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Original Article

Genetic variation in *Mineus* group of *Mycalesis* (Nymphalidae: Satyrinae) in peninsular Thailand: Inferred from mitochondrial and nuclear genes

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Abstract

The aim of this study was to investigate genetic variation and phylogenetic relationships of the *Mineus* group of *Mycalesis* in peninsular Thailand inferred from mitochondrial and nuclear gene markers (*COI*, *EF-1α* and *wingless)*. Seven species and 214 individuals of the genus *Mycalesis* were collected in six provinces in peninsular Thailand. In this study, two distinct seasonal morphs were found as wet season form and dry season form. Phylogenetic relationships based on combined gene sequences indicated that the *Mineus* group of *Mycalesis* was monophyletic group. However, *M. intermedia*, and *M. perseoides* might be species complex. Five haplotype patterns were observed in *M. perseoides* indicating that Chumphon province was the origin of *M. perseoides*. Divergence time of the *Mineus* group in peninsular Thailand was 23.03 mya during Oligocene period.

Keywords: *Mycalesis*, genetic differentiation, haplotype network, peninsular Thailand, phylogeny

1. Introduction

Butterflies of the subtribe Mycalesina (Nymphalidae: Satyrinae) are described and grouped into 7 genera based on molecular and morphological evidences consisting of the genus *Lohora*, *Heteropsis*, *Mycalesis*, *Mydosama*, *Culapa*, *Bicyclus* and *Hallelesis* (Aduse-Poku *et al*., 2015). This subtribe is restricted in the Old World tropics in which *Mycalesis* is a dominant genus with over 100 estimated described species. Moreover, this genus has been widely dispersed in Indo-Australian region such as in Sri Lanka, India, Southeast Asia, Australia, New Guinea and the east of Solomon Islands (Kodandaramaiah *et al*., 2010). The majority of genus *Mycalesis* prefers lowland habitats and can be found in many kinds of habitat such as forest edges, grasslands and savannas. The butterflies are low-flight and prefer understory shade (Monastyrskii, 2005). The food plants were found in the family of Poaceae and Cyperaceae (Ackery, 1988; Torres *et al*., 2001). This genus shows striking polymorphism in wing pattern, as an important character for identification.

Butterflies in subtribe Mycalesina are an important model to understand the evolutionary study, ecology, developmental genetics and phenotypic plasticity of tropical butterflies (Braby, 1994; Brakefield, 1984, 2012; Islam *et al*., 2010; Torres *et al*., 2001). For instance, the study of seasonal polymorphism of *Mycalesis* in Australia indicated that the mechanism of survival between wet and dry seasonal change in their habitat (Braby, 1994). In addition, physical factors such as temperature, rainfall, humidity and photoperiod can determine the variation of wet and dry season forms (Islam *et al*., 2010). The wet season forms are characterized by con-

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spicuous eyespot. However, the dry season forms are rather dull (Braby, 1994). Interestingly, phenotypic variation of tropical butterfly in terms of morphological characters in the dry the wet season forms are important. However, the phenotypic character in the dry season form are difficult to identify because of less colorations of wings and eyespots (ocelli) reduced. In a previous study, morphometric characterization of *Mycalesis* butterflies in Sri-Lanka showed that phenotypic characters are different, for examples, coloration of wings and dorsal forewing ocelli of adult *Mycalesis* species (Goonesekera, Poorten, & Ranawaka, 2014). Furthermore, genetic variation in *Mycalesis* from Sri Lanka based on analysis of *COI* gene indicated that the variation of haplotype network in *Mycalesis* was not different and was restricted on their location. Genetic distances were significantly different among local species (Goonesekera & Rawanaka, 2013).

Peninsular Thailand is placed on the Sundaic shelf, which is an important transition zone between the Indo-Chinese and Sundaic sub-regions. Both sub-regions are separated by the Isthmus of Kra which was proposed a natural barrier for dispersal of fauna (Lohman *et al*., 2011). According to vegetation structure in the peninsular Thailand, two types of forest community consist of evergreen rain forest and mixed moist deciduous forest (the southern part of Thailand, Thai type) and evergreen rain forest (Kangar-Pattani line, Malayan type)(Santisook, 2012). However, the study on *Mycalesis* was not attended, especially in terms of diversity and abundance. Moreover, Ek-Amnuay (2012) reported that there are 27 species spreading throughout Thailand. Among these, *M. fusca*, *M. janardana*, *M. perseus*, *M. mineus*, *M. visala*, *M. orseis* were restricted to the peninsular Thailand. *Mycalesis* species was divided into five groups, i.e., *Gotama*, *Mineus*, *Oroatis*, *Nicotia*, and *Patnia*. The members of *Mineus* group are *M. perseus*, *M. mineus*, *M. igilia*, *M. visala*, *M. perseoides*, *M. subtida*, *M. mercea*, *M. khasia*, *M. rama*, *M. evansii*, *M. mystes*, *M. adolphei*, *M. intermedia* and *M. oculus* (Evan, 1932; Talbot & Corbet, 1939). Nowadays, the phylogenetic relationship of the subtribe Mycalesina based on molecular data was established as monophyly. *Mycalesis* species are clustered into two clades as *Mycalesis* I consists of the taxa from mainland Southeast Asia and *Mycalesis* II belongs the taxa from Indo-Australia. However, both clusters are not monophyletic group (Aduse-Poku *et al*., 2015; Kodandaramaiah *et al*., 2010). In addition, there is lack of knowledge on phylogenetic relationships and genetic variation of *Mycalesis Mineus* group in peninsular Thailand.

