Genetic Variation of the Long-Legged Flies *Phacaspis mitis* Complex (Diptera: Dolichopodidae) in Peninsular Thailand Inferred From Three Mitochondrial Genes

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Subject Editor: Juan Rull

Received 1 June 2017; Editorial decision 27 February 2018

Abstract

*Phacaspis* (Meuffels and Grootaert 1988) is a true marine dolichopodid fly genus. They are common on the mud flats in the front of mangroves where they deal with extreme conditions. The genus is represented in southern Thailand by *Phacaspis mitis* (Grootaert and Meuffels 2001) (Diptera: Dolichopodidae). Previous studies have focused on both taxonomy and classification of this genus, but there are a few studies focusing on this species in terms of molecular genetics. The objective of the present study was to investigate genetic variation and phylogenetic relationships of *P. mitis* using ribosomal DNA subunit 12S, ribosomal DNA subunit 16S, and cytochrome oxidase subunit I of mitochondrial genes. The specimens were collected in six coastal provinces from the Andaman Sea and the Gulf of Thailand. The phylogenetic relationship of combined mitochondrial genes revealed that *P. mitis* in peninsular Thailand is a monophyletic group that can be divided into two distinct clades. According to the haplotype network, 16 haplotype patterns were observed in *P. mitis*, but *P. mitis* was separated into two major haplotype networks. In addition, an positive correlation between genetic distance ($F_{st}$) and geographical distance (km) was found among the populations of peninsular Thailand. The level of genetic differentiation between populations is influenced by geographic isolation. Moreover, *P. mitis* arose in late Eocene (35.5 Ma) and it diversified during the Plio-Pleistocene (3.14 Ma). Although, *P. mitis* is divided into two populations in this study, it is a well-supported monophyletic group.

Key words: genetic variation, haplotype network, phylogeny, peninsular Thailand, mitochondrial gene

The Dolichopodidae Latreille is one of the largest family in the order Diptera Linnaeus. They are classified into 230 genera and over 7,100 described species (Yang et al. 2006). Most larvae and adults play an important ecological role in predation (Pollet et al. 2004). Long-legged flies can be found in all terrestrial habitats such as damp soil, riverbanks, and tree trunks. Whereas some species are only found in marine habitats. One of the true marine long-legged fly genera is *Phacaspis* (Meuffels and Grootaert 1988). It is interesting that they are able to live in extreme salty conditions and fully sun exposed areas such as mangroves (Grootaert and Meuffels 2004). There are several publications about this genus but most of them are focused on taxonomy and classification. Meuffels and Grootaert (1988) considered that this genus is incertae sedis because they were not able to classify it in an existing subfamily. In the past decade, Yang et al. (2006) proposed in the world catalog of long-legged flies that *Phacaspis* species should be classified in a new subfamily, Kowmunginae. In the year 2010, Lim et al. studied the phylogenetic relationship using mitochondrial and nuclear markers (Lim et al. 2010). Only one species of *Phacaspis* species from Singapore was used as representative of the Kowmunginae Yang, Zhu, Wang & Zhang to construct the phylogenetic relationship and it is still a controversial classification. Nowadays, three species of *Phacaspis* have been described: *Phacaspis petiolata* Meuffels & Grootaert, *Phacaspis ornata* Meuffels & Grootaert (Meuffels and Grootaert 1988), and *Phacaspis mitis* Grootaert & Meuffels (Grootaert and Meuffels 2001) (Diptera: Dolichopodidae). Interestingly, *P. mitis* is a small-bodied species (about 1.2 mm of body length) and is only found on the mudflats in the front of mangroves (Grootaert and Meuffels 2001). Moreover, this species has a fairly wide distribution in Southeast Asia when compared with others.

Mangroves are an important ecosystem since it is the nesting and breeding site and contains food sources for several species. The distribution of mangroves is located in tropical regions and subsists daily fluctuations in sea level. Mangroves are widespread around the world and can be found in the Americas, Africa, Asia, Australia, and Oceania (Ward et al. 2016). Thailand is situated in Southeast Asia and has a high diversity of plant species and structure of mangroves,
particularly in the peninsular Thailand. It is surrounded by Gulf of Thailand to the east and the Andaman Sea to the west. Plathong et al. (2011) reported that 172,922 ha of mangrove area was found in coastal provinces of peninsular Thailand. However, it is fragmented by human activities: shrimp farming, seaport, and agriculture. The decline of mangrove ecosystems might lead to decrease in overall suitable habitat fragmented into small patches. In addition, the influence of fragmentation has obvious effects on the gene flow, genetic diversity, and genetic variation among many populations of plants and animals (Young et al. 1996, Eckert et al. 2008).

