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

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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, a 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 Mya) and it diversified during the Plio-Pleistocene (3.14 Mya). 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 specimen 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 of *P. mitis* 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 for 30 min. After centrifugation at 13,400 rpm for 15 min, the supernatant were transferred to a new sterile micro-centrifuge tube. One hundred microliters of 95% ethanol were added and centrifuged at 13,400 rpm for 15 min. Subsequently, the supernatant was discarded and the DNA precipitate was washed with 100 µl of 70% ethanol. Following centrifugation at 13,400 rpm for 15 min, waste was discarded. DNA was dried for 2–3 hr. TAE buffer was added (50 µl) to dissolve DNA before storage at –20°C.

DNA Amplification and Sequencing
Polymerase chain reaction was conducted using three mitochondrial genes. The amplification and sequencing primers of the cytochrome
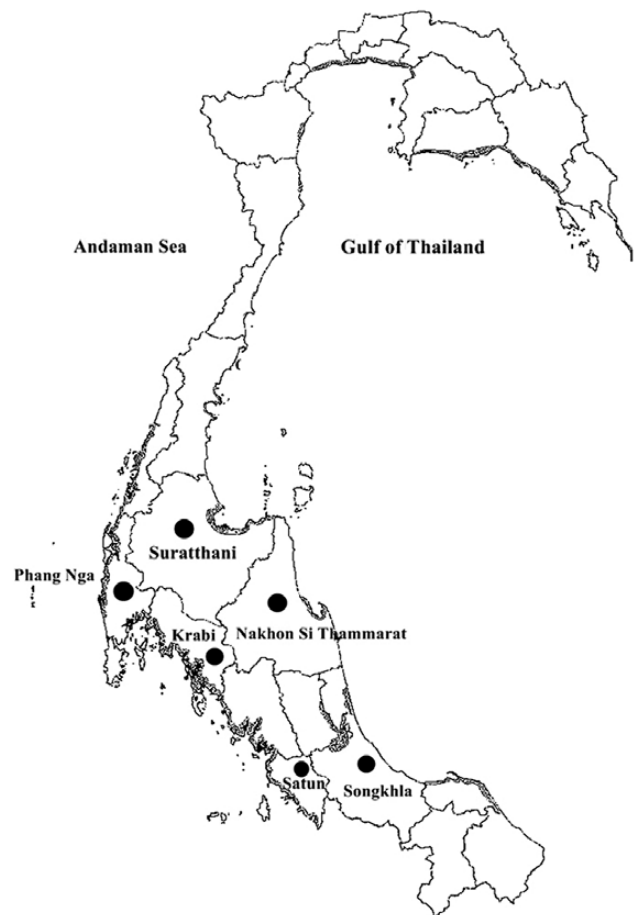

Fig. 1. Map of collection sites in peninsular Thailand (modified from Aksornkoae 2002).

oxidase subunit I (mtCOI), 12S rDNA, and 16S rDNA are listed in Table 2. The amplifications were performed in 50 µl reactions containing 25 µl of Dream*Taq* 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 (F_{ST}) 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. *Nanothinophilus hoplites* and *Thinophilus* sp.

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

Collection sites				GenBank accession number		
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	MF944152	MF928542	MF928563
			SK.2	MF944153	MF928543	MF928564
			SK.3	MF944154	MF928544	MF928565
			SK.4	MF944155	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′-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′		

were used as outgroups. *N. hoplites* and *Thinophilus* sp. are the true marine dolicopodid 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 ver. 3.2.6 (Ronquist et al. 2012). The analysis was carried out with Markov Chain Monte Carlo simulations of 3×10^6 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 ver. 1.4.2.(Rambaut 2014). Moreover, the divergence time of *COI* sequences of *P. mitis* was estimated by fossil records of genus *Thinophilus* Wahlberg about 37.2–33.9 Mya (Million years ago) (Pollet et al. 2004) and phylogram was calculated by uncorrected pairwise distance using Neighbor-joining analysis.

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 (*Pi*) were 580, 11, 20, 13, 0.989, 0.00918, respectively, in population A. However, the parameters of population B were 598, 1, 2, 3, 0.524, and 0.00127, respectively. 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 (*Pi*) for 12S 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 12S rDNA was 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

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

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, H_d	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, H_d	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.