This study aimed to investigate the phylogenetic relationship and genetic variation of *Mycalesis Mineus* group inferred from one mitochondrial and two nuclear genes. Within the hypothesis of phylogenetic study, divergence times of *Mycalesis* group have been estimated. The finding can contribute and clarify the *Mycalesis Mineus* group in peninsular Thailand.

2. Materials and Methods

2.1 Taxon sampling

Butterflies of the genus *Mycalesis* were collected in six provinces along the mountain ranges of peninsular Thailand. Six sampling sites were at Chumphon province (10°45.79'N 99°23.03'E), Surat Thani province (09°23.34'N 99°15.24'E), Nakhon Si Thammarat province (08°20.02'N 100°09.56'E), Phang-Nga province (08°90.24'N 98°40.50'N), Trang province (07°33.36'N 99°46.72'E) and Narathiwat province (05°58.65'N 101°54.39'E) during February 2015-July 2016. Adult butterflies were collected using insect nets and baited traps with 10 fruit-baited traps set up along 1 km. line transect at each sampling site. Each trap was randomly placed on the line transect at a distance 100 m. The baited-trap was sampled during seven consecutively days at each sampling site. Adult butterflies were preserved in triangle paper. A pair of middle legs were removed from the specimens and placed in 95% ethanol for DNA extraction. The voucher specimens were deposited in Princess Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University.

2.2 Species identification

Mycalesis species were identified to species level using identification keys by Corbet, Pendlebury and Eloit (1978), Ek-Amnuay (2012), Monastyrskii (2005), Pinratana (1988) and Talbot (1947).

2.3 DNA extraction, PCR amplification and sequencing

Total DNA was extracted using modified protocol from Collins *et al*. (1987). The sample was homogenized with a pestles in a 1.5 micro centrifuge tube with 50 µl of lysis buffer including 0.8 M NaCl, 0.16 M sucrose, 0.06 M EDTA, 0.5% SDS and 0.1 M Tris-HCl pH 8.6. Two µl of Proteinase K was added and the homogenate was incubated at 65°C overnight. Seven µl of 8 M CH_3CO_2K was added and the tube was incubated for 30 min at -20°C. Then it was centrifuged at room temperature for 15 min at 13,400 rpm. Supernatant was removed to a sterile tube and 95% ethanol added and centrifuged at 13,400 rpm for 15 min. After discarding supernatant, the pellet was washed with 70% ethanol and centrifuge. The pellet was dried and suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and maintained at -20°C.

DNA was amplified from a mitochondrial gene Cytochrome Oxidase subunit I (*COI*) and two nuclear genes, Elongation factor 1 alpha (*EF-1α*) and *wingless* genes. The polymerase chain reaction was done using 2X Blue/Red mix DNA polymerase master mix (RBC Bioscience, Taiwan) following manufacturer's protocol. The primers of three genes follow Kodandaramaiah *et al*. (2010) with *COI* using primer LCO – HCO (LCO ; 5' GGTCAACAAATCATAAAGATA TTGG 3' F and HCO : 5' TAAACTTCAGGGTGACCA AAAAATCA 3 'R) with following protocol : 95 °C 7 min for initial denature, 40 cycles for 95 °C 30 s, 50 °C 30 s and 72 °C 1 min, final extension at 72 °C for 10 min. *EF-1α* using primer Starsky-Luke (Starsky : 5'CACATYAAC ATTGTCG TSATYGG 3' F and Luke : 5'CATRTTGTCKCCGTGCC AKCC 3' R) with following protocol : 95 °C 7 min for initial denature, 40 cycles for 95 °C 30 s, 55 °C 30 s and 72 °C 1 min, final extension at 72 °C for 10 min. *wingless* using primer Wingnut 1A – Wingnut-3 (Wingnut 1A : 5'-GAAAT GCGNCARGARTGYAA-3' F and Wingnut-3 : 5'-ACYTCR CARCACCARTGRAA-3' R) with following protocol : 80 °C 1 min for initial denature, 40 cycling for 94 °C 1 min, 52 °C 2 min and 72 °C 1 min, final extension at 72 °C for 10 min.

PCR products were checked by 1.5% agarose gel electrophoresis. DNA sequencing was done by Sanger's method with ABI 3730XL sequencer (Bioneer Corporation, South Korea).

2.4 Genetic variation and phylogenetic analysis

Sequences were viewed by Chromas Lite version 2.5.1 software and aligned using Bioedit version 7.1.3 software (Hall, 1999). The aligned sequences were arranged by MEGA version 6.0 to analyze nucleotide composition and pairwise genetic distance (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). DnaSP version 5.10.01 (Rozas & Rozas, 1999) was use to estimate number of haplotype (*h)*, haplotype diversity (*Hd*), nucleotide diversity (π), invariable site, parsimony informative site, number of polymorphic sites (*S*) and Tajima's *D* test (Nei & Kumar, 2000). Sequences were submitted to GenBank for accession numbers (Table 1).