Mitochondrial DNA (mtDNA) has been widely used to study evolutionary history, molecular phylogeny, phylogeography, and genetic variation of insects (Avise 1987, 1994, 2000; Harrison 1989; Simon et al. 1994; Caterino et al. 2000; Simmons and Weller 2001). There are several advantages of mitochondrial genes such as a wide availability of primers for amplification. In addition, the evolutionary rate of mitochondrial genes is higher than nuclear genes (DeSalle et al. 1987, Moriyama and Powell 1997, Monteiro and Pierce 2001, Lin and Danforth 2004). According to previous studies, there is paucity of information on this molecular approach and evolutionary genetics studies for Phacaspis.

In this study, cytochrome oxidase subunit I, 12S rDNA and 16S rDNA mitochondrial DNA markers were used to investigate genetic divergence and phylogeny of P. mitis in coastal provinces along peninsular Thailand. The phylogenetic tree was carried out by Bayesian inference methods. Genetic variation was compared among P. mitis found in different study sites. The correlation between genetic distance and geographic distance was calculated. Haplotype networks and molecular clocks were analyzed by divergence time.

Materials and Methods

Specimen Collections

Specimens were collected from mangroves in six coastal provinces along peninsular Thailand (Suratthani, Nakhon Si Thammarat, Songkhla, Satun, Phang Nga, and Krabi) (Fig. 1; Table 1). In total, 21 specimens were sampled using three methods as follows: Malaise traps, hand-collecting with plastic bottles, and net sweeping. All fresh specimens were preserved in 95% ethanol and stored at −4°C until molecular processing.

DNA Extraction

Male specimens were subjected to DNA extraction following methods in (Bebee et al. 2005) with modifications. For each specimen, the whole body was placed in a 1.5 ml micro-centrifuge tube and added with 100 µl of lysis buffer (0.08 M NaCl/0.06 M EDTA, pH 8/0.5% SDS/0.01 M Tris–HCl, pH 8.6/0.16 M sucrose). The body was grinded with a micro pestle and 2 µl of proteinase K was added before incubating at 60°C for 24 hr. Next, the specimen was placed in 7 µl of 8 M potassium acetate and stored at −20°C until molecular processing.

DNA Amplification and Sequencing

Polymerase chain reaction was conducted using three mitochondrial genes. The amplification and sequencing primers of the cytochrome oxidase subunit I (mtCOI), 12S rDNA, and 16S rDNA are listed in Table 2. The amplifications were performed in 50 µl reactions containing 2.5 µl of DreamTaq Green PCR Master Mix, 1 µl of each primer, 18 µl of water or nuclease-free, and 5 µl of DNA template. Thermocycling conditions of mtCOI were as follows: an initial denaturation step at 95°C for 3 min, followed by 40 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min 30 s, and a final extension at 72°C for 5 min. The PCR conditions of 12S rDNA and 16S rDNA were as follows: an initial denaturation step at 95°C for 4 min, followed by 35 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were verified on 1.5% agarose–TAE gels before sequencing at First BASE Laboratories in Malaysia.

Genetic Analyses

All sequences were aligned and edited using BioEdit ver.7.2 software (Hall 1999). Uncorrected pairwise distance was calculated using MEGA6 (Tamura et al. 2013). Number of polymorphic sites, number of haplotypes (h), nucleotide diversity (Pi), and haplotype diversity (Hd) were analyzed using DNAsp software ver. 5.10.01 (Librado and Rozas 2009). The correlation between genetic distance (Dst) and geographic distance (km) was carried out with a Mantel test using R software ver. 3.3.2. A statistical parsimony haplotype network was conducted with COI sequences using TCS1.21 software (Clement et al. 2000). In addition, the combined sequence (COI, 12S and 16S) was constructed for the phylogenetic tree using the Bayesian inference method. Nanothisphilus hoplitae and Thinophilus sp.
were used as outgroups, N. bopites and Thinophilus sp. are the true marine dolichopodid flies closely related to the genus Phacaspis Meuffels & Grootaert in terms of phylogeny (Lim et al. 2010). The phylogenetic tree was inferred by Bayesian inference using MrBayes version 3.2.6 (Ronquist et al. 2012). The analysis was carried out with Markov Chain Monte Carlo simulations of $3 \times 10^6$ generations and sampling tree every 100 generations. The rate of variation among sites was determined using gamma models, or gamma + invariant sites models (Swofford et al. 1996). The standard deviation of split frequencies was 0.05 or 0.01, the first 25% of generation was discarded as burn-in. Bayesian phylogram was visualized in Figtree