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 Surattani (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 (F_{ST}) of *P. mitis* in peninsular Thailand was analyzed to investigate the genetic variation using cytochrome oxidase subunit I gene. The lowest F_{ST} index was 0.000 between Krabi and Satun provinces. Conversely, the highest F_{ST} index was 0.152 and was found between Krabi and Songkhla provinces (Table 5). Mantel test showed a significant correlation between genetic distance (F_{ST}) and geographical distance among populations ($r = 0.3799$, $P < 0.01$) (Fig. 5). The scatterplot of F_{ST} and geographical distance can be explained by the genetic drift and gene flow.
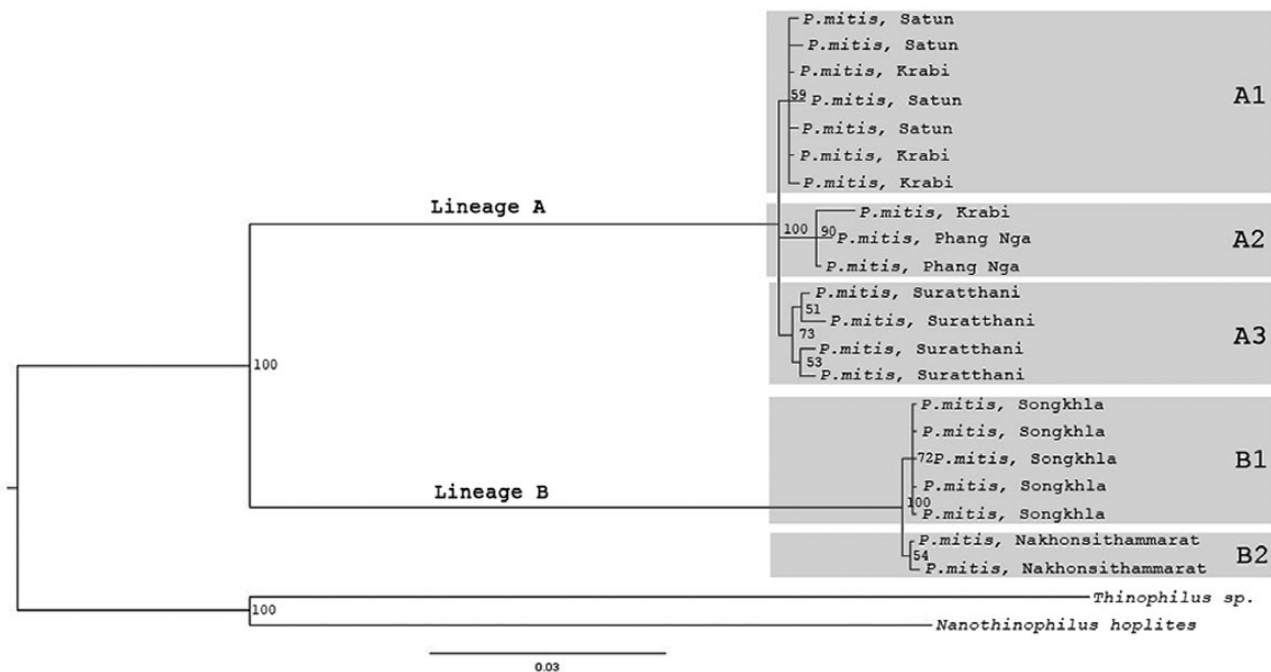