Genetic differentiation and phylogenetic tree of the butterfly genus *Mycalesis* in peninsular Thailand using *COI*, *EF-1α* and *wingless* genes were determined. Kimura 2-parameter model was used to calculate the genetic distance. Haplotype network of the *M. perseoides* was generated using TCS program version 1.7 (Clement, Posada, & Crandall, 2000). Phylogenetic analysis of the butterfly genus *Mycalesis* was conducted for *COI* sequence and for the combined dataset (*COI*, *EF-1α* and *wingless*). Bayesian analysis with MrBayes version 3.2.6 using General Time Reversible (GTR) model by GAMMA distribution was done for 10,000,000 generations with a sampling frequency of 100 generations (Huelsenbeck & Ronquist, 2001). Phylogeny was viewed and edited by FigTree version 1.3.1 (Rambaut, 2009). Species divergence time was investigated by the molecular clock using fossil record of *Lethe corbieri* by Neighbor-joining method (NJ) in MEGA program version 6.0.

3. Result

The butterflies of genus *Mycalesis* were sampled during February 2015 to July 2016 with a total of 214 individuals, seven species have been described into five members of *Mineus* group consisting of *M. intermedia*, *M. visala*, *M. perseoides*, *M. mineus* and *M. perseus* (Table 2). The rest of *Mycalesis* were *M. orseis* and *M. janardana*. The species distribution of *Mycalesis* in each province showed that *Mycalesis* in Chumphon province (CHP) consisted of *M. mineus* and *M. perseoides*. Four *Mycalesis* species (*M. perseus*, *M. mineus*, *M. perseoides* and *M. visala*) were collected at Phang-Nga province (PHG) in Phuket mountain range. Three species of *Mycalesis* including *M. mineus*, *M. intermedia* and *M. perseoides* were sampled at Surat Thani province (STN). Furthermore, four *Mycalesis* species (*M. perseus*, *M. intermedia*, *M. perseoides* and *M. visala*) were collected at Nakhon Si Thammarat province (NST). In addition, six species of *Mycalesis* (*M. orseis*, *M. janardana*, *M. mineus*, *M. intermedia*, *M. perseoides* and *M. visala*) were sampled at Trang province (TRG) in Nakhon Si Thammarat

Table 1. List of *Mycalesis* species sequences obtained for this study.

Species	Localities	Coordinate	Accession Number		
			COI	*EF-1α*	*wingless*
M. perseoides	CHP	10°45.79'N 99°23.03'E	MG461862	MG461892	MG461922
			MG461863	MG461893	MG461923
			MG461864	MG461894	MG461924
			MG461865	MG461895	MG461925
M. perseoides	STN	09°23.34'N 99°15.24'E	MG461871	MG461901	MG461931
			MG461872	MG461902	MG461932
M. perseoides	NST	08°20.02'N 100°09.56'E	MG461875	MG461905	MG461935
			MG461880	MG461910	MG461940
M. perseoides	NTW	05°58.65'N 101°54.39'E	MG461887	MG461917	MG461947
			MG461891	MG461921	MG461951
M. intermedia	STN	09°23.34'N 99°15.24'E	MG461873	MG461903	MG461933
M. intermedia	NST	08°20.02'N 100°09.56'E	MG461874	MG461904	MG461934
			MG461877	MG461907	MG461937
M. intermedia	TRG	07°33.36'N 99°46.72'E	MG461882	MG461912	MG461942
			MG461885	MG461915	MG461945
			MG461886	MG461916	MG461946
M. intermedia	NTW	05°58.65'N 101°54.39'E	MG461888	MG461918	MG461948
			MG461889	MG461919	MG461949
			MG461890	MG461920	MG461950
M. mineus	STN	09°23.34'N 99°15.24'E	MG461869	MG461899	MG461929
			MG461870	MG461900	MG461930
M. mineus	PNG	08°20.02'N 100°09.56'E	MG461866	MG461896	MG461926
			MG461867	MG461897	MG461927
M. visala	NST	08°20.02'N 100°09.56'E	MG461876	MG461906	MG461936
M. visala	TRG	07°33.36'N 99°46.72'E	MG461883	MG461913	MG461943
M. perseus	NST	08°20.02'N 100°09.56'E	MG461878	MG461908	MG461938
			MG461879	MG461909	MG461939
M. orseis	TRG	07°33.36'N 99°46.72'E	MG461881	MG461914	MG461944
M. janardana	TRG	07°33.36'N 99°46.72'E	MG461884	MG461911	MG461941

Table 2. The number of individuals in six sampling sites.