**Results**

**Genetic Variation Analyses**

Sequences from three mitochondrial DNA genes with the final length of 600 bp for COI, 250 bp for 12S rDNA and 410 bp for 16S rDNA were included for data analyses. The genetic diversity indexes for two populations of *P. mitis* were calculated for COI gene. The COI gene is a good gene marker to discriminate among closely related species and investigate the evolutionary history and population genetics of organisms (Hebert et al. 2003). Population A (lineage A) consisted of *P. mitis* from Suratthani, Phangnga, Krabi, and Satun provinces, whereas population B (lineage B) was composed of *P. mitis* from Songkhla and Nakhon Si Thammarat provinces. The values of invariable (monomorphic) sites, parsimony informative sites, number of polymorphic sites ($S$), number of haplotypes ($h$), haplotype diversity ($H_d$), and nucleotide diversity ($P_i$) were 375, 28, 30, 7, 0.762, 0.03185. The results of 16S rDNA were 215, 29, 29, 4, 0.633, 0.05578, respectively, whereas for 16S rDNA they were 375, 28, 30, 7, 0.762, 0.03185. The results of Tajima's $D$-test revealed that population A was not significantly different ($D = -0.52081$; ns) and also population B was not significantly different ($D = -0.27492$; ns) (Table 3). In addition, the results of invariable (monomorphic) sites, parsimony informative sites, number of polymorphic sites ($S$), number of haplotypes ($h$), haplotype diversity ($H_d$), and nucleotide diversity ($P_i$) were 381, 20, 13, 0.989, 0.00918, respectively, in population A. However, the parameters of population B were 398, 1, 2, 3, 0.324, and 0.00127, respectively. Tajima's $D$-test revealed that population A was not significantly different ($D = 1.93172$; ns) (Table 4). However, 16S rDNA was not significantly different ($D = 2.66331$; $P < 0.01$). However, 16S rDNA was not significantly different ($D = 1.93172$; ns) (Table 4).

**Phylogenetic Analyses**

The Bayesian inference of phylogeny was performed using combined genes of mitochondrial DNA. The phylogenetic relationship of

### Table 1. Locality coordinates and accession number of GenBank in each individual at different provinces

| Provinces       | Locality          | Coordinates       | Animal number | COI    | 12S rDNA   | 16S rDNA   |
|-----------------|-------------------|-------------------|---------------|--------|------------|------------|
| Suratthani      | Chaiya            | 9°22′33.6″N, 99°16′00.3″E | S.1           | MF944146 | MF928536   | MF928557   |
|                 |                   |                   | S.2           | MF944147 | MF928537   | MF928558   |
|                 |                   |                   | S.3           | MF944148 | MF928538   | MF928559   |
|                 |                   |                   | S.4           | MF944149 | MF928539   | MF928560   |
| Nakhon Si-Thammarat | Pak-Phanang     | 8°24′09.4″N, 100°11′29.9″E | NK.1          | MF944150 | MF928540   | MF928561   |
|                 |                   |                   | NK.2          | MF944151 | MF928541   | MF928562   |
| Songkhla        | Chana             | 7°01′20.1″N, 100°42′59.4″E | SK.1          | MF944132 | MF928542   | MF928563   |
|                 |                   |                   | SK.2          | MF944133 | MF928543   | MF928564   |
|                 |                   |                   | SK.3          | MF944134 | MF928544   | MF928565   |
|                 |                   |                   | SK.4          | MF944135 | MF928545   | MF928566   |
|                 |                   |                   | SK.5          | MF944156 | MF928546   | MF928567   |
| Phang Nga       | Takua Pa          | 8°55′46.5″N, 98°23′22.0″E | PG.1          | MF944157 | MF928547   | MF928568   |
|                 |                   |                   | PG.2          | MF944158 | MF928548   | MF928569   |
| Krabi           | Mueang Krabi      | 8°03′23.5″N, 98°53′38.2″E | KB.1          | MF944159 | MF928549   | MF928570   |
|                 |                   |                   | KB.2          | MF944160 | MF928550   | MF928571   |
|                 |                   |                   | KB.3          | MF944161 | MF928551   | MF928572   |
|                 |                   |                   | KB.4          | MF944162 | MF928552   | MF928573   |
| Satun           | La-ngu            | 6°47′29.8″N, 99°48′53.5″E | ST.1          | MF944163 | MF928553   | MF928574   |
|                 |                   |                   | ST.2          | MF944164 | MF928554   | MF928575   |
|                 |                   |                   | ST.3          | MF944165 | MF928555   | MF928576   |
|                 |                   |                   | ST.4          | MF944166 | MF928556   | MF928577   |