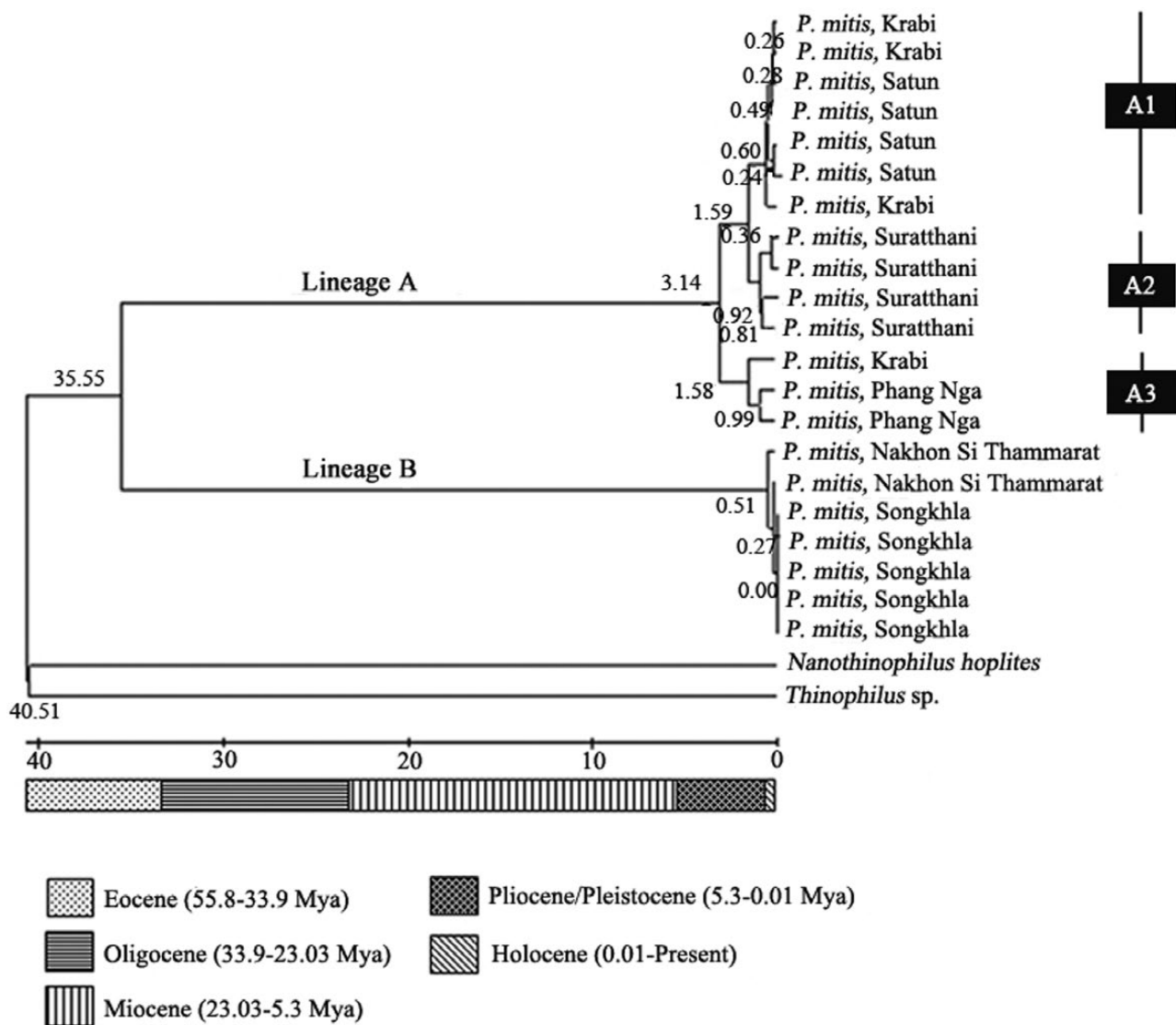**Fig. 2.** Bayesian inference phylogenetic tree based on combined genes. Posterior probability was shown on the branches.

Fig. 3. Divergence time of *P. mitis* based on COI gene obtained by Neighbor-joining analysis.

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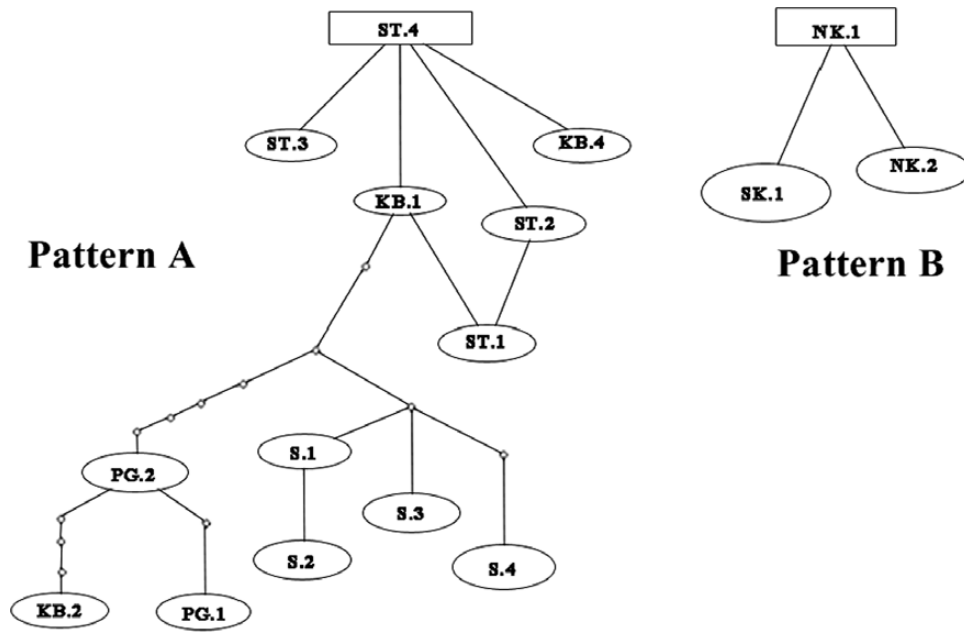
Fig. 4. Haplotype networks of *P. mitis* in peninsular Thailand.