Species	CHP		STN		NST		PNG		TRG		NTW	
	M	F	M	F	M	F	M	F	M	F	M	F
M. janardana	-	-	-	-	-	-	-	-	1	1	-	-
M. orseis	-	-	-	-	-	-	-	-	1	-	-	-
M. perseus	-	-	-	-	10	7	5	3	-	-	1	4
M. mineus	3	1	5	3	-	-	6	1	-	2	5	3
M. perseoides	26	11	15	6	9	-	-	2	1	-	17	6
M. intermedia	-	-	1	-	2	-	-	-	11	18	3	3
M. visala	-	-	-	-	-	1	1	-	2	1	1	1

Note: CHP: Chumphon province; STN; Surat Thani province; NST: Nakhon Si Thammarat province; PNG: Phang-Nga province; TRG: Trang province; NTW: Narathiwat province; M: male; F: female

mountain range. However, five species of *Mycalesis* consisted of *M. mineus*, *M. intermedia*, *M. perseoides*, *M. visala* and *M. perseus* occurred at Narathiwat province (NTW) in San kala khiri mountain range. The result showed that *M. perseoides* was dominant species in all sampling sites across peninsular Thailand (Figure 1). Interestingly, *Mycalesis* species in peninsular Thailand showed two distinct seasonal morphs; wet season form and dry season form, such as *M. mineus* and *M. perseoides*. This shows relationship between genetic variation and phenotypic plasticity (Figure 2). The phenotypic character of *M. mineus* and *M. perseoides* in the dry season form and the wet season form are different, especially in dry season forms are difficult to identify because the characters such as less colorations of wings and eyespots reduced. On the other hand, in wet season form the colorations of wings and eyespots are prominent characters.

3.1 Genetic variation

The aligned sequences were 632 bp (*COI*), 485 bp (*EF-1α*) and 379 bp (*wingless*). The *COI* sequence showed higher invariant sites than *EF-1α* and *wingless*. Among 632 bp of *COI* sequence, 73 sites were parsimony informative. The *EF-1α* sequence contained 24 variable parsimony informative sites while *wingless* contained only 16 variable parsimony informative sites. Genetic diversity indices of the three genes showed *COI* gene had 0.04019 nucleotide diversity (π) and 125 polymorphic sites (*S*). Furthermore, the nucleotide diversity and number of polymorphic sites of *EF-1α* and *wingless* were 0.02293, 88 and 0.01591, 52 respectively. However, the number of haplotype (*h*) and haplotype diversity (*Hd*) were high in *wingless* gene, these belonging to 23 and 0.980. The result of Tajima's *D*-test revealed that all genes were not significant (Table 3). *M. perseoides* and *M. perseus* possessed the greatest intraspecific genetic differentiation in the nuclear gene (*EF-1α* values were 1.76 and 4.70, and *wingless* values were 0.80 and 1.10, respectively). However, intraspecific genetic divergence for *M. intermedia* and *M.visala* were high in *COI* gene (2.70 and 6.20, respectively) (Table 4).

According to haplotype network of *Mycalesis* species in peninsular Thailand, the haplotype network of *M. perseoides* based on *EF-1α* gene in four provinces (CHP, STN, NST and NTW) was generated. The result showed that five haplotype patterns were found and the haplotype diversity

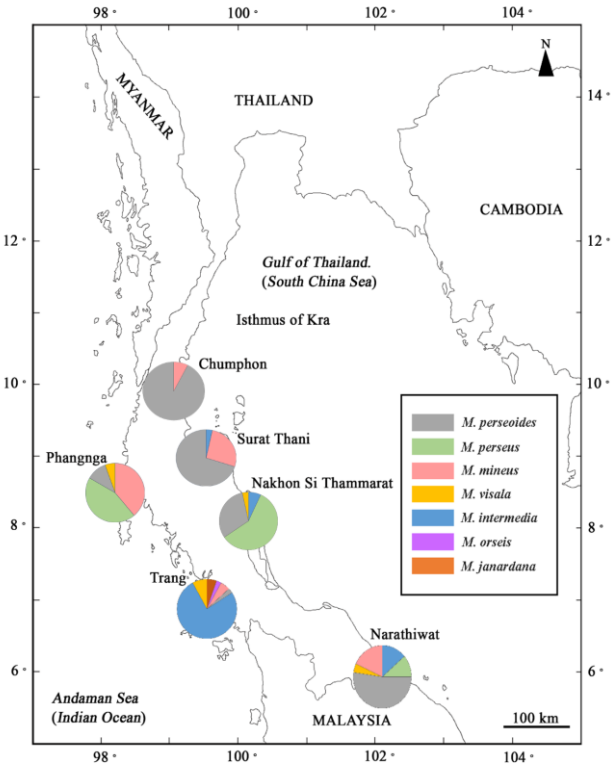

Figure 1. Distribution of *Mycalesis* species in six study sites in peninsular Thailand. Pie chart segments are proportional to the number of individuals collected at each location

was 0.667. Furthermore, number of polymorphic sites and nucleotide diversity were 22 and 0.00949, respectively (Table 5). The haplotype relationship of five haplotype is shown in Figure 3. Haplotype 1 of *M. perseoides* (n = 6) indicated that Chumphon province might be the origin of *M. perseoides* in peninsular Thailand. However, the haplotype of *M. perseoides* in Chumphon is different from the Surat Thani (haplotype 3, n = 1) in one mutation step of nucleotide substitution. Moreover, haplotype 4 (Nakhon Si Thammarat, n = 1) is 19 mutation steps of nucleotide substitution away from Chumphon haplotype pattern. However, the haplotype 5 (Narathiwat, n = 1) is different in two mutation steps of nucleotide substitution from Chumphon haplotype pattern.