### Table 2. Summary of oligonucleotide primers used in this study

| Primer names | Strand | Sequences                     | Sizes of regions (bp) | References      |
|--------------|--------|-------------------------------|-----------------------|-----------------|
| LCO1490      | Forward| 5′-GGTCAACAAATCATAAAGATTTGG-3′| 710                   | Folmer et al. (1994) |
| HCO2198      | Reverse| 5′-TAACTTCAGGTTGACAAAAAATCA-3′| 355                   | Germann et al. (2011) |
| SR-J-14233   | Major  | 5′-AAGGAGGCAGCGCCATGTTG-3′    |                       |                 |
| SR-N-14588   | Minor  | 5′-AAACTAGGATTAGATACCCCTAT-3′| 511                   | Germann et al. (2011) |
| LR-J-12887   | Major  | 5′-CGGTTTTGAACAGCAGTATCGT-3′  |                       |                 |
| LR-N-13398   | Minor  | 5′-CGCCTGTTTTACAAAAACAT-3′    |                       |                 |
P. mitis in peninsular Thailand was a monophyletic group and it was divided into two distinct clades (Fig. 2). Lineage A consisted of populations from Krabi, Satun, Suratthani, as well as Phang Nga provinces. Three subclades such as A1, A2, and A3 were found in lineage A. The populations from Krabi and Satun were grouped together in subclade A1. Subclade A2 was composed of populations from Krabi and Phang Nga provinces and populations from Suratthani were only found in subclade A3. Lineage B contained 2 subclades: B1 and B2. Subclade B1 was composed of the population from Songkhla and subclade B2 was the population from Nakhon Si Thammarat. Estimating divergence times of P. mitis in peninsular Thailand based on the aligned sequences of COI. Neighbor-joining tree showed that P. mitis was divided into two distinct clades; lineage A and lineage B about 35.5 Mya in late Eocene (Fig. 3). The divergence time within lineage A was approximately 3.14 Mya during Pliocene and lineage B diverged about 0.51 Mya in the Pleistocene. P. mitis from Krabi was separated from Phang Nga about 1.58 Mya in the Pleistocene (subclade A3). P. mitis from Suratthani (subclade A2) diverged approximately 1.59 Mya in the Pleistocene. P. mitis from Satun and Krabi (subclade A1) were subsequently separated about 0.60–0.24 Mya in the Pleistocene as well. On the other hand, P. mitis from Songkhla originated from Nakhon Si Thammarat in the Holocene.

A statistical parsimony haplotype network of P. mitis in peninsular Thailand revealed 16 patterns divided into two networks (Fig. 4). Haplotype network A was composed of 13 distinct haplotype patterns. The origin of this network was Satun (ST.4) and it derived to the Krabi haplotype pattern (KB.1 and KB.4). Subsequently, Krabi (KB.1) divided into two sub-patterns. The first sub-pattern consisted of Phang Nga (PG.1 and PG.2) and Krabi (KB.2). The second sub-pattern was only found in Suratthani (S.1, S.2, S.3, and S.4). On the other hand, haplotype network B arose into three distinct haplotype patterns. Nakhon Si Thammarat (NK.1) was the origin of this network and then became the Songkhla pattern (SK.1). The pairwise genetic distance (FST) of P. mitis in peninsular Thailand was analyzed to investigate the genetic variation using cytochrome oxidase subunit I gene. The lowest FST index was 0.000 between Krabi and Satun provinces. Conversely, the highest FST index was 0.152 and was found between Krabi and Songkhla provinces (Table 5). Mantel test showed a significant correlation between genetic distance (FST) and geographical distance among populations (r = 0.3799, P < 0.01) (Fig. 5). The scatterplot of FST and geographical distance can be explained by the genetic drift and gene flow.