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																					
S.2	0.002																				
S.3	0.003	0.005																			
S.4	0.005	0.007	0.005																		
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	0.148	0.147	0.148	0.150	0.150	0.150	0.148	0.148	0.148	0.148	0.150	0.152							
SK.2	0.145	0.147	0.148	0.147	0.148	0.150	0.150	0.150	0.148	0.148	0.148	0.148	0.150	0.152	0.000						
SK.3	0.145	0.147	0.148	0.147	0.148	0.150	0.150	0.150	0.148	0.148	0.148	0.148	0.150	0.152	0.000	0.000					
SK.4	0.145	0.147	0.148	0.147	0.148	0.150	0.150	0.150	0.148	0.148	0.148	0.148	0.150	0.152	0.000	0.000	0.000				
SK.5	0.145	0.147	0.148	0.147	0.148	0.150	0.150	0.150	0.148	0.148	0.148	0.148	0.150	0.152	0.000	0.000	0.000	0.000			
NK.1	0.143	0.145	0.147	0.145	0.147	0.148	0.148	0.148	0.147	0.147	0.147	0.147	0.148	0.150	0.002	0.002	0.002	0.002	0.002		
NK.2	0.143	0.145	0.147	0.145	0.147	0.148	0.148	0.148	0.147	0.147	0.147	0.147	0.148	0.150	0.003	0.003	0.003	0.003	0.003	0.002	

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
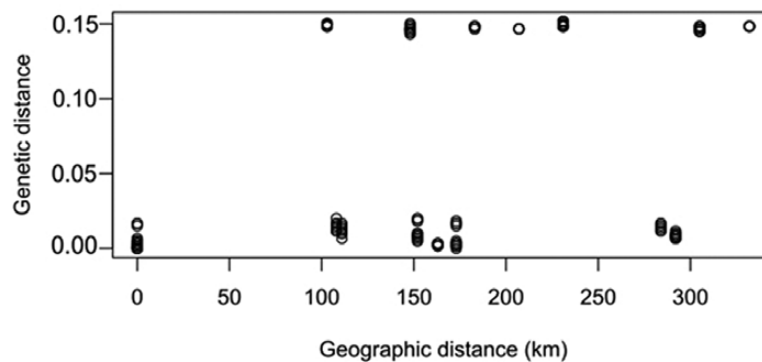
Fig. 5. The pairwise comparison of genetic (F_{ST}) and geographic distance among *P. mitis* in peninsular Thailand inferred from *COI* gene ($r = 0.3799$, $P < 0.01$).

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 Plio-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

References Cited

Alley, R., P. A. Mayewski, T. Sowers, M. Stuiver, K. C. Taylor, and P. U. Clark. 1997. Holocene climate instability: a prominent, widespread event 8200 yr ago. Geology. 25: 483–486.

Avise, J. C. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. 18: 489–522.

Avise, J. C. 1994. Molecular markers, natural history, and evolution. Chapman & Hall, New York.

Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA.

Bascompte, J., H. Possingham, and J. Roughgarden. 2002. Patchy populations in stochastic environments: critical number of patches for persistence. Am. Nat. 159: 128–137.