Figure 2. Two species of *Mycalesis* in peninsular Thailand: *M. mineus* (left column) and *M. perseoides* (right column); A) representative examples of wet season form (top row) and dry season form (bottom row); B) male genitalia. All specimens are males. Adult butterfly (scale = 1 cm).

Table 3. Statistic for individual gene segments.

Parameters	COI	EF-1α	wingless
Number of samples	29	29	29
Aligned positions	632	485	379
Best-fit model	GTR+I+R	GTR+I+R	GTR+I+R
Polymorphic site			
- Invariable sites	505	389	327
- Singleton variable sites	52	64	36
- Parsimony informative sites	73	24	16
- Nucleotide diversity (π)	0.04019	0.02293	0.01591
- Number of polymorphic sites (*S*)	125	88	52
Haplotype diversity			
- Number of haplotype (*h*)	14	19	23
- Haplotype diversity (*Hd*)	0.771	0.887	0.980
Tajima's Test			
- Tajima's *D*	-1.23492*	-2.08307*	-2.20456*

Note: * not significant

Table 4. Intraspecific genetic divergence among the members of *Mycalesis Mineus* group in peninsular Thailand based on the Kimura 2-parameter model.

Species	***COI***	***EF-1α***	***Wingless***
M. perseoides	0.33 (0.000-0.006)	1.76 (0.000-0.042)	0.80 (0.003-0.013)
M. intermedia	2.70 (0.000-0.066)	1.00 (0.000-0.019)	0.67 (0.000-0.013)
M. mineus	0.25 (0.002-0.003)	0.40 (0.000-0.004)	0.65 (0.005-0.008)
M. visala	6.20 (0.000-0.062)	1.00 (0.002-0.015)	0.40 (0.003-0.005)
M. perseus	0.20 (0.002)	4.70 (0.047)	1.10 (0.011)

Note: The analyses involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There are a total of 360 positions for *COI*, 476 positions for *EF-1α* and 379 positions for *wingless*. All values are mean (%) (min.-max.). For *M. perseus* had only one value.

Table 5. Statistic of *M. perseoides* based on *EF-1α* sequence.

Parameters	*EF-1α*
Number of samples	10
Aligned positions	485
Best-fit model	GTR+I+R
Polymorphic site	
Invariable sites	458
Singleton variable sites	21
Parsimony informative sites	1
Nucleotide diversity (*Pi*)	0.00949
Number of polymorphic sites (*S*)	22
Number of haplotype (*h*)	5
Haplotype diversity (*Hd*)	0.667
Tajima's D	-1.95824*

Note: * not significant

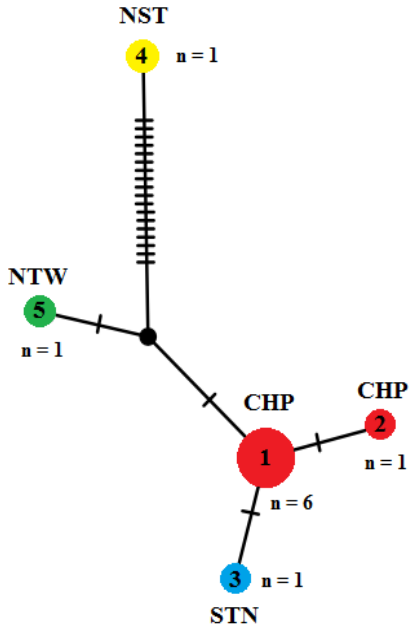

Figure 3. Haplotype network for *EF-1α* sequence of *M. perseoides* in peninsular Thailand. Solid lines on branches refer to mutation step.

3.2 Phylogenetic relationships

Phylogenetic analyses based on the combined dataset included 1,496 bp from 29 samples of *Mycalesis* species and one outgroup *O. medus* sequences from GenBank, DQ338766 (*COI*), DQ338906 (*EF-1α*) and DQ338633 (*wingless*). The phylogenetic tree of Bayesian approach showed that the *Mineus* group of *Mycalesis* in peninsular Thailand was monophyletic with a strongly support (posterior probability, BI = 100). This tree was divided into two major clades, A and B (Figure 4). Clade A consisting of *M. perseoides*, *M. intermedia*, *M. visala* and *M. mineus* was strongly supported as monophyly (posterior probability, BI = 100). Clade A can be divided into two subclades: A1 and A2. In subclade A1, *M. visala* from Nakhon Si Thammarat province and Trang province were grouped together. However, the result indicated that *M. perseoides* from Chumphon, Surat Thani, Nakhon Si Thammarat and Narathiwat provinces and *M. intermedia* from Surat Thani, Nakhon Si Thammarat and Narathiwat provinces in subclade A1 were grouped together. The result indicated that this might be species complex of *Mineus* group. Subclade A2 consisted of *M. intermedia* from Trang province and Narathiwat province and *M. mineus* from Surat Thani province and Phang-Nga province. The result showed that each species was clearly separated in different branch. Furthermore, clade B composed of *M. perseus* from Nakhon Si Thammarat province and it was strongly supported as monophyly (BI = 100). The rest of *Mycalesis* (*M. janardana* of *Janardana* group and *M. orseis* of *Francisca* group from Trang province) were collected.