### Table 3. Variability indices of genetic variation between two populations estimates in cytochrome oxidase subunit I gene

|                | Lineage A | Lineage B |
|----------------|-----------|-----------|
| Total base pair| 600       | 600       |
| Invariable (monomorphic) sites | 580       | 598       |
| Parsimony informative sites | 11        | 1         |
| Number of polymorphic sites, S | 20        | 2         |
| Number of haplotypes, h | 13        | 3         |
| Haplotype (gene) diversity, Hd | 0.989     | 0.524     |
| Nucleotide diversity, Pi | 0.00918   | 0.00127   |
| Tajima’s test, D | −0.52081ns | −0.27492ns |

ns (not significant).

### Table 4. Variability indices of genetic variation estimates in 12S rDNA and 16S rDNA genes

|                | 12S rDNA | 16S rDNA |
|----------------|----------|----------|
| Total base pair| 250      | 410      |
| Invariable (monomorphic) sites | 215       | 375       |
| Parsimony informative sites | 29       | 28       |
| Number of polymorphic sites, S | 29        | 30       |
| Number of haplotypes, h | 4        | 7        |
| Haplotype (gene) diversity, Hd | 0.633     | 0.762     |
| Nucleotide diversity, Pi | 0.05578   | 0.03185   |
| Tajima’s test, D | 2.66331** | 1.93172 ns|

Asterisk indicates significant differences, **P < 0.01, ns = not significant.
Discussion

This is the first analysis of genetic variation and phylogeny of the *P. mitis* complex in two coastal regions along peninsular Thailand. The phylogenetic relationship was constructed by Bayesian inference based on the combined genes 12S rDNA, 16S rDNA, and cytochrome oxidase subunit I and revealed that *P. mitis* are a monophyletic group divided into two distinct clades: Andaman clade and Gulf of Thailand clade. Similarly, according to the Mantel tests, *P. mitis* in peninsular Thailand is composed of two populations. Interestingly, the populations of *P. mitis* from Andaman Sea are similar in terms of external morphology to the population from the Gulf of Thailand according to morphological traits such as male genitalia, wing venation, etc. However, there is a high degree of genetic variability between the two populations. This supports the hypothesis that the *P. mitis* in peninsular Thailand is a species complex. Genetic differentiation of the two populations may be the result of different selective forces or a series of range expansions and contractions resulting in a series of interruptions of gene flow and secondary contact. Perhaps, historical variation in sea level (Bosio et al. 2005). Interestingly, *P. mitis* plays an important role as a predator in mangrove ecosystems and it is a true species. They occupy a unique microhabitat with sunlight exposure on the mudflats in front of mangroves and high-salinity (P.G., personal observations). Microhabitats such as those occupied by *P. mitis* can result in the evolution of metapopulations. Metapopulations are a set of local populations which occupy suitable habitats on a patch and each suitable patch is separated by unsuitable terrain (Levins 1969, Yuttham et al. 2003). Viability and size of populations are 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 metapopulations is affected by dispersal and extinction processes between local habitats in such landscapes (Hanski 1997, 1999). Hanski and Ovaskainen (2000) suggested that the connectivity of habitats within a patch network could be explained by metapopulation capacity.

Haplotype networks illustrate genetic relationships among individuals at each sampling site and have also been used for investigation of the phylogeographic and evolutionary history of organisms (Clement et al. 2000, Leigh et al. 2015). Gorostiza et al. (2012) proposed that the oldest haplotype is probably the original among populations. In this study, the haplotype pattern of Satun province

![Fig. 3. Divergence time of *P. mitis* based on COI gene obtained by Neighbor-joining analysis.](image)

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might be assumed to be the original haplotype pattern in peninsular Thailand and from which haplotype pattern A, including Krabi, Phang Nga, and Suratthani provinces derived. The mangroves of the coastal provinces along the Andaman region compose the most extensive area in the peninsula. The forest structure and geomorphic character of mangroves in this region are homogenous. The prevailing type of mangrove is estuary and deep mudflat (Lugo and Snedaker 1974, Twilley et al. 1998, Plathong and Plathong 2011).