Bebee, N. W., P. I. Whelan, A. Van Den Hurk, S. A. Ritchie, and R. D. Cooper. 2005. Genetic diversity of the dengue vector *Aedes aegypti* in Australia and implications for future surveillance and mainland incursion monitoring. Commun. Dis. Intell. 29: 299–304.

Berggren, W. A. 1972. Late pliocene–pleistocene glaciation, in initial reports of the deep sea drilling project 12. U.S. Government Printing Office, Washington, DC.

Bosio, C. F., L. C. Harrington, J. W. Jones, R. Sithiprasasna, D. E. Norris, and T. W. Scott. 2005. Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA. Am. J. Trop. Med. Hyg. 72: 434–442.

Caterino, M. S., S. Cho, and F. A. Sperling. 2000. The current state of insect molecular systematics: a thriving Tower of Babel. Annu. Rev. Entomol. 45: 1–54.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9: 1657–1659.

DeSalle, R., T. Freedman, E. M. Prager, and A. C. Wilson. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J. Mol. Evol. 26: 157–164.

Eckert, C. G., K. E. Samis, and S. C. Lougheed. 2008. Genetic variation across species' geographical ranges: the central–marginal hypothesis and beyond. Mol. Ecol. 17: 1170–1188.

Eiamsa-Ard, M., and S. Amornchairojkul. 1997. The marine fisheries of Thailand, with emphasis on the Gulf of Thailand trawl fishery, pp. 85–95. *In* G. T. Silvestre and D. Pauly (eds.), Proceedings, Status and management of tropical coastal fisheries in Asia. International Center for Living Aquatic Resources Management, 2–5 July 1996, Manila, Asian Development Bank. International Center for Living Aquatic Resources Management, Makati.

Etienne, R. S., and J. A. Heesterbeek. 2000. On optimal size and number of reserves for metapopulation persistence. J. Theor. Biol. 203: 33–50.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294–299.

Germann, C., M. Pollet, C. Wimmer, and M. V. Bernasconi. 2011. Molecular data sheds light on the classification of long-legged flies (Diptera: Dolichopodidae). Invertebr. Syst. 25: 303–321.

Gorostiza, A., V. Acunha-Alonzo, L. Regalado-Liu, S. Tirado, J. Granados, D. Sámano, H. Rangel-Villalobos, and A. González-Martín. 2012. Reconstructing the history of Mesoamerican populations through the study of the mitochondrial DNA control region. PLoS One. 7: e44666.

Grootaert, P., and H. J. G. Meuffels. 2001. Note on marine dolichopodid flies from Thailand (Insecta: Diptera: Dolichopodidae). Raffles Bull. Zool. 49: 339–353.

Grootaert, P., and H. J. G. Meuffels. 2004. Insecta: Diptera, Dolichopodidae, pp. 810–817. *In* C. M. Yule and H. S. Yong (eds.), Freshwater invertebrates of the Malaysian region. Academy of Sciences Malaysia and Monash University, Kuala Lumpur, Malaysia.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95–98.

Hanski, I. 1997. Metapopulation dynamics, from concepts and observations to predictive models, pp. 69–91. *In* I. A. Hanski and M. E. Gilpin (eds.), Metapopulation biology: ecology, genetics, and evolution. Academic Press, San Diego, CA.

Hanski, I. 1999. Metapopulation dynamics. Nature. 396: 41–49.

Hanski, I., and O. Ovaskainen. 2000. The metapopulation capacity of a fragmented landscape. Nature. 404: 755–758.

Harrison, R. G. 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends Ecol. Evol. 4: 6–11.

Hebert, P. D., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270: 313–321.

Hoorn, C., J. Straathof, H. A. Abels, Y. Xu, T. Utescher, and G. Dupont-Nivet. 2012. A late Eocene palynological record of climate change and Tibetan Plateau uplift (Xining Basin, China). Palaeogeogr. Palaeoclimatol. Palaeoecol. 344–345: 16–38.

Leigh, J. W., and D. Bryant. 2015. PopART: full-feature software for haplotype network construction. Methods Ecol. Evol. 6: 1110–1116.

Levins, R. 1969. Some demographic and genetic consequences of environmental heterogeneity for biological control. Bull. Entomol. Soc. Am. 15: 237–240.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25: 1451–1452.