3.3 Divergence time estimates

The divergence time for the genus *Mycalesis* in this study was estimated from age of fossil record of *Lethe corbieri* during the Oligocene in southeast France, approximately 28.4 to 23.03 million years ago (mya) (Nel, Nel, & Balme, 1993). The analysis in Neighbor-joining (NJ) indicated that *Mycalesis* in peninsular Thailand diverged between 32.47 mya during Oligocene period (Figure 5). Initial split of the *Janardana* group and *Mineus* group arised ca. 23.03 mya during Oligocene period. The molecular phylogram showed that it was divided into two clades (A and B) ca. 11.76 mya during Miocene period. In clade A, *Mycalesis* species were separated approximately 10.66 mya into two subclades (A1 and A2). Subclade A1 arised ca. 9.72 mya and is composed of *M. perseoides* from Chumphon, Surat Thani, Nakhon Si Thammarat and Narathiwat provinces, *M. intermedia* from Surat Thani, Nakhon Si Thammarat and Narathiwat provinces, *M. visala* from Nakhon Si Thammarat and Trang provinces colonizing around 0.78 mya during Pleistocene period. Subclade A2 consists of *M. mineus* from Surat Thani and Phang-Nga provinces (0.65 mya) during Pleistocene. However, Clade B consisted of *M. intermedia* from Trang and Narathiwat provinces colonizing around 0.29 mya during Pleistocene period.

4. Discussion

Molecular technique provides a powerful tool to investigate population dynamics of organism and also enable more detailed understanding on the relationship between populations (Sum *et al*., 2014). Various genetic markers have been used to illustrate the genetic differentiation that occurs in butterfly populations, especially mitochondrial and nuclear gene markers. In this study, a good candidate gene marker is revealed as the mitochondrial gene (*COI*) with high variability in polymorphic sites (invariable sites, singleton variable sites, parsimony informative sites, nucleotide diversity and number of polymorphic sites of interspecific genetic differentiation). However, the number of haplotype and haplotype diversity were high in nuclear genes (*EF-1α* and *wingless*). Four *Mycalesis Mineus* groups (*M. visala, M. intermedia, M. perseus* and *M. perseoides*) were highly different in intraspecific genetic divergence in each gene. Intraspecific genetic variation of *M. visala* had high diversity in *COI* and *M. perseus* in *EF-1α* and *wingless*. *M. mineus* had low intraspecific genetic diversity in all genes, indicating high degree of gene flow within population.