The tidal characteristic of Andaman coastline is a semi-diurnal cycle and also the tidal amplitude ranges from 3 to 4 m. Consequently, space and resources of mangroves are open to recruit aquatic invertebrates for resource partitioning (Macintosh et al. 1991, Plathong and Plathong 2011). In this ecosystem, *P. mitis* occupies the predator niche, where numerous large populations are found thriving on abundant resources and suitable habitats. In addition, the coastal provinces of the Andaman region are large patches of connected mangrove areas (Eiamsa-ard and Amornchairojkul 1997). Therefore, genetic connectivity within the population is facilitated by the distribution of individuals across structured habitats via corridors. Eventually, the populations of *P. mitis* in Suratthani, Phang Nga, Krabi, and Satun provinces were grouped together in lineage A.

On the other hand, lineage B consists of *P. mitis* from Nakhon Si Thammarat and Songkhla provinces. This haplotype network

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**Fig. 4.** Haplotype networks of *P. mitis* in peninsular Thailand.

**Table 5.** Matrix of genetic distance (*FST*) among *P. mitis* in Suratthani (S); Satun (ST); Phang Nga (PG); Krabi (KB); Songkhla (SK), and Nakhon Si Thammarat (NK) provinces using cytochrome oxidase subunit I gene.

|     | S.1 | S.2 | S.3 | S.4 | ST.1 | ST.2 | ST.3 | ST.4 | PG.1 | PG.2 | KB.1 | KB.2 | KB.3 | KB.4 | SK.1 | SK.2 | SK.3 | SK.4 | SK.5 | NK.1 | NK.2 |
|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| S.1 | 0.002 |     |     |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| S.2 | 0.003 | 0.005 |     |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| S.3 | 0.005 | 0.007 | 0.005 |     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| S.4 | 0.008 | 0.010 | 0.008 | 0.010 | 0.002 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| ST.1 | 0.007 | 0.008 | 0.007 | 0.012 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| ST.2 | 0.008 | 0.010 | 0.008 | 0.010 | 0.002 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| ST.3 | 0.008 | 0.010 | 0.008 | 0.010 | 0.005 | 0.003 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
| ST.4 | 0.007 | 0.008 | 0.007 | 0.008 | 0.003 | 0.002 | 0.002 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
| PG.1 | 0.015 | 0.017 | 0.015 | 0.020 | 0.015 | 0.017 | 0.017 | 0.015 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| PG.2 | 0.012 | 0.013 | 0.012 | 0.017 | 0.012 | 0.013 | 0.013 | 0.012 | 0.003 |     |     |     |     |     |     |     |     |     |     |     |      |
| KB.1 | 0.005 | 0.007 | 0.005 | 0.010 | 0.002 | 0.003 | 0.003 | 0.002 | 0.013 | 0.010 |      |      |      |      |      |      |      |      |      |      |      |
| KB.2 | 0.018 | 0.020 | 0.018 | 0.020 | 0.018 | 0.017 | 0.017 | 0.015 | 0.010 | 0.007 | 0.017 |      |      |      |      |      |      |      |      |      |      |
| KB.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 |      |      |      |      |      |      |      |      |      |
| KB.4 | 0.008 | 0.010 | 0.008 | 0.010 | 0.005 | 0.003 | 0.003 | 0.002 | 0.017 | 0.013 | 0.003 | 0.017 | 0.002 |      |      |      |      |      |      |      |      |
| SK.1 | 0.145 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.150 | 0.148 | 0.148 | 0.150 | 0.152 | 0.000 |      |      |      |      |      |      |      |      |      |      |
| SK.2 | 0.145 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.150 | 0.148 | 0.148 | 0.150 | 0.152 | 0.000 | 0.000 |      |      |      |      |      |      |      |      |      |
| SK.3 | 0.145 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.150 | 0.148 | 0.148 | 0.150 | 0.152 | 0.000 | 0.000 | 0.000 |      |      |      |      |      |      |      |      |
| SK.4 | 0.145 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.150 | 0.148 | 0.148 | 0.150 | 0.152 | 0.000 | 0.000 | 0.000 | 0.000 |      |      |      |      |      |      |
| SK.5 | 0.145 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.150 | 0.148 | 0.148 | 0.150 | 0.152 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |      |      |      |      |      |
| NK.1 | 0.143 | 0.145 | 0.147 | 0.147 | 0.148 | 0.148 | 0.148 | 0.148 | 0.148 | 0.147 | 0.147 | 0.148 | 0.150 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 |
| NK.2 | 0.143 | 0.145 | 0.147 | 0.147 | 0.148 | 0.148 | 0.148 | 0.148 | 0.147 | 0.147 | 0.147 | 0.148 | 0.150 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 |