Lim, G. S., W. S. Hwang, S. Kutty, R. Meier, and P. Grootaert. 2010. Mitochondrial and nuclear markers support the monophyly of dolichopodidae and suggest a rapid origin of the subfamilies (Diptera). Syst. Entomol. 35: 59–70.

Lin, C. P., and B. N. Danforth. 2004. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. Mol. Phylogenet. Evol. 30: 686–702.

Lugo, A. E., and S. C. Snedaker. 1974. The ecology of mangroves. Annu. Rev. Ecol. Syst. 5: 39–64.

Macintosh, D. J., S. Aksornkoae, M. Vannucci, D. C. Field, B. F. Clough, B. Kjerve, N. Paphavasit, and G. Wattayakorn. 1991. Integrated multidisciplinary survey and research programme of the Ranong mangrove ecosystem. UNDP/UNESCO regional project: research and its application in the management of the mangroves of Asia and the Pacific (RAS/86/120). National Research Council of Thailand, Bangkok.

Meuffels, H. J. G., and P. Grootaert. 1988. Dolichopodidae (Diptera) from Papua New Guinea VIII. *Phacaspis*, a new genus incertae sedis from the mangrove. Indo-Malayan Zool. 5: 311–319.

Monteiro, A., and N. E. Pierce. 2001. Phylogeny of bicyclus (Lepidoptera: Nymphalidae) inferred from COI, COII, and EF-1alpha gene sequences. Mol. Phylogenet. Evol. 18: 264–281.

Moriyama, E. N., and J. R. Powell. 1997. Synonymous substitution rates in *Drosophila*: mitochondrial versus nuclear genes. J. Mol. Evol. 45: 378–391.

Plathong, S., and J. Plathong. 2011. The environmental setting of mangroves in peninsular Thailand. (http://www. sc.psu.ac.th/Units/Cbipt) (accessed 23 February 2017).

Pollet, M., S. E. Brooks, and J. M. Cumming. 2004. Catalog of the Dolichopodidae (Diptera) of America north of Mexico. Bull. Am. Mus. Nat. Hist. 283: 1–114.

Rambaut, A. 2014. FigTree: Tree figure drawing tool, version 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/) (accessed 13 November 2016).

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 539–542.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Simmons, R. B., and S. J. Weller. 2001. Utility and evolution of cytochrome b in insects. Mol. Phylogenet. Evol. 20: 196–210.

Swofford, D. L., G. J. Olsen, P. J. Waddell, and D. M. Hillis. 1996. Phylogenetic inference, pp.407–514. *In* D. M. Hillis, C. Moritz, and B. K. Mable (eds.), Molecular systematics. Sinauer Associates, Sunderland, MA.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis, version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thampanya, U., J. E. Vermaat, S. Sinsakul, and N. Panapitukkul. 2006. Coastal erosion and mangrove progradation of Southern Thailand. Estuar. Coast. Shelf Sci. 68: 75–85.

Twilley, R. R., V. H. Rivera-Monroy, R. Chen, and L. Botero. 1998. Adapting an ecological mangrove model to simulate trajectories in restoration ecology. Mar. Poll. Bull. 37: 404–419.

Umitsu, M., P. Pramojanee, A. Ohira, and K. Kawase. 1999. Late Holocene mangrove habitat and evolution of coastal lowlands in Southern Thailand. Tropics. 8: 317–328.

Ward, R. D., D. A. Friess, R. H. Day, and R. A. MacKenzie. 2016. Impacts of climate change on mangrove ecosystems: a region by region overview. Ecosyst. Health Sustain. 2: 1–25.

Watanabe, K., M. T. Monaghan, Y. Takemon, and T. Omura. 2010. Dispersal ability determines the genetic effects of habitat fragmentation in three species of aquatic insect. Aquatic Conserv Mar. Freshw. Ecosyst. 20: 574–579.

Yang, D., Y. Zhu, M. Wang, and L. Zhang. 2006. World catalog of Dolichopodidae (Insecta: Diptera). China Agricultural University Press, Beijing.

Young, A., T. Boyle, and T. Brown. 1996. The population genetic consequences of habitat fragmentation for plants. Trends Ecol. Evol. 11: 413–418.

Yuttham, K., M. Jaroensutasinee, and K. Jaroensutasinee. 2003. Metapopulation and its applications in conservation biology. Songklanakarin J. Sci. Technol. 25: 395–40.