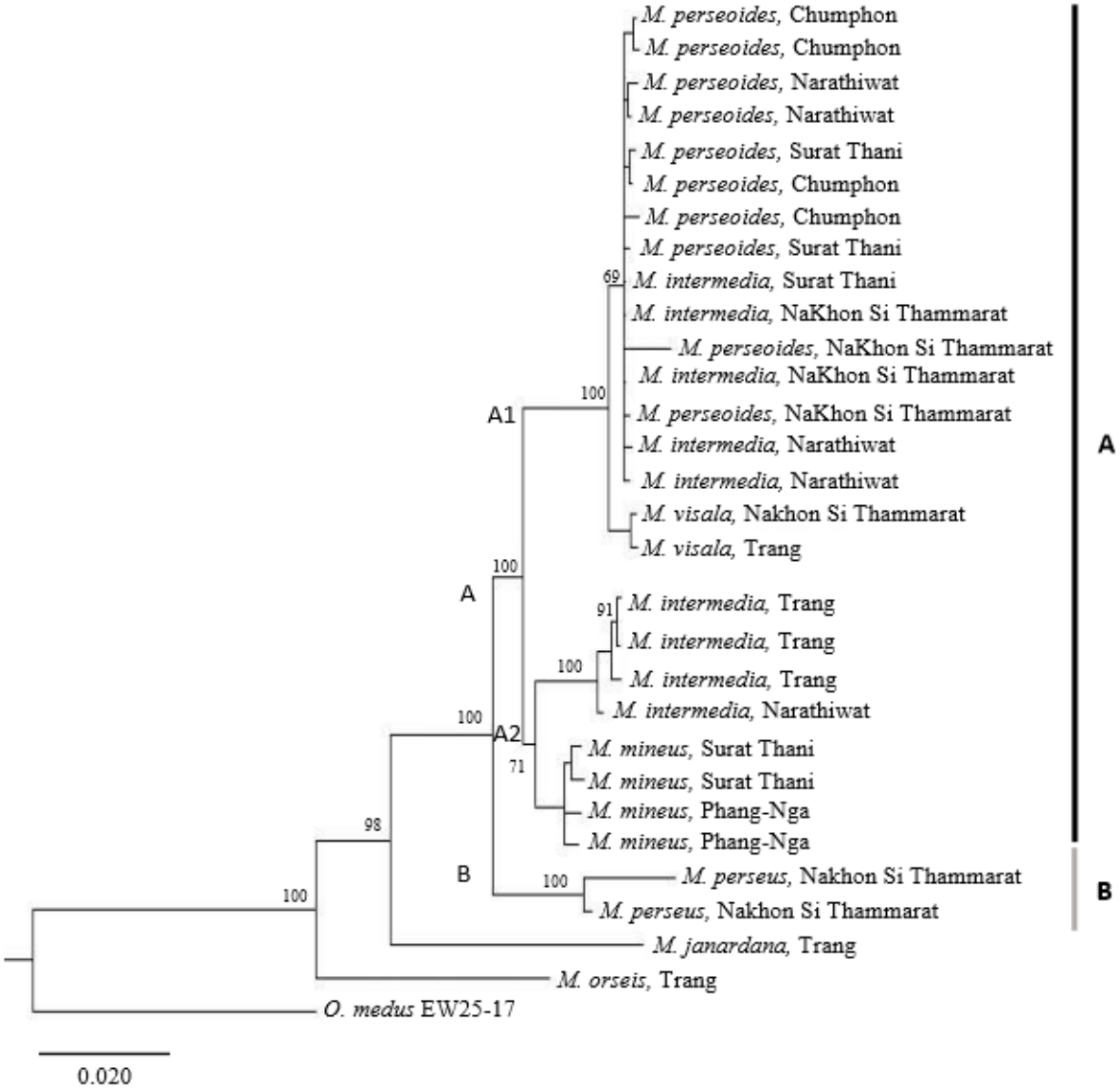

Figure 4. Bayesian tree for the combined dataset of *COI, EF1-α* and *wingless* sequences of *Mycalesis* species in peninsular Thailand. Posterior probabilities are based on Likelihood ratio test for Maximum likelihood.

Phylogenetic relationship of *Mycalesis Mineus* group in peninsular Thailand was monophyletic with strong support based on Bayesian analysis. *Mycalesis Mineus* group can be divided into two major clades (clade A and B). At species level, *M. mineus* of Surat Thani province and Phang-Nga provinces were grouped together in clade A2, consistent with intraspecific genetic distance which was low in all genes. Therefore, their genetic relationship is closely related within population. However, *M. intermedia* and *M. perseoides* were species complex. However, those species were not clearly related in this study. These two species were different in terms of morphological characters of male genitalia. On the other hand, the genetic divergence among species of *M. intermedia* and *M. perseoides* was low. This result suggested that the gene introgression in mtDNA and nuclear gene likely happened during species expansion. According to species delimitation, their connecting populations have been influenced by gene flow. Intraspecific genetic differentiation (introgression) would affect species integrity. Recent studies have proposed that the correlation between intraspecific genetic differentiation and gene flow was a negative correlation. Therefore, species delimitation might be more effective with markers experiencing high degree of gene flow (Petit & Excoffier, 2009).

This finding of the monophyly of *Mineus* group in peninsular Thailand is consistent with the scenario of Kodandaramaiah *et al*. (2010) indicating that the *Mycalesis* species was clustered into two clades as *Mycalesis* I and *Mycalesis* II. *Mycalesis* I belonged to *Mycalesis* species from mainland Southeast Asia (China, Vietnam, Laos, Bangladesh and Thailand) and *Mycalesis* II belonged to the taxa from Indo-Australia (Indonesia, Australia, Papua New Guinea and