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indicated that the oldest haplotype of pattern B was Nakhon Si Thammarat province. Thampanya et al. (2006) reported that the coastal erosion and forest fragmentation in mangrove ecosystems in the Gulf of Thailand have been influenced by anthropogenic activities. Consequently, genetic differentiation of populations was produced. In this study, the correlation between genetic distance \( (F_{ST}) \) and geographical distance (km) was positive indicating that the level of genetic differentiation among populations increased in relation to geographic distance. This result could be explained by natural barriers to gene flow. The mangroves from Gulf of Thailand region are more fragmented than those of the Andaman region. After patching, dispersal routes for \( P. \) mitis are interrupted by geographic isolation. Hence, gene flow interruption within populations of \( P. \) mitis in Gulf of Thailand coast has been more influential in producing genetic variation than the population of \( P. \) mitis in the Andaman coast. Likewise, Watanabe et al. (2010) concluded that dispersal ability determined the genetic effects of habitat fragmentation on three species of aquatic insects. Their results showed that the effect of distance between fragmented habitats influenced genetic differentiation.

The finding of two distinct groups of \( P. \) mitis from different regions 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. Unfortunately, no fossil record of the genus Phacaspis is available at present. However, Thinophilus contains also many true marine long-legged flies and they are closely related with Phacaspis in terms of phylogeny (Lim et al. 2010). The result showed that \( P. \) mitis was divided into two lineages approximately 35.5 Mya during the late the Eocene. The most significant event of this epoch was sea-level falling due to climate characteristics which tended to be cooler and drier 36.4–33.5 Mya (Hoorn et al. 2012). Consequently, sea-level falling might have affected the distribution and fragmentation of mangroves in peninsular Thailand. Divergence time showed that lineage A derived about 3.14 Mya during the Pliocene while lineage B originated in the Pleistocene (0.51 Mya).

In addition, \( P. \) mitis from Satun, Krabi, Suratthani, and Phang Nga separated the Pleistocene. \( P. \) mitis from Songkhla was recently separated from Nakhon Si Thammarat in the Holocene. The Pleistocene and Holocene epochs are known as glacial periods (ice age) (Berggren 1972, Alley et al.1997). During these epochs, the sea level fluctuated rapidly and was also lower than in present time. Under this scenario, severe effects on mangroves in peninsular Thailand, especially, occurred such as fluctuation of sea level leading to the rapid expansion and fragmentation of mangroves. It could be assumed that there were several suitable habitats in mangroves for occupying. Consequently, \( P. \) mitis could have dispersed at that time.

Moreover, our result suggests that Satun province might be the origin of \( P. \) mitis in peninsular Thailand in accordance with the research of Umitsu et al. (1999) that the main formation of mangroves in Satun province coincides with Pleistocene and late Holocene. Our result suggest that the formation of mangroves during the Pleistocene and late Holocene resulted in the occurrence of the several suitable microhabitats and, hence, \( P. \) mitis in Satun province was first established in peninsular Thailand.

Acknowledgments

We thank Dr. Patamarerk Engsontia, Mr. Abdullah Samoh, Ms. Oratip Waranusit, Ms. Pimpanit Kongruang, and all members in Entomology research unit, Department of Biology, Faculty of Science, PSU for help and support in the field. This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (SC540531M/2013), the graduate school of Prince of Songkla University and the Department of Biology, Faculty of Science, PSU.

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