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Molecular Phylogeny of Indonesian Armyworm *Mythimna* Guenée (Lepidoptera: Noctuidae: Hadeninae) Based on CO I Gene Sequences

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Armyworm *Mythimna* Guenée is one of the most important pests on graminaceous crops and pastures in South East Asia (i.e. *M. separata* Walker is well known to cause serious damages on rice in Indonesia). Like of most other genera of moths, the systematic of this genus is still in dispute, especially on the taxonomy and classification within this genus due to their morphological characters that are very difficult to distinguish from one to others. Molecular approaches such as using CO I gene sequence to differentiate among species has been recommended since this gene has ability to reveal the character identity at the specific level. In order to populate the genetic characters of Indonesian *Mythimna*, to clarify the clasification within the genus *Mythimna* and to reveal the phylogenetic relationship among them, we analyzed 14 species of *Mythimna* and two species outgroups (*Spodoptera litura* dan *S. exigua*) based on nucleotide sequence variation across a 649 bp region in the CO I gene. Over entire 649 bp region 72% of the nucleotide positions were constant, 10.6% were uninformtive (i.e. any variants were found in a single sequence) and 16.9% were parsimony informative. The informative site constituted in the 3rd codon position was the highest, whereas in 2nd codon position was the lowest. The results also showed that the base composition of this region was low A + T biased. The results showed that the monophyly of *Mytimna* was supported by 95% bootstrap test at any tree building methods. The three subgenera based on morphology were recovered but *M.* (*Mythimna*) shown to be a paraphyletic group in term of *M.* (*Hyphilare*), and *M.* (*Pseudalteia*); *M.* (*Mythimna*) was branched off first then followed by *M.* (*Pseudaletia*) and *M.* (*Hyphilare*). However, all internal nodes were least support except for the monophyly of subgenus *M.* (*Hyphilare*). It indicates that the relationships among internal nodes proposed here were least valid due to the number of species included in the analysis which may not be enough to represent the real number of species in the nature. More investigation was needed by including more species and other genes.

Key words: *Mythimna*, phylogenetic relationships, CO I gene

INTRODUCTION

Armyworm is one of the most important pests on Graminaceous crops in Indonesia. There are two groups of armyworm, i.e. the genus *Spodoptera* and *Mythimna*. The last one comprises species involved in what are referred to as 'armyworm' outbreaks (