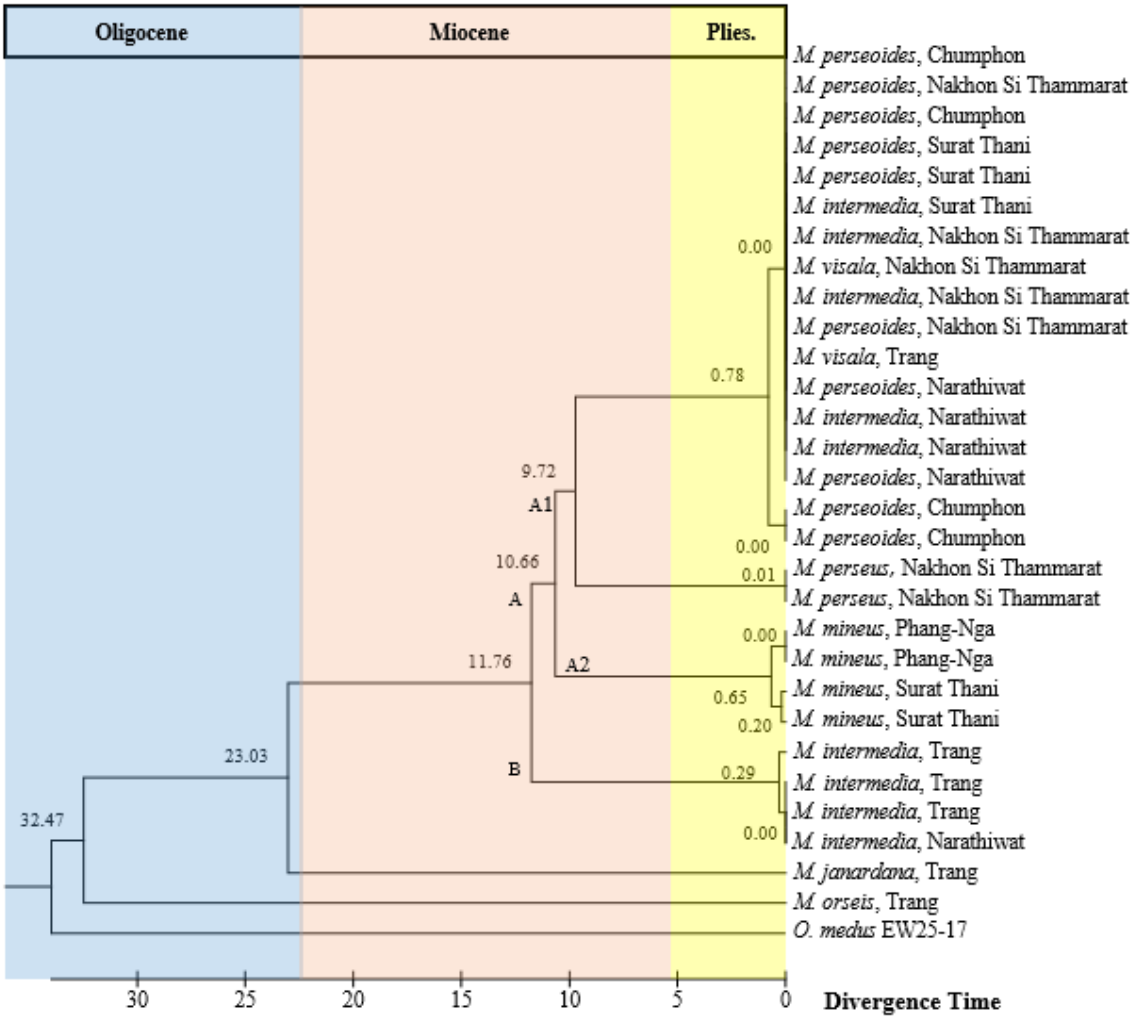

Figure 5. The neighbor-joining tree is obtained from *COI* gene of *Mycalesis* species and out group in peninsular Thailand. Values on branches refer to divergence times. Scale bar indicates million years ago (mya) for divergence time molecular clock.

Solomon Island). In this study, all members of *Mycalesis* in peninsular Thailand might be clustered in clade *Mycalesis* I. The finding of the monophyletic group of *Mycalesis Mineus* group in peninsular Thailand was supported by divergence time as well. The result showed that *Mycalesis* species in peninsular Thailand diverged ca. 32.47 mya during Oligocene period. *Mycalesis Mineus* group arised at approximately 23.03 mya in Oligocene period, consistent with the reported of Aduse-Poku *et al*. (2015) proposing that the butterfly subtribe Mycalesina and Lethina diverged from each other about 39.8 mya during Eocene period. Furthermore, divergence time of *Mycalesis* in Southeast Asia was observed to have occurred around the Oligocene and Eocene boundary, roughly at the same time as diverged the endemic African genera *Bicyclus* and *Hallesis*. Furthermore, the origin of the subtribe Mycalesina was in Asia and later distributed to other regions (Aduse-Poku *et al*., 2015). However, *Mycalesis Mineus* group was divided into two clades approximately 11.76 mya in mid Miocene period.

According to Pena and Wahlberg (2008), the fossil record of butterfly reconstructed a divergence time of sub-family Satyrinae and the result showed that subfamily Satyrinae might dispersed to other continental with their adaptive radiation of food plants approximately 25 mya during Oligocene epoch (Brakefield, 2012; Cerling *et al*., 1997; Sage, 2004). The larva of these butterflies feed on grasses (Poaceae) that are dominant in shaded and open habitats, especially the tropical rain forest (Pena & Wahlberg, 2008). Interestingly, *M. perseoides* is a good example to answer the haplotype network and migration route of *Mycalesis* species. The haplotype network of the *EF-1α* sequence of *M. perseoides* suggested that the haplotype pattern of Chumphun (n = 6) is assumed to be the origin then it was divided into Surat Thani pattern (n = 1), Narathiwat pattern (n =1) and Nakhon Si Thammarat pattern (n = 1). In this study, the result indicated that *M. perseoides* should has a genetic connectivity microhabitat between populations of *M. perseoides* in peninsular Thailand. Furthermore, this study has determined relationship between genetic variation and phenotypic plasticity of *Mycalesis* populations in Thailand. *Mycalesis* species in peninsular Thailand were found in two distinct seasonal morphs, wet and dry season forms such as in *M. mineus* and *M. perseoides*. The

morphology of male genitalia was distinguishing character to identify species in this genus. The butterfly in subtribe Mycalesina is well known an important model to understand development, genetics and evolution of phenotypic plasticity as a response to wet and dry seasons environment in their habitat. This phenomena has been influenced on seasonal fluctuation and reproductive dormancy. Temperature and humidity plays an important role to regulate diel activity (Islam *et al*., 2010). Furthermore, the dry season form was usually reproductively inactive, long-lived, and has less diel activity. By contrast, wet season form was reproductively active more diel activity (Braby, 1994; Islam *et al*., 2010).

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