3 (Fig. 11). Subclade A1 was composed of *P. mitis* from Satun and Krabi. Subclade A2 consists of Phang Nga and Krabi provinces. The rest of Surat Thani was grouped together in subclade A3. In addition, clade B was found *P. mitis* of Songkhla and *P. mitis* of Nakhon Si Thammarat.

Figure 12 demonstrated the result of 12S rDNA gene. It showed that the monophyletic tree and it divided into 2 distinct clades. Clade A was separated into 2 subclades: A1 and A2. *P. mitis* from Surat Thani, Satun, Krabi and Phang Nga were grouped together in subclade A1, whereas *P. mitis* from Satun and Surat Thani were found in subclade A2. Clade B consists of specimens from Songkhla and Nakhon Si-Thammarat provinces.

The result of 16S rDNA gene showed that *Phacaspis mitis* in peninsular Thailand was monophyletic group and it can be divided into 2 distinct clades. Clade A consists of *P. mitis* from Surat Thani, Satun, Krabi and Phang Nga provinces. The rest specimens of Songkhla and Nakhon Si Thammarat were grouped together in clade B (Fig. 13).

In addition, The Bayesian inference phylogenetic tree based on combined genes (12S rDNA, 16S rDNA and cytochrome oxidase subunit I genes) showed that *Phacaspis mitis* in peninsular Thailand was monophyletic and it was divided into 2 clades (Fig14). Clade A consists of Krabi, Satun, Surat Thani as well as Phang Nga provinces. There are 3 subclades: A1, A2 and A3 were found in clade A. *P. mitis* of Satun and Krabi were grouped together in subclade A1. *P. mitis* of Krabi and Phang Nga were grouped together in subclade A2. Meanwhile, subclade A3 contained *P. mitis* of Surat Thani. Clade B consists of 2 subclades such as subclade of *P. mitis* from Songkhla and subclade of *P. mitis* from Nakhon Si Thammarat.

The phylogenetic trees of maximum likelihood analysis and Bayesian inference based on 3 mitochondrial genes and combined genes were congruent. The results showed that *Phacaspis mitis* from 6 provinces in peninsular Thailand was monophyletic group and it divided into 2 distinct clades. The representatives of Krabi, Satun, Surat Thani and Phang Nga provinces were grouped together in clade A. Meanwhile, the representatives of Songkhla and Nakhon Si Thammarat provinces were grouped together in clade B.

6.3. Estimating the date of divergence time

The neighbor-joining tree obtained from cytochrome oxidase subunit I gene showed that *Phacaspis mitis* in peninsular Thailand was separated into 2 distinct clades; clade A and clade B that it was originated about 35.55 Mya in late Eocene (Fig. 15). Clade A consists of *P. mitis* from Krabi, Satun, Surat Thani and Phang Nga provinces. The divergent time of this clade was approximately 3.14 million years ago (Mya) in the Pliocene. Clade B consists of Songkhla and Nakhon Si Thammarat provinces, which has diverged approximately 0.51 Mya in the Pleistocene. However, the result of clade A revealed *P. mitis* of Phang Nga separated from Krabi about 1.58 Mya in the Pleistocene (subclade A3), whereas *P. mitis* of Surat Thani (subclade A2) separated from Satun and Krabi (subclade A1) about 1.59 Mya in the Pleistocene. Moreover, *P. mitis* of Satun and Krabi were subsequently separated about 0.60-0.24 Mya in the Pleistocene. Meanwhile, *P. mitis* of Songkhla was recently separated from Nakhon Si Thammarat in the Holocene.

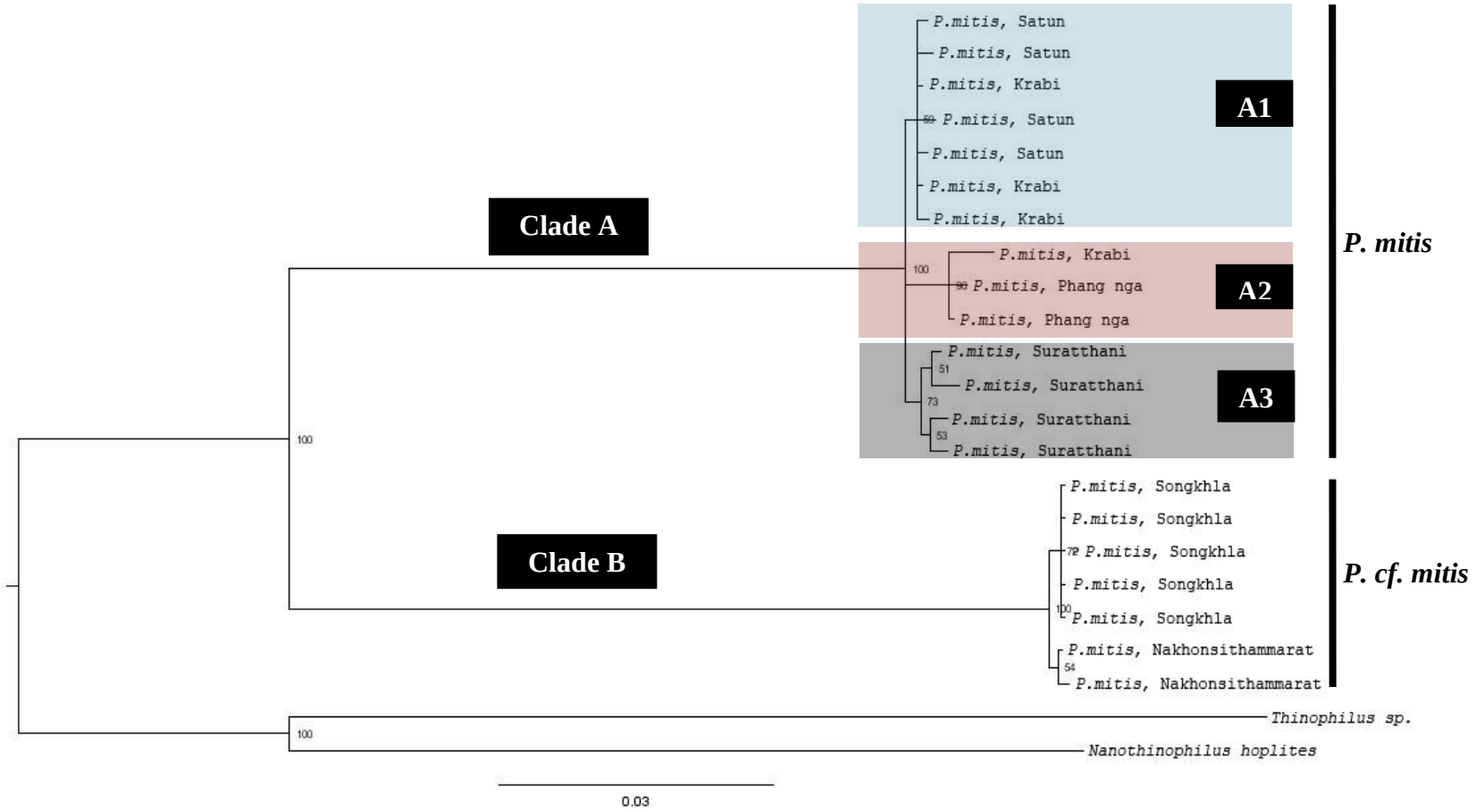


Figure 11. Bayesian inference tree obtained from *COI* gene of *Phacaspis mitis* in peninsular Thailand. Posterior probability was shown on the branches.

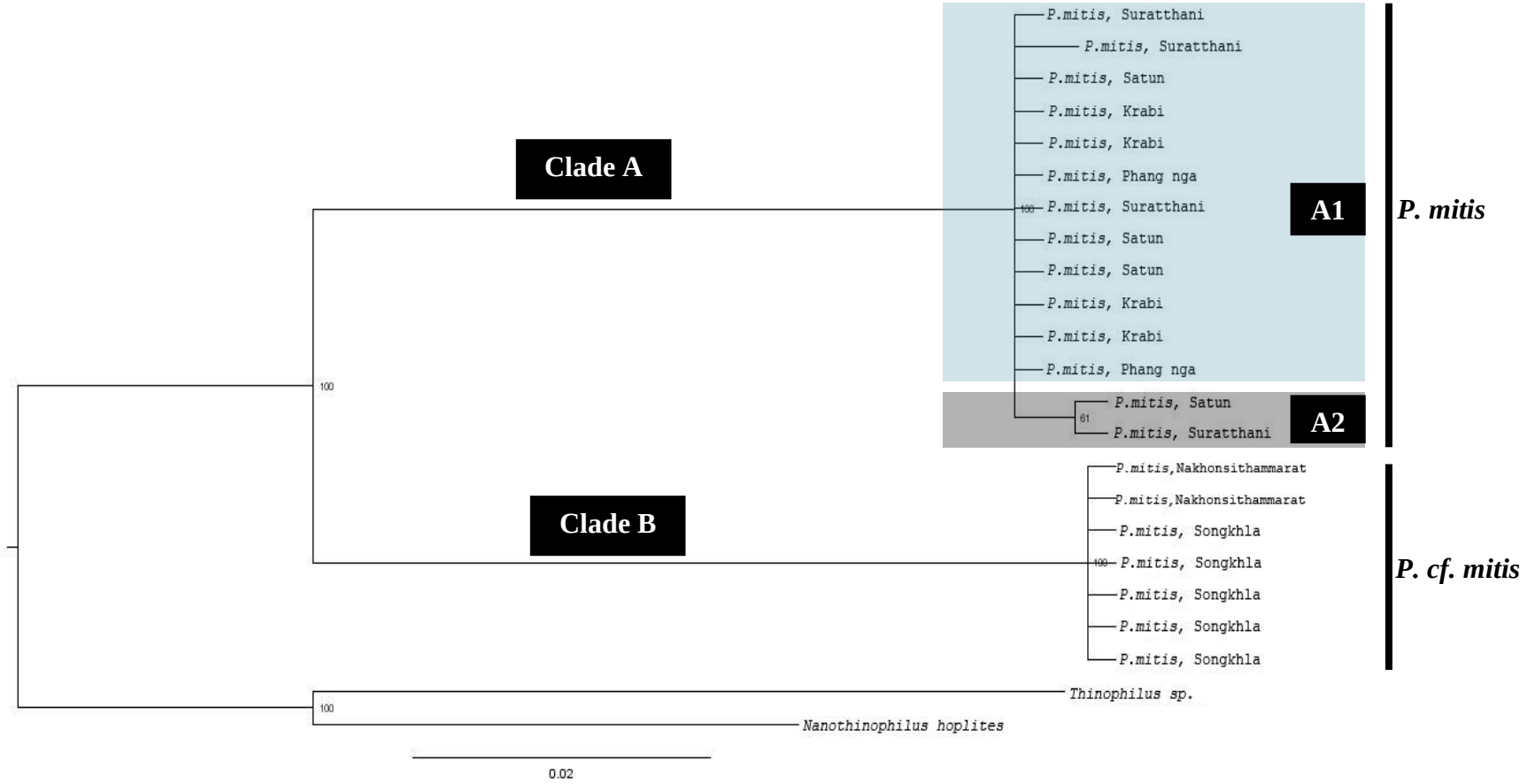


Figure 12. Bayesian inference tree obtained from 12S rDNA gene of *Phacaspis mitis* in peninsular Thailand. Posterior probability was shown on the branches.

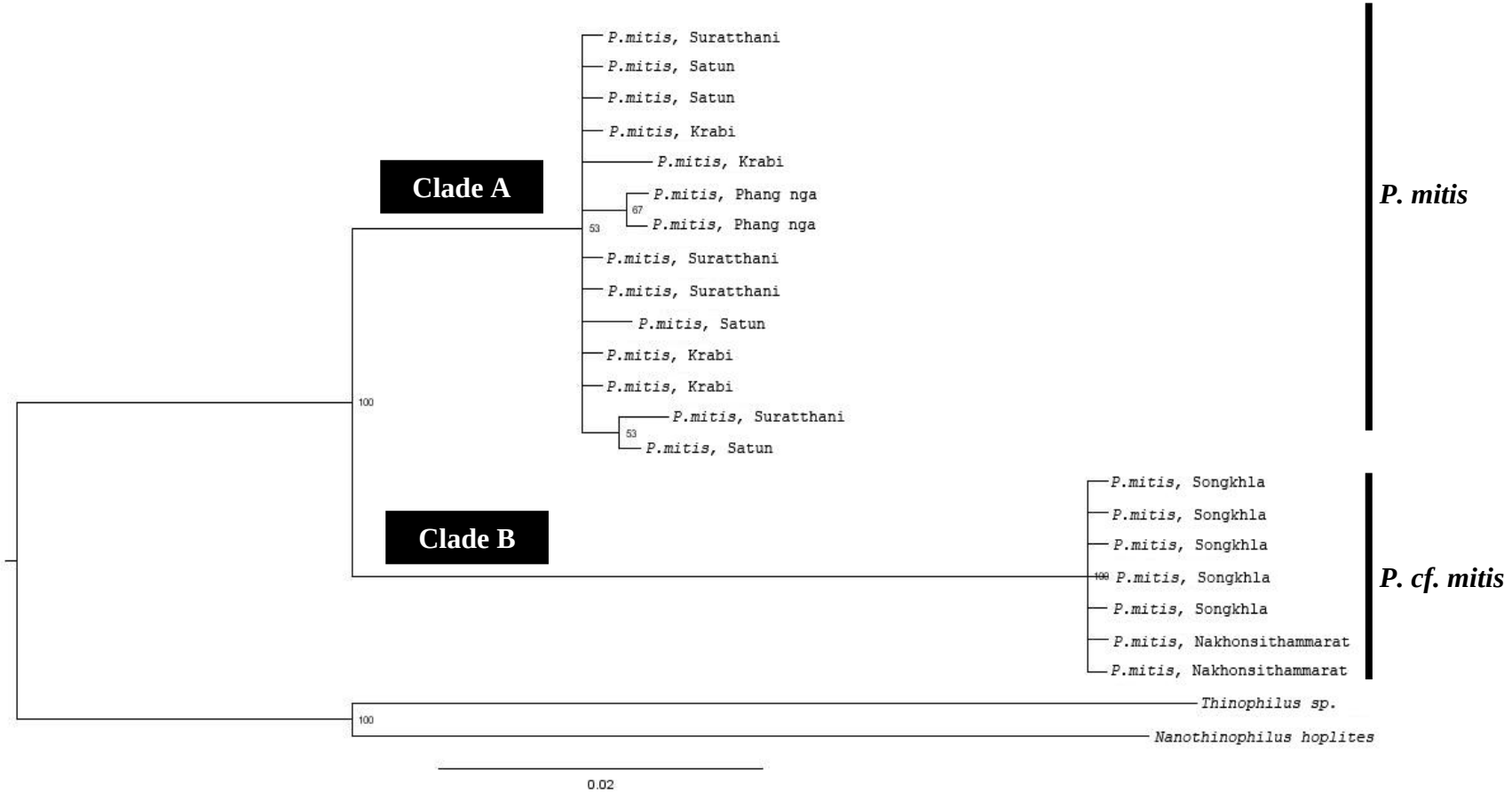


Figure 13. Bayesian inference tree obtained from 16S rDNA gene of *Phacaspis mitis* in peninsular Thailand. Posterior probability was shown on the branches.

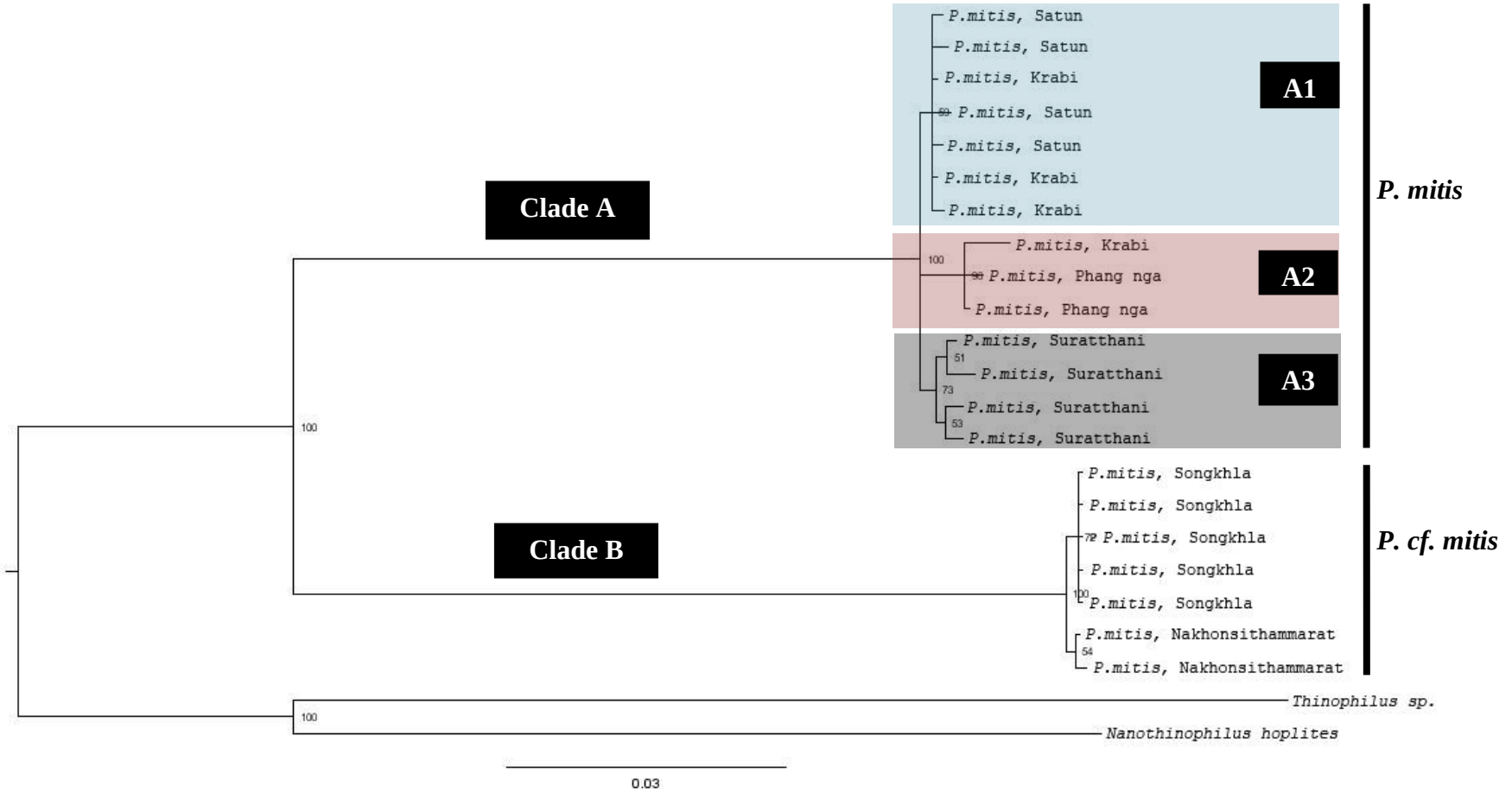


Figure 14. Bayesian inference tree obtained from combined genes of *Phacaspis mitis* in peninsular Thailand. Posterior probability was shown on the branches.

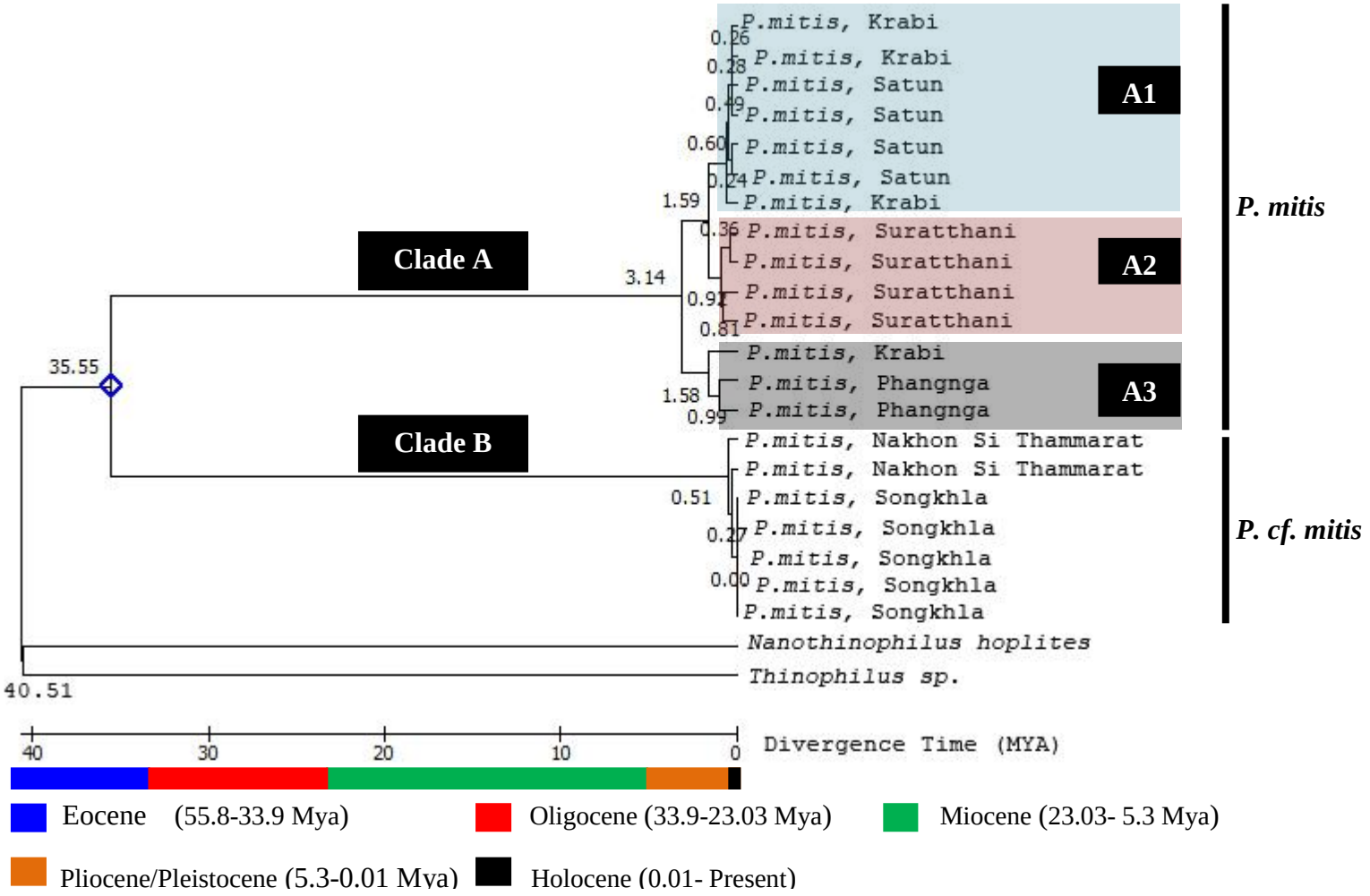


Figure 15. Divergence time of *Phacaspis mitis* in peninsular Thailand based on *COI* gene.

7. The relationship between genetic distances and geographical distances

The results of Mantel's test in 3 mitochondrial genes revealed that there were the significant association between genetic (F_{ST}) and geographical distances among the populations of *Phacaspis mitis* in peninsular Thailand. The result of *COI* showed that the correlation between F_{ST} values and geographic distance (km) was positively significant (r = 0.3799, $p < 0.01$) (Fig.16). Moreover, the scatterplots of F_{ST} showed that there was lack of regional equilibrium with genetic drift more influential than gene flow. The analysis of 12S rDNA gene showed that there was positively significant relationship between F_{ST} values and geographic distance (r = 0.3691, $p < 0.01$) (Fig.17). In addition, the scatterplots of F_{ST} showed that there was lack of regional equilibrium with genetic drift more influential than gene flow. Likewise, there was a positively significant relationship between F_{ST} values and geographic distance in 16S rDNA gene (r = 0.373, $p < 0.001$) (Fig.18). Moreover, the scatterplots of F_{ST} showed that there was lack of regional equilibrium with genetic drift more influential than gene flow.

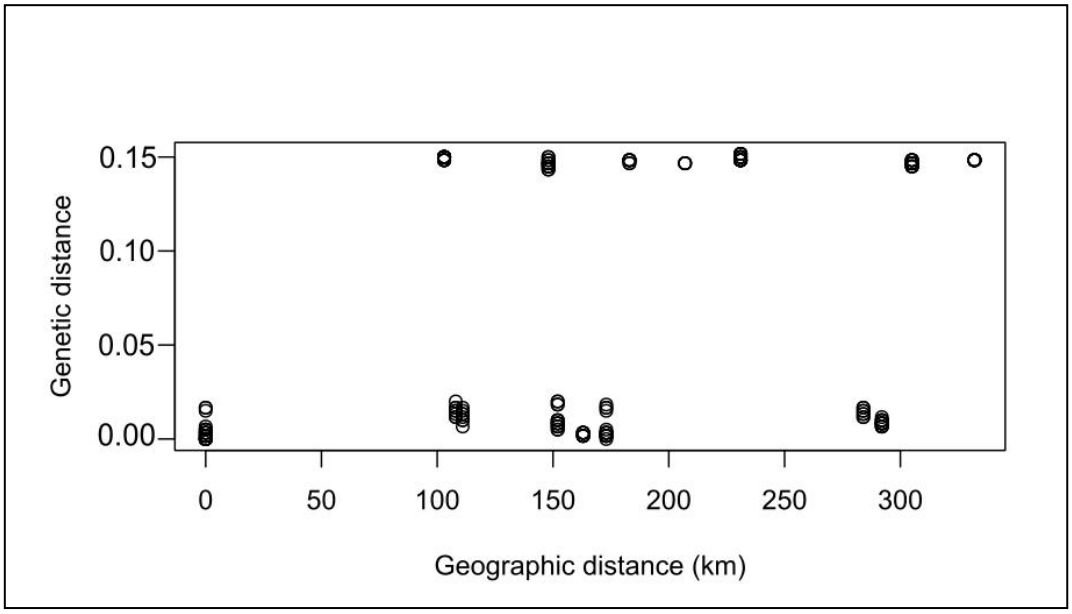


Figure 16. The pairwise comparison of genetic (F_{ST}) and geographic distance among *Phacaspis mitis* in peninsular Thailand inferred from *COI* gene (r = 0.3799, $p < 0.01$).

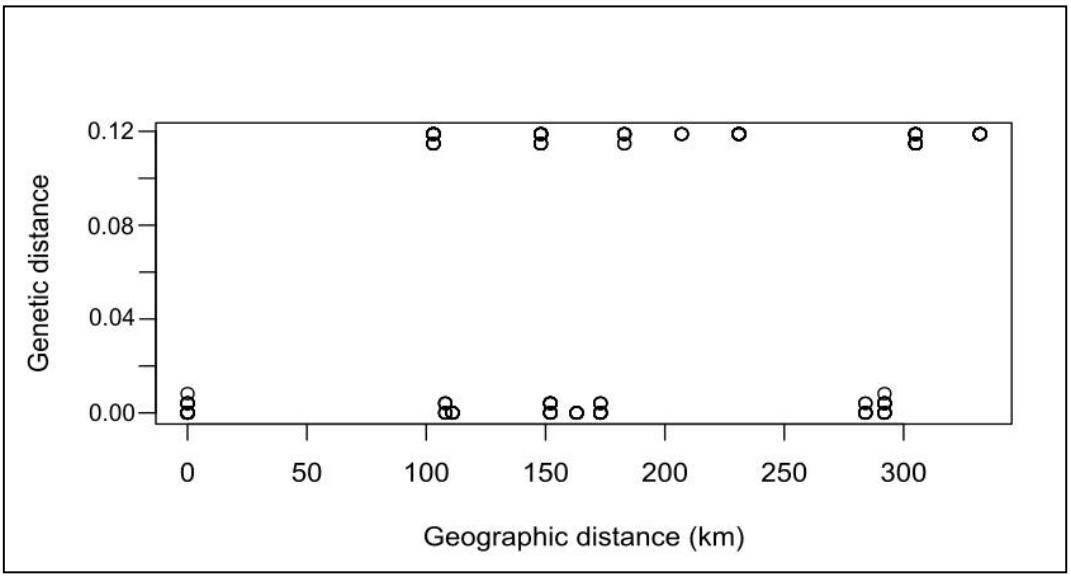


Figure 17. The pairwise comparison of genetic (F_{ST}) and geographic distance among *Phacaspis mitis* in peninsular Thailand inferred from 12S rDNA gene (r = 0.3691, $p < 0.01$)

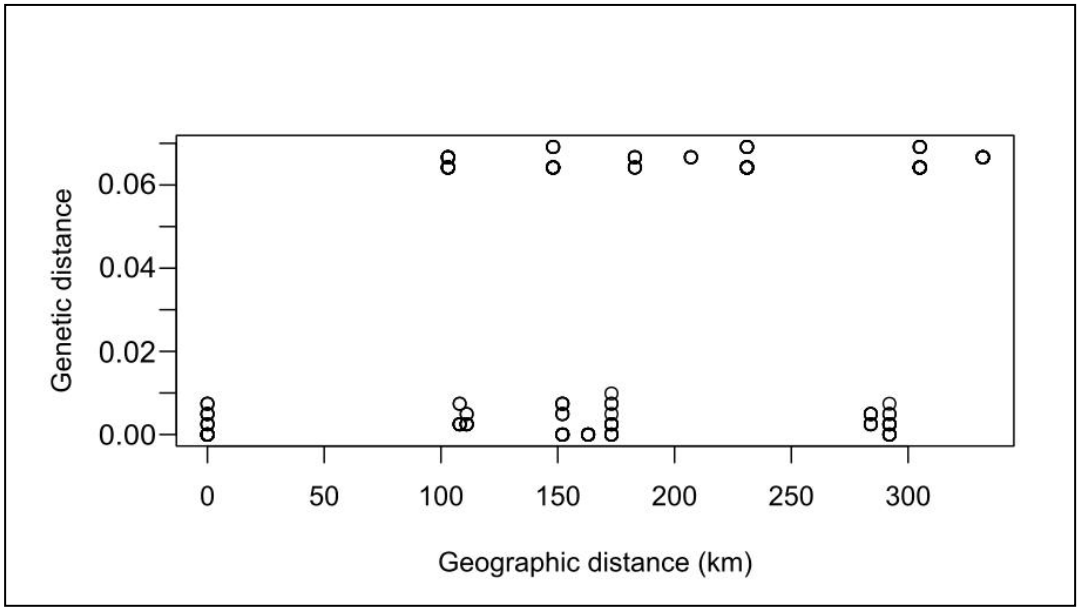


Figure 18. The pairwise comparison of genetic (F_{ST}) and geographic distances among *Phacaspis mitis* in peninsular Thailand inferred from 16S rDNA gene (r = 0.373, $p < 0.001$).

8. Phylogenetic tree of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei

According to the research question 2, the 21 specimens of *Phacaspis mitis* were representative for 6 provinces in peninsular Thailand, 3 specimens from Singapore and 1 specimen from Brunei including *Phacaspis ornata* from Singapore (4 specimens) and *Ornamenta* sp. from Brunei (3 specimens) were analyzed and constructed the phylogenetic tree. In this study, only universal primer of *COI* gene was only used to analyze. Because cytochrome oxidase subunit I gene is the effective marker for explain about evolutionary history. Moreover, the sequences database from Brunei and Singapore was performed using *COI* gene as well. The aligned sequences of *COI* gene with 560 bp including analyzed gaps were performed by molecular analyses by the maximum likelihood and Bayesian inference.

The result of phylogenetic tree by maximum likelihood analysis showed that *Phacaspis* in peninsular Thailand with adjacent area were monophyletic group and it was divided into 4 distinct clades (Fig. 19). *Phacaspis mitis* was separated into 2 clades such as clade A and clade C. Clade A with 98 % of bootstrap support consists of *Phacaspis mitis* of 4 provinces in peninsular Thailand, Singapore and Brunei. However, clade A was separated into 5 subclades: A1, A2, A3, A4 and A5. Subclade A1 was the specimens from Krabi and Satun that it was grouped together in this subclade. *P. mitis* from Singapore was grouped together with *P. mitis* from Krabi in subclade A2. Subclade A3 was only found *P. mitis* from Surat Thani. Moreover, subclade A4 was composed of *P. mitis* from Krabi and Phang Nga. Eventually, subclade A5 was only found *P. mitis* from Brunei. However, *Phacaspis mitis* from Nakhon Si Thammarat and Songkhla were grouped together in clade C with 100 % of bootstrap support. In addition, the specimens of *Phacaspis ornata* from Singapore were grouped together in clade D with 99 % of bootstrap support. Apart from genus *Phacaspis*, *Ornamenta* sp. from Brunei was grouped together in clade B.

The Bayesian inference phylogenetic tree based on universal cytochrome oxidase subunit I gene revealed the relationship of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei were monophyletic group. It can be divided into 4 distinct clades (Fig. 20).

Phacaspis mitis was separated into 2 clades; clade A and C. Clade A consists of *P. mitis* from Thailand, Brunei and Singapore. They were divided into 4 subclades; A1, A2, A3 and A4. *P. mitis* from Krabi and Phang Nga were grouped together in subclade A1. Subclade A2 contained *P. mitis* from Satun, Krabi and Singapore. *P. mitis* from Surat Thani was only found in subclade A3 and then *P. mitis* from Brunei was separated into subclade A4. However, *P. mitis* from Nakhon Si-Thammarat and Songkhla in Thailand were separated from the others in clade C. Additional, *Phacaspis Ornata* from Singapore was included together in clade D and *Ornamenta* sp. from Brunei was separated to be clade B.

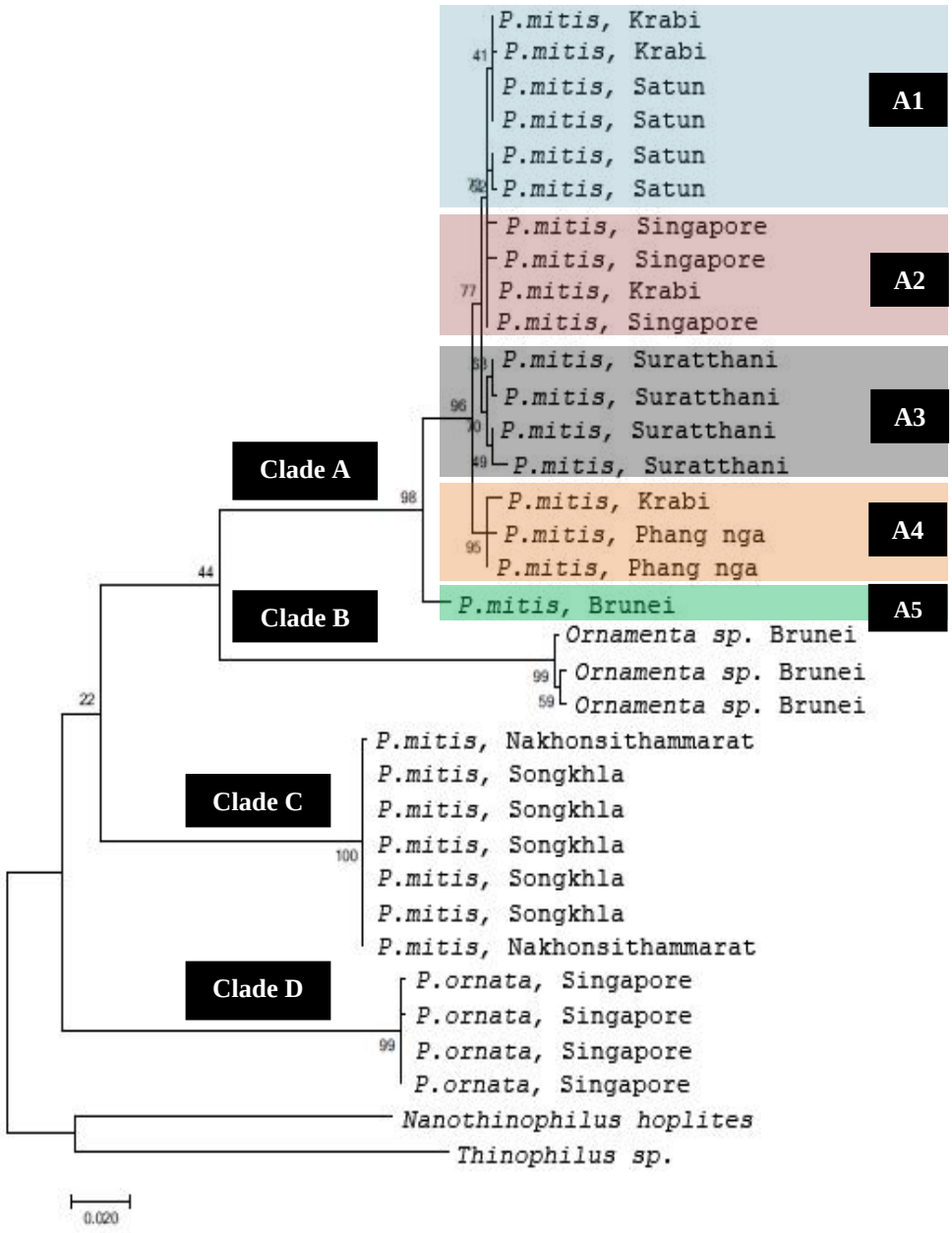


Figure 19. Maximum likelihood tree obtained from *COI* gene of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei.

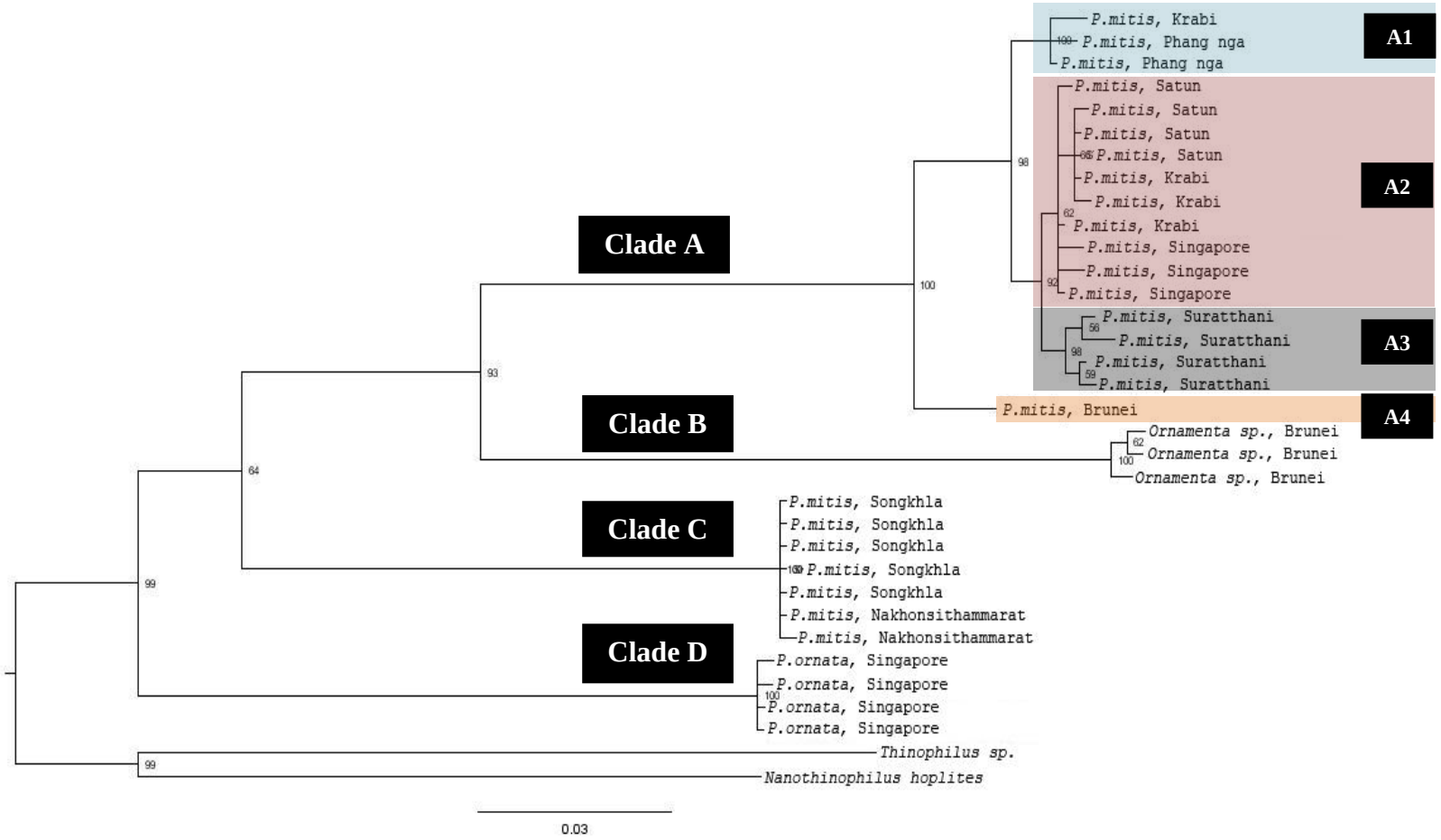


Figure 20. Bayesian inference tree obtained from *COI* gene of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei. Posterior probability was shown on the branches.

9. Haplotype networks of *Phacaspis mitis* in peninsular Thailand, Singapore and Brunei

The aligned sequences of cytochrome oxidase subunit I gene, 600 bp were analyzed by DNAsp program based on 25 sequences of *Phacaspis mitis* in peninsular Thailand, Singapore and Brunei. The result revealed 18 distinct haplotype patterns that haplotype pattern 1, 2, 9 and 10 comprises sequence of Surat Thani. Haplotype pattern 8 and 14 were found in sequence of Phang Nga. Haplotype pattern 4, 5 and 11 consists of sequence of Satun. Haplotype pattern 7 and 13 were aligned sequence of Krabi. Haplotype pattern 15 and 16 consists of sequence of Nakhon Si Thammarat and Brunei, respectively. Moreover, haplotype pattern 17 and 18 consists of sequence of Singapore. Interestingly, haplotype pattern 3, 6 and 12 showed that there was more haplotype pattern than one sequence in each conformation. Sequences of Songkhla and Nakhon Si Thammarat were combined together in same pattern (haplotype pattern 3). Haplotype pattern 6 was composed of sequences of Krabi and Singapore. Haplotype pattern 12 consists of sequences of Satun and Krabi (Table 11).

A statistical parsimony network was analyzed by TCS program. The results showed that there were 18 haplotype patterns and they were divided into 2 haplotype networks (Fig. 21). Haplotype network A contained 15 distinct haplotype patterns. The origin of this group was found in Satun and it was firstly separated into group of Krabi. Moreover, Krabi can be divided into 3 sub-patterns. The first sub-pattern (1^{st} sub-pattern) was only found in Singapore. The second sub-pattern (2^{nd} sub-pattern) was Surat Thani and the last sub-pattern (3^{rd} sub-pattern) consists of Phang Nga and Krabi. Conversely, the result of haplotype network B showed that Nakhon Si Thammarat was separated from Songkhla. However, the sequence of Brunei was not grouped with the other haplotype patterns in peninsular Thailand and Singapore.

Table 11. Haplotype variation among *Phacaspis mitis* in peninsular Thailand, Singapore and Brunei.

Haplotype	Sequences	10 20 30 40 50 * * * * *
Haplotype1	SNI.1	TAAACCTATATTAATTTTCCCAATTGTAATTCAACTTTATAATATATTATTATTA
Haplotype2	SNI.2	.....
Haplotype3	SKA.1,2,3,4,5, NRT.1	ATTTATAGATACCTAACCTT.TTCCTC.GCCTT.TA.CTA.TCTATC.TA.CACG
Haplotype4	STN.1	.T.....A.....G.C.....
Haplotype5	STN.2	.T.....A.....G....G.C.....
Haplotype6	KBI.1 ,Singapore	.T.....A.....C.....
Haplotype7	KBI.2	.....T.....T.....A.....C...G.....
Haplotype8	PNA.1	.....T....A.....C.....
Haplotype9	SNI.3	.....C....
Haplotype10	SNI.4	.....G.....C....
Haplotype11	STN.3	.TG.....A.....G.....C.....
Haplotype12	STN.4,KBI.3	.T.....A.....G.....C.....
Haplotype13	KBI.4	.T.....A.....G.....C.....
Haplotype14	PNA.2	.....A.....C.....
Haplotype15	NRT.2	ATTTATAGATGCCTAACCTT.TTCCTC.GCCTT.TA.CTA.TCTATC.TA.CACG
Haplotype16	Brunei	.....T.....C.....T.G....C...C.....G.....
Haplotype17	Singapore	.T.....A.....C.....
Haplotype18	Singapore	.T.....A.....C.....C...G...

Table 11(Continued). Haplotype variation among *Phacaspis mitis* in peninsular Thailand, Singapore and Brunei.

Haplotype	Sequences	60 70 80 90 100
		* * * * *
Haplotype1	SNI.1	CTTACCTTAAACTTTTCATAAATTATTTTCTTCTTATATACTATCTACT
Haplotype2	SNI.2	.C.....
Haplotype3	SKA.1,2,3,4,5,NRT.1	T.C.TA.CTGTTCAAC.TCTT.AATAAACTA..AAT.T.TTATC.GCT.
Haplotype4	STN.1	C
Haplotype5	STN.2	C
Haplotype6	KBI.1,Singapore	C
Haplotype7	KBI.2	T..G.....G.....C.....C.....C
Haplotype8	PNA.1	T.....G.....G.....C.....C.....C
Haplotype9	SNI.3	C
Haplotype10	SNI.4	.....C.....
Haplotype11	STN.3	C
Haplotype12	STN.4,KBI.3	C
Haplotype13	KBI.4	.....C.....C
Haplotype14	PNA.2	T.....G.....C.....C.....C
Haplotype15	NRT.2	T.C.TA.CTGTTCAAC.TCTT.AATAAACTA..AAT.T.TTATC.GCT.
Haplotype16	Brunei	TC..T.....TC.....C.....CA.....TA.TC
Haplotype17	Singapore	.....T.....C.....C
Haplotype18	Singapore	C

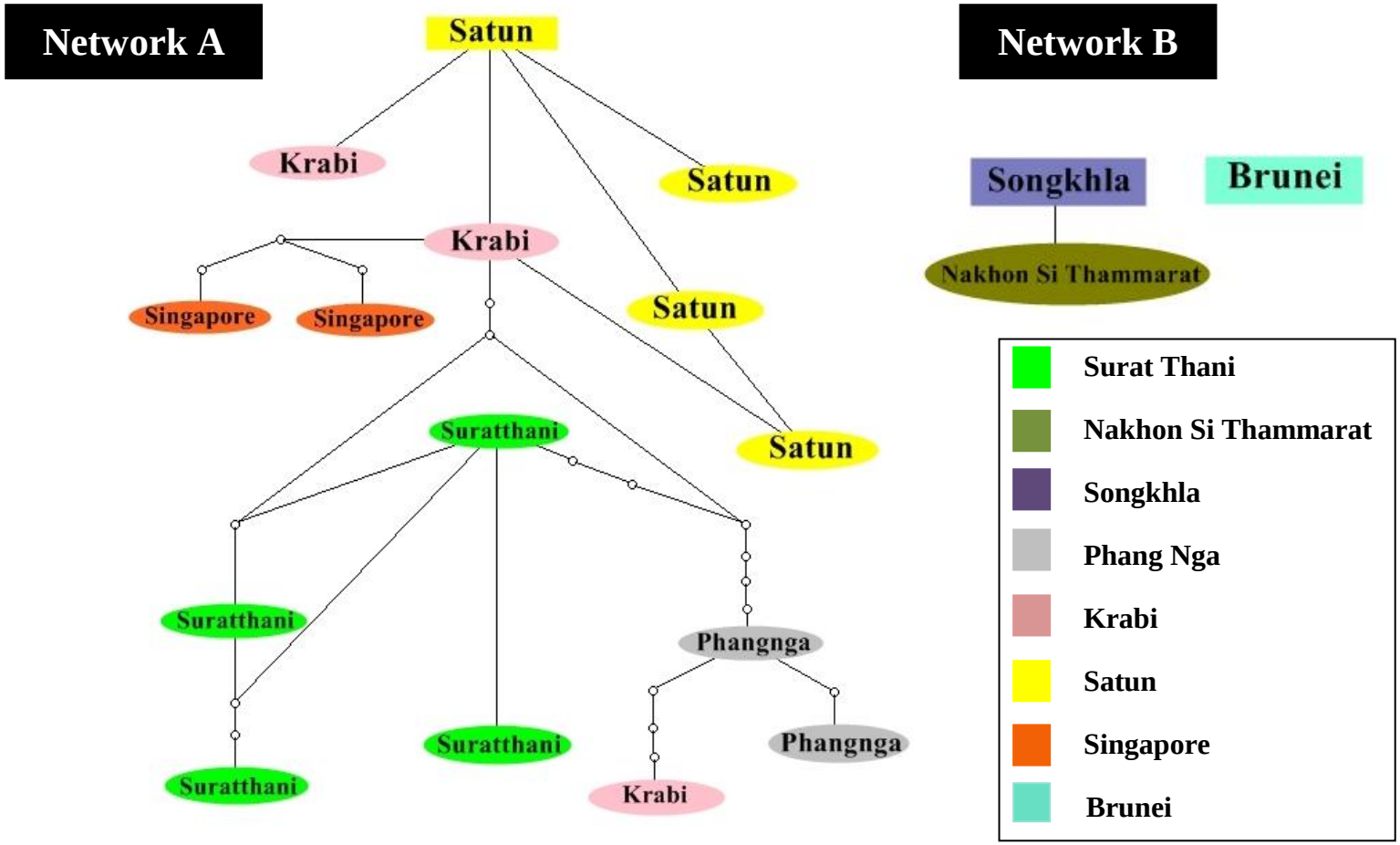


Figure 21. Haplotype networks of *Phacaspis mitis* in peninsular Thailand, Singapore and Brunei.

CHAPTER 4

DISCUSSION

Species composition

In this study, 4 genera, 6 species and 235 individuals of long-legged flies were identified from peninsular Thailand, Singapore and Brunei. In peninsular Thailand, *Phacaspis mitis* was found in 6 coastal provinces such as Surat Thani, Nakhon Si Thammarat, Songkhla, Phang Nga, Krabi and Satun provinces. In addition, 40 individuals were found in the mangrove of the Gulf of Thailand whereas 106 individuals were collected from the Andaman Sea. Owing to the extensive mangroves from the Andaman Sea are large patch and continuity of area. Conversely, the mangroves from the Gulf of Thailand are fragmentation (Thampanya *et al.,* 2006). Consequently, the suitable habitats for occupying of *P.mitis* in the Andaman Sea were more number of individuals than the Gulf of Thailand. However, *Phacaspis mitis* was not found in Chumphon and Pattani provinces. Because the topology of mangrove habitat from both provinces was not characterized of suitable habitat that there was not deep mudflat, high salinity (brackish waters and estuary) and open forest (*pers com,* Grootaert).

The phylogenetic relationships among *Phacaspis mitis* in peninsular Thailand

In this study the phylogenetic relationship of *P. mitis* from 6 coastal provinces along peninsular Thailand was investigated using Cytochrome oxidase subunit I, ribosomal DNA subunit 12S, ribosomal DNA subunit 16S of mitochondrial DNA genes and combined genes. Maximum likelihood method and Bayesian inference were used to construct the phylogenetic tree. The maximum likelihood tree from *COI*, 12S rDNA, 16S rDNA as well as combined genes (Fig. 7, 8, 9 and 10 respectively) indicates that *P. mitis* in peninsular Thailand is monophyletic group and it can be divided into 2 distinct clades.

Likewise, the results of Bayesian inference from *COI*, 12S rDNA, 16S rDNA and combined genes (Fig. 11, 12, 13 and 14 respectively) revealed that *P. mitis* in peninsular Thailand is monophyletic group and they are divided into 2 clades as well; the Andaman clade and Gulf of Thailand clade. It was proposed that the genetic differentiation has coincidence in the both population of *P. mitis* in peninsular Thailand. Because the results of phylogenetic tree suggested that it was clearly separated between the population of Andaman Sea and Gulf of Thailand. However, the *P. mitis* in Surat Thani has been transition zone between both populations. According to the Mantel test analysis, the results from 3 mitochondrial genes showed strong evidence supporting that there are two populations of *P. mitis* in peninsular Thailand. In addition, the two main clades of phylogenetic tree represent not only the two major populations of *P. mitis*, but also shown that they consisted of several sub-populations in each clade. Interestingly, *P. mitis* plays an important role as predator in mangrove ecosystem and it was the true marine long-legged flies. Moreover, they occupy in unique habitat which the microhabitat of *P. mitis* has high-salinity environment and sunlight exposure on the mudflats in front of mangrove (*pers com*, Grootaert). The organism capacity for living in microhabitat has been influenced on the metapopulation existence. Metapopulation is a set of local populations which occupy a suitable habitat on a patch and each suitable patch is separated by unsuitable terrain (Levins 1969; Yuttham *et al.,* 2003). The viability of local population and size of population are the important factors relating to habitat necessary for metapopulation survival (Etienne and Heesterbeek 2000; Bascompte *et al.,* 2002; Yuttham *et al.,* 2003). Moreover, the existence of metapopulation is affected by dispersal and extinction processes between local habitats in such landscapes (Hanski 1997, 1999). According to Hanski and Ovaskainen (2000) suggested that the connectivity of habitat within a patch network could be explained by metapopulation capacity.

The result of haplotype network would be illustrated genetic relationship between individual in each sampling site and it also has been used for investigation of the phylogeography and evolutionary history of organisms (Clement *et al.,* 2000; Leigh *et al.,* 2015). Gorostiza and colleagues (2012) suggested that the

oldest haplotype is probably to be the original among the population. In this study, the haplotype network of *P. mitis* in peninsular Thailand was analyzed based on *COI* gene. The result revealed that there were two haplotype networks in peninsular Thailand. In addition, the haplotype pattern of Satun province might be assumed to be the original haplotype pattern in peninsular Thailand and it also was derived to be the haplotype network A, including Krabi, Phang Nga and Surat Thani provinces. The finding was supported by the characteristics of mangroves in Andaman coast. The mangroves of the coastal provinces along Andaman region are the most extensive area. The forest structure and geomorphic character of mangrove in this region are similar. Moreover, the type of mangroves is estuary and deep mudflat (Lugo and Snekdaker 1974; Twilley *et al.*, 1998; Plathong and Plathong 2011). The tidal characteristic of Andaman coastline is a semi-diurnal cycle and the tidal amplitude was ranging from 3 to 4 meters. Consequently, the aquatic invertebrate was recruited into the mangrove ecosystem (Macintosh *et al.*, 1991; Plathong and Plathong 2011). According to the role of *P. mitis* in ecosystem as a predator, *P. mitis* would be promoted the numerous of population in this area because there are a good resources and suitable habitats to contribute for adaptation of *P. mitis*. In addition, the coastal provinces from Andaman region are large patches of connected mangrove area (Eiamsa-Ard and Amornchairojkul 1997). Therefore, they had genetic connectivity among the population due to there was distribution of individuals across structured habitat via corridor. Eventually, the populations of *P. mitis* in Surat Thani, Phang Nga, Krabi and Satun provinces were grouped together in haplotype network A.

On the other hand, haplotype network B was composed of *P. mitis* from Nakhon Si Thammarat and Songkhla provinces. This haplotype network indicated that the oldest haplotype of pattern B was Nakhon Si Thammarat province. Previously, the fragmentation of mangroves ecosystem was effected by sea level change and climate change. Currently, Thampanya and colleagues (2006) reported that the coastal erosion and forest fragmentation in mangroves ecosystem in Gulf of Thailand have been influenced by anthropogenic activities. Consequently, genetic differentiation in their populations was determined. In this study, the correlation between genetic distance (F_{ST}) and geographical distance (km) was analyzed using

COI, 12S rDNA and 16S rDNA (Fig. 16, 17 and 18 respectively). The result of genetic differentiation between populations was severely evolutionary force following by geographic distance. The similar result from 3 mitochondrial genes demonstrated that the correlation between genetic distances and geographical distance was determined by genetic drift and gene flow that the effect of genetic drift has more influential than gene flow. However, the forest fragmentation has played an important role on both of genetic drift and gene flow. Although the genetic drift has previously occurred in the genetic structure of *P. mitis* in both regions, a result of gene flow has subsequently influenced on population of *P. mitis*. However, the mangrove from Gulf of Thailand region was fragmented more than the Andaman region. After patching, the migration route of *P. mitis* was interrupted by geographic isolation. Hence, a result of gene flow within populations of *P. mitis* in Gulf of Thailand coast has been more influential than the population of *P. mitis* in Andaman coast. This study was congruent with the result of Hutchison and Templeton (1999) that positive correlation between genetic distance and habitat distance indicated the level of genetic differentiation between populations was increased following by geographic distance.

Although the external morphological characteristic among the populations of *Phacaspis mitis* in peninsular Thailand is similar, the population was separated into 2 groups such as the Gulf of Thailand and the Andaman Sea in term of phylogeny. Unfortunately, there is a good opportunity that the 2 populations of *P.mitis* in peninsular Thailand will be classified to the different species or new species in the future.

The divergence time estimates of *P. mitis* in peninsular Thailand

The finding of two populations of *P. mitis* from different region in peninsular Thailand was supported by divergence time as well. The divergence time of *P. mitis* was estimated and inferred by the fossil record of genus *Thinophilus* about 37.2-33.9 Mya (Pollet *et al.,* 2004). Unfortunately, the fossil of genus *Phacaspis* was not record at the present. The genus *Thinophilus* is the one of true marine long-legged flies and they were closely related to *Phacaspis* in terms of phylogeny (Lim *et al.,* 2010). The result in Figure 15 showed that *P. mitis* was still divided into two lineages at approximately 35.55 Mya in late Eocene epoch. The most significant event of this epoch was sea-level falling due to climate characteristic tends to be cooler and drier during 36.4 to 33.5 Mya (Hoorn *et al.,* 2012). Consequently, the sea-level falling might affect to the distribution and fragmentation of mangroves in peninsular Thailand. Moreover, the divergence time showed that lineage A has derived about 3.14 Mya during the Pliocene while lineage B was originated in the Pleistocene (0.51 Mya). In addition, *P. mitis* from Satun and Krabi have diverged about 0.60 Mya in the Pleistocene. *P.mtis* from Surat Thani was separated from the other provinces approximately 1.59 Mya in the Pleistocene. *P. mitis* from Krabi was separated from Phang Nga about 1.58 Mya in the Pleistocene as well. Conversely, *P. mitis* from Songkhla was recently separated from Nakhon Si Thammarat in the Holocene. The Plio-Pleistocene and Holocene epochs are known as glacial period (ice age) (Berggren 1972; Alley *et al.,* 1997). During these epochs the sea level fluctuated rapidly and also was lower than in present time. Our result was speculated that this scenario has severe effects on mangrove in peninsular Thailand, especially, the fluctuation of sea level leading to the expanding rapidly of mangrove expansion and fragmentation. It could be assumed that there were several suitable habitats in the mangrove for occupying. Consequently, *P. mitis* can be dispersed at that time. Moreover, our result proposed that Satun province might be the origin of *P. mitis* in peninsular Thailand. It was coincidence with the research of Umitsu and colleagues (1999) that the mainly formation of mangroves in Satun province was relativeness with Pleistocene and late Holocene. Our result proposed that the formation of mangroves during the Pleistocene

and late Holocene played an important role to occur the several suitable microhabitats and hence, *P. mitis* in Satun province was firstly established in peninsular Thailand.

The phylogenetic relationships of genus *Phacaspis* in peninsular Thailand, Singapore and Brunei

In this study, the long-legged flies genus *Phacaspis* from peninsular Thailand, Singapore and Brunei were constructed the phylogenetic relationship based on cytochrome oxidase subunit I gene and also included the *Ornamenta* sp. from Brunei. The result of maximum likelihood analysis and Bayesian inference were congruent. The phylogenetic trees consistently suggested that genus *Phacaspis* in peninsular Thailand and adjacent areas was monophyletic group (Fig. 19 and 20). In addition, all of *Phacaspis ornata* from Singapore was grouped together within basal clade that *P. ornata* should be the primitive group of genus *Phacaspis* in this region. Although the specimens of *P. mitis* were separated into 2 distinct clades; clade A and clade C. *P. mitis* from Brunei, Singapore, Krabi, Phang Nga, Satun, Surat Thani, Nakhon Si Thammarat and Songkhla from peninsular Thailand was shared an common ancestor. According to sea-level changes of Southeast Asia, the Plio-Pleistocene plays an important role to connect between mainland and island in the periods of glaciation (Voris, 2000). Moreover, the changing of sea level has influenced on the distribution of plant community, aquatic and terrestrial organisms in Sundaland (Molengraaff and Weber, 1921; Darlington, 1957; Wallace, 1881; Voris , 2000). Consequently, the expansion of mangroves ecosystem within the peninsular Thailand, Singapore and Brunei have been affected by sea-level changes. Key factor of distribution of *P. mitis* in Sundaland was expansion and forest fragmentation of mangroves ecosystems in the period of Plio-Pleistocene and then the connectivity between patches of mangroves was a major role to occurrence of genetic variation and gene flow in this species. Moreover, the results of phylogenetic tree and haplotype network were coincidence (Fig. 21). In this study, the haplotype network of genus *Phacaspis* from peninsular Thailand, Singapore and Brunei was analyzed based on *COI* gene. The result revealed that there were 2 distinct haplotype networks; A and B in peninsular Thailand, Singapore and Brunei. Haplotype network A, the result indicated that there were 15 haplotype patterns that it was indicated that Satun province might be origin of this network. On the other hand, haplotype network B was composed of Nakhon Si Thammarat and Songkhla provinces.

Interestingly, this haplotype network indicated that the oldest haplotype of pattern B was Songkhla province. Meanwhile, the Brunei haplotype pattern was ungroup with the other haplotype patterns.

CHAPTER 5

CONCLUSIONS

The long-legged flies *Phacaspis mitis* was collected in six coastal provinces along the Gulf of Thailand and the Andaman Sea. There are 3 techniques were used to collected the specimens such as the malaise traps, the hand-collecting and the net sweeping. The hand collection using a plastic bottle is the best technique to collect the small body size of long-legged flies in unique habitat, especially *P. mitis*. Conversely, the malaise traps and the net sweeping are not suitable technique for the small population of *Phacaspis mitis*.

The phylogenetic relationship of this genus in peninsular Thailand was investigated using Cytochrome oxidase subunit I, ribosomal DNA subunit 12S, ribosomal DNA subunit 16S and combined mitochondrial DNA genes. The phylogenetic tree of maximum likelihood analysis and Bayesian inference revealed that all of *P. mitis* in peninsular Thailand was monophyletic group and also were divided into 2 distinct clades. *P. mitis* from Surat Thani, Krabi, Phang Nga and Satun provinces was grouped together in the same clade. On the other hand, *P. mitis* from Nakhon Si Thammarat and Songkhla provinces was assembled in the other clade. Moreover, the haplotype network was analyzed based on *COI* gene. The result indicated that the populations of *P. mitis* in peninsular Thailand were composed of 16 haplotype patterns within two networks. The result of haplotype network was not only congruent with the phylogenetic tree but also showed the oldest of *P. mitis* in peninsular Thailand. Although the first network consists of *P. mitis* from Surat Thani, Krabi, Phang Nga and Satun province, Satun province was also the oldest in this network. Conversely, *P. mitis* from Nakhon Si Thammarat might be older than Songkhla province in the second network. The genetic drift and gene flow have influenced on two major population groups of *P. mitis* in peninsular Thailand. In addition, it was supported by the occurrence of metapopulation in microhabitat.

According to the divergence time, *P. mitis* in peninsular Thailand can be divided into 2 distinct clades in the late Eocene epoch. The effect of sea-level

changes in that period played an important role to formulate the distribution and expansion of mangroves. Moreover, the result also showed that *P. mitis* from Surat Thani, Krabi, Phang Nga and Satun has derived and they were diversely during Plio-Pleistocene. On the other hand, *P. mitis* from Nakhon Si Thammarat and Songkhla was originated during Pleistocene and Holocene. The fluctuation of sea-level in the Plio-Pleistocene and Holocene epochs has severe effects on mangrove expansion and fragmentation. Consequently, there were several diversified the microhabitats of *P. mitis* at that time. In this study, the result proposed that Satun province might be the origin of *P. mitis* in peninsular Thailand. Since the formation of mangroves in Satun was crucial event to regulate relatively by Pleistocene and late Holocene epochs (Umitsu *et al*., 1999).

Furthermore, the long-legged flies genus *Phacaspis* in peninsular Thailand, Brunei and Singapore was constructed the phylogenetic tree based on *COI* gene. The results revealed that *P. ornata* from Singapore were a basal clade. It might be the primitive group of genus *Phacaspis* in these regions. Although the specimens of *P. mitis* from peninsular Thailand, Brunei and Singapore were separated into 2 distinct clades, they were shared a common ancestor. The distribution of *P. mitis* in Sundaland was under the influence of the mangrove expansion and fragmentation during Plio-Pleistocene periods. However, the connectivity between patches of mangroves was a major role to occurrence of genetic variation and gene flow in this species.

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APPENDICES

Appendix 1. Protocol for preparing lysis buffer and TAE buffer

Lysis buffer was made up with the following reagents

- 0.4 M NaCl	4 ml
- 2% SDS	10 ml
- 2 mM EDTA, pH 8.0	0.2 ml
- 0.01 M Tris-HCL, pH 7.5	1 ml
- Deionized water	34.8 ml
Total volume	**50 ml**

TAE buffer was made up with the following reagents

- 10 mM Tris-acetate	1 ml
- 1 mM EDTA	0.1 ml
- Deionized water	48.9 ml
Total volume	**50 ml**

Appendix 2. Nuclotide sequence alignments of the *COI* gene.

***Phacaspis mitis*_Surat Thani_1**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTGCCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTCC
TCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGATCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Surat Thani_2**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTGCCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTCC
TCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTCCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGATCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Surat Thani_3**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTGCCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTCC
TCTATCTGCAGGAATTGCCCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Surat Thani_4**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTGCCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
TCTATCTGCAGGAATTGCCCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATCACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGATCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Nakhon Si Thammarat_1**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGTGATCCTATT

***Phacaspis mitis*_Nakhon Si Thammarat_2**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGGCACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGTGATCCTATT

***Phacaspis mitis*_Songkhla_1**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGGGATCCTATT

***Phacaspis mitis*_Songkhla_2**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGGGATCCTATT

***Phacaspis mitis*_Songkhla_3**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGGGATCCTATT

***Phacaspis mitis*_Songkhla_4**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGGGATCCTATT

***Phacaspis mitis*_Songkhla_5**

GGTACTTCATTAAGAGTTATTATTCGAGCTGAACTTGGACACCCCGGTGCC
CTAATTGGAGATGACCAAATTTACAATGTAATTGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAAC
TGACTAGTTCCACTAATATTAGGGGCCCCTGATATAGCCTTCCCCCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCATTAACTCTTCTTCTTGCTA
GAAGAATAGTAGAAAATGGAGCTGGAACCGGTTGAACAGTTTATCCCCCT
CTTTCAGCAGGAATTGCTCATGGAGGAGCCTCAGTTGACTTAGCGATTTTT
TCTCTTCACTTAGCAGGAATTTCATCAATTCTTGGGGCTGTAAATTTCATT
ACAACAGTAATTAATATACGATCCACTGGAATCACTTTTGATCGTATACCA
TTATTTGTATGATCTGTTGTAATTACAGCAATTCTTCTCTTATTATCCCTAC
CAGTTTTAGCAGGTGCTATTACTATATTATTAACTGACCGAAACCTGAATA
CTTCCTTTTTTGATCCTGCTGGAGGGGGGGATCCTATT

***Phacaspis mitis*_Phang Nga_1**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTTTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACCCTTCTTTTAGC
TAGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTC
CTCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTT
TTTCTCTTCATTTAGCAGGAATCTCCTCAATTTTAGGGGCAGTAAACTTTA
TTACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATGC
CTTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTCCTTTTATCCCTT
CCTGTATTAGCAGGTGCAATCACAATACTTTTAACAGATCGAAACCTTAAT
ACTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Phang Nga_2**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACCCTTCTTTTAGC
TAGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTC
CTCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTT
TTTCTCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTA
TTACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATGC
CTTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTCCTTTTATCCCTT
CCTGTATTAGCAGGTGCAATCACAATACTTTTAACAGATCGAAACCTTAAT
ACTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Krabi_1**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTATATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Krabi_2**

GGAACATCCTTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGCC
CTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCATGCTTTT
ATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAAT
TGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAATA
AATAATATAAGATTCTGAATATTACCACCTTCACTTACCCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
TCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCTCTTCATTTAGCGGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATGCC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTCCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATCACAATACTTTTAACAGATCGAAACCTTAAT
ACTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Krabi_3**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Krabi_4**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATCTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Satun_1**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGAGCAGGAACTGGATGAACTGTGTATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Satun_2**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTGTATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Satun_3**

GGGACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

***Phacaspis mitis*_Satun_4**

GGAACATCCCTAAGTATTATTATTCGTGCAGAACTTGGTCATCCAGGAGC
CCTTATTGGTGATGATCAAATTTATAATGTAATTGTAACAGCCCACGCTTT
TATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAA
TTGATTAGTACCATTAATATTAGGAGCCCCTGATATAGCCTTTCCTCGAAT
AAATAATATAAGATTCTGAATATTACCACCTTCACTTACTCTTCTTTTAGCT
AGAAGAATAGTTGAAAATGGGGCAGGAACTGGATGAACTGTATATCCTCC
CCTATCTGCAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCAATTTT
TTCCCTTCATTTAGCAGGAATCTCCTCAATTTTAGGAGCAGTAAACTTTAT
TACTACTGTAATTAATATACGATCTACAGGAATTACATTTGATCGAATACC
TTTATTTGTTTGATCAGTTGTTATTACTGCTATTCTTCTTCTTTTATCCCTTC
CTGTATTAGCAGGTGCAATTACAATACTTTTAACAGATCGAAACCTTAATA
CTTCATTCTTTGACCCTGCAGGAGGAGGTGATCCTATT

Nanothinophilus hoplites

GGTACATCTCTGAGAATTATCGTACGAGCTGAACTTGGACATCCTGGTGCT
TTAATTGGTGACGATCAAATTTACAATGTTGTAGTAACAGCTCATGCTTTT
ATTATAATTTTCTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATT
GACTTGTTCCACTGATATTAGGAGCCCCTGATATAGCATTTCCACGAATAA
ATAATATAAGTTTCTGACTACTTCCCCCCTCACTAACTCTTTTACTAGCTA
GTAGAATGGTAGAAAATGGGGCTGGTACAGGATGAACTGTTTATCCTCCT
CTATCTAGAGGAATTGCTCATGGAGGAGCATCTGTTGATTTAGCTATTTTC
TCTCTTCATTTAGCTGGTGTATCTTCAATTTTAGGAGCTGTAAATTTCATTA
CAACAGTAATTAATATACGATCTACAGGTATTACATTTGACCGAATACCTT
TATTTGTTTGATCTGTAGTAATTACAGCAATTCTTTTGTTACTATCTCTACC
CGTTTTAGCAGGAGCTATTACTATATTACTTACTGATCGTAACCTAAATAC
ATCTTTCTTTGATCCAGCTGGAGGAGGAGACCCAATT

***Thinophilus* sp.**

GGAACCTCCCTGAGTATTATTGTCCGGGCTGAACTTGGTCACCCCGGAGC
CCTTATTGGAGATGATCAAATTTATAATGTTGTAGTAACAGCACATGCATT
TGTTATAATTTTTTTTATAGTAATACCCATTATAATTGGTGGATTTGGGAAT
TGACTAGTACCCCTAATACTAGGAGCCCCCGATATAGCCTTCCCACGAAT
AAATAATATAAGTTTTTGATTACTTCCCCCATCATTAACCCTTCTACTTGCC
AGTAGAATAGTAGAAAACGGAGCAGGTACAGGCTGAACAGTTTACCCCC
CGCTTTCTTCAGGAATTGCTCACGGAGGTGCTTCAGTAGATCTAGCAATTT
TCTCTCTTCATTTAGCAGGAGTATCTTCAATTTTAGGAGCAGTAAATTTTA
TTACCACAGTAATCAATATACGATCCACAGGAATTACATTTGACCGAATA
CCATTATTTGTGTGATCAGTTGTAATTACTGCTATTCTGTTGCTTCTCTCCT
TACCAGTTTTAGCTGGAGCTATTACAATACTCCTAACAGACCGAAACTTA
AATACTTCATTCTTCGACCCCGCTGGAGGTGGAGATCCTATT

Appendix 3. Nuclotide sequence alignments of the 12S rDNA gene.

***Phacaspis mitis*_Surat Thani_1**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Surat Thani_2**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAAACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Surat Thani_3**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Surat Thani_4**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTAAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Nakhon Si Thammarat_1**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Nakhon Si Thammarat_2**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Songkhla_1**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Songkhla_2**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Songkhla_3**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Songkhla_4**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Songkhla_5**

ATTTCATGTAAAAATCCATAT--
AAATAAATTTATTGTAACCCATGTAAACCTTAACTATAAGCTGCACCTTGA
CTTGATTTATTTTTTA--
ATAAAAACTTCTGAAAATTATTGTCCTTTTAAAATTTTCAAATAACAGCGA
TATACAAACTGAAAAA-
CAAAATTAAGTAAGATCCATCGTGGATTATCAATTACATCACAGGTTCCTC
TAAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Phang Nga_1**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTTT-
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Phang Nga_2**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA

CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Krabi_1**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTTTTATAAAAATTTTTGAAAATTATTATTCTTATAAAA
TTTTCAAATAACAGCGGTATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Krabi_2**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTTT-
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Krabi_3**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Krabi_4**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Satun_1**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Satun_2**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTAAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Satun_3**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA

CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

***Phacaspis mitis*_Satun_4**

ATTTAAATTTAAAATCCGCAT--
AAATAAATTTATTGTAACCCATATATTACTTAATCATAAGCTGCACCTTGA
CTTGATTTATTTTTTT--
ATAAAAATTTTTGAAAATTATTATTCTTATAAAATTTTCAAATAACAGCGG
TATACAAACTGTTTA--
CAAGACTAAGTGAGGTTCATCGTGGATTATCAATTACATTACAGGTTCCTC
TGAATAGACTAAAATACCGCCAAATTTTTTGAGTTTCAA

Nanothinophilus hoplites

ATTTCAAACTTAATTCCGTTTTTAAATAATTTTATTGTAACCCATATTATAC
TTAAACATAAGCTGCACCTTGACCTGATTTACTTTTTT--
ATTAAAAATTATGAAAATTATTTTTCTCCTAAAATTCTCAAATAACGGCGA
TATACAAACTGAATAAACAAATTTAAGTAAGGTCCATCGTGGATTATCAA
TTACATTACAGGTTCCTCTGAATAGTCTAAAATACCGCCAAATTTTTTGAG
TTTCAA

***Thinophilus* sp.**

ATTTCAAACATCAATTCGTTT--
AAATACATTTATTGTAGCCCATGTAATTCTTAAATATAAGCTGCACCTTGA
CCTGATTTACTTTTTT--
ATAAAAAATTTTGAAAATTATTTTCCTCTAAAAATCTTCTAATAACGGCGG
TATACAAACTGAA-
AGACAAACTTAAGTAAGGTCCAACGTGGATTATCAATTACATCACAGGCT
CCTCTGAATAGTCTAAATTACCGCCAAATTTTTTGAGTTTCAA

Appendix 4. Nuclotide sequence alignments of the 16S rDNA gene.

***Phacaspis mitis*_Surat Thani_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTAAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATGTCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Surat Thani_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Surat Thani_3**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
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TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Surat Thani_4**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Nakhon Si Thammarat_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Nakhon Si Thammarat_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA

AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
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TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Songkhla_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Songkhla_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Songkhla_3**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG

GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Songkhla_4**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Songkhla_5**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATTTGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAACTAATTCATTAATTTATGTTTAGTA-
TAATTGAAAGTTCATTAAATTTCAATATCACCCCAATAAAATAATTTATAA
AATAAATTCAAAAATAATCTTTATAATAA-ATAATTTATAAATT---
ATTAAGATCTATAGGGTCTTCTCGTCTTTCTATAATATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATAATTTTTAATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Phang Nga_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTTTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Phang Nga_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
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GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTTTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Krabi_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
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TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA

TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Krabi_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTTTAAAATAATCCTTATAATAA-ATACTAATTAAATT---
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TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Krabi_3**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
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TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Krabi_4**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---

ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Satun_1**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCGTTAATAACG
GATCAAATTCATCATTAATTTATGTAAATAA-
TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Satun_2**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
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TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Satun_3**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
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GATCAAATTCATCATTAATCTATGTAAATAA-

TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTGAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

***Phacaspis mitis*_Satun_4**

TAATCCAACATCGAGGTCGCAACCTATTTTATCGATATGAACTCTCCAAAA
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TAATTGAAAGTTAATTAAATTTCAATATCACCCCAATAAAATAATTTAATT
AATAAATTCTAAAATAATCCTTATAATAA-ATATTAATTAAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTTAAAAAATTTTAGCTTTTTAAC
TAAAAAATAAAATTCTATTAATTTTTATGAGACAGCTTATATCTCGTCCAA
TCATTCATACAAGCCTTCAATTAAAAGACTAATTATTATGCTACCTTTGCA
CAGTTAAAATACTGCGGCCATTTA

Nanothinophilus hoplites

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TAATTACGCTGTTATCCCTAAGGTAACTTAAATTTTTAATCACTAAAAATG
GATCAA-
TAATTCATTAATATATGTTACAATTAAACTGAAAGTTTATTAAATTTCATT
ATCACCCCAATAAAATA-TAAAATTAATTATAATTAAAATAAATCTAA-
AATAATCTATAACTACAATTTATATAAAGATCTATAGGGTCTTCTCGTCTT
TTAAAATAATTTTAGCTTTTTAACTAAAAAATAAAATTCTAAAATTTAATT
TGAGACAGCTTATATCTCGTCCAATCATTCATACAAGCCTTCAATTAAAAG
ACTAATTATTATGCTACCTTTGCACAGTCAAAATACTGCGGCCCTTTA

***Thinophilus* sp.**

TAATCCAACATCGAGGTCGCAATCTATTTTATCGATAAGAACTCTCCAAA
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GGATCAA-
TAATTCATAAATTAATGTATAAAAATAATTGAAAGTTAAATAAATTTCAAT
ATCACCCCAATACAATAATAAAAATAATATAAATATAAGTCAACCAAATA
ATCTTATATTAATTTAATT---
ATAAAGATCTATAGGGTCTTCTCGTCTTTTAAATAAATTTTAGCTTTTTAAC
TAAAAAATAAAATTCTAAAATTTTTATTGAGACAGTTTATATCTCGTCCAA
TCATTCATACAAGCCCTCAATTAAAAGACTAATGATTATGCTACCTTTGCA
CAGTCAAAATACTGCGGCCCTTCA

Appendix 5. Photos of *Phacaspis* used in this study.

Figure 1. *Phacaspis mitis*

Figure 2. *Phacaspis ornata*

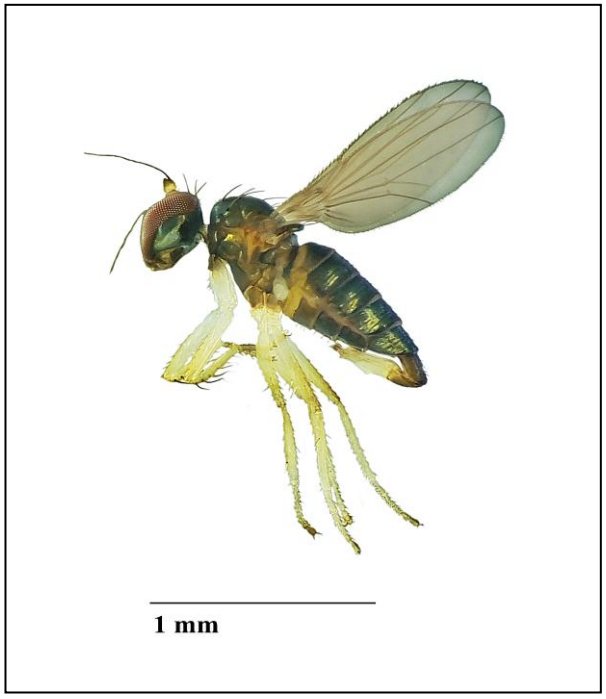

Figure 3. *Nanothinophilus hoplites*

Figure 4. *Thinophilus* sp.

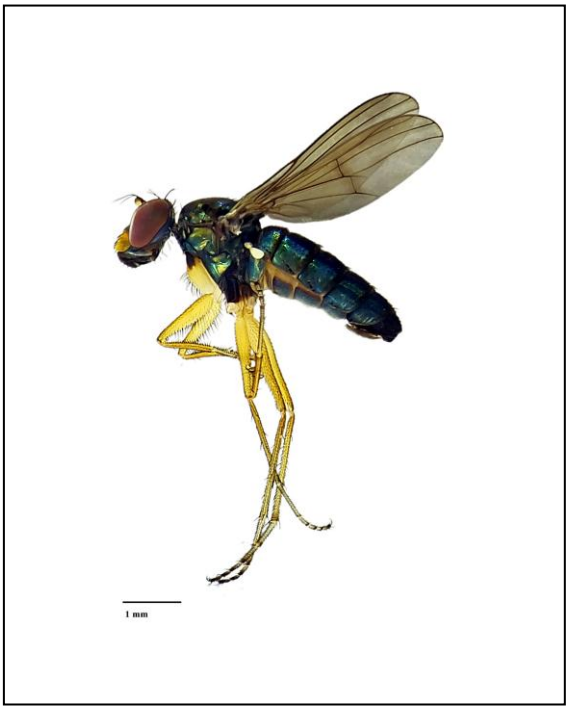

Figure 5. *Thinophilus chaetolosus*

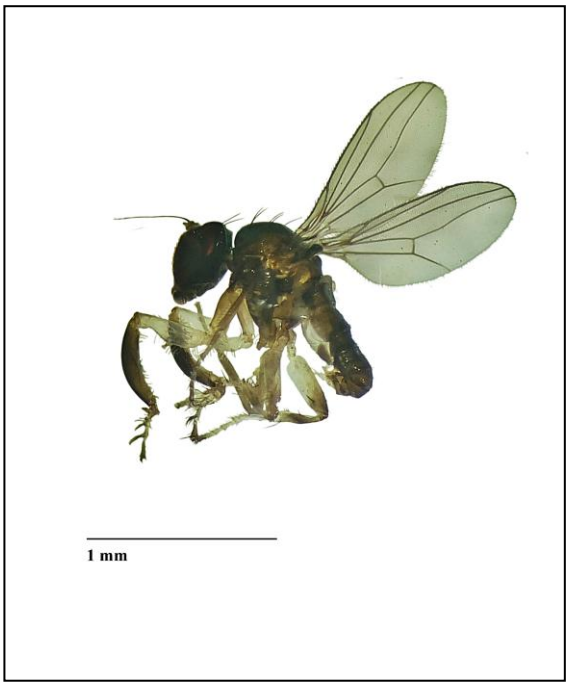

Figure 6. *Ornamenta* sp.

VITAE

Name Miss Natcha Kaewkrajang

Student ID 5610220040

Educational Attainment

Degree	Name of Institution	Year of Graduation
B. Sc. (Biology)	Prince of Songkla University	2013

Scholarship Awards during Enrolment

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- The graduate school of Prince of Songkla University.
- Department of Biology, Faculty of Science, Prince of Songkla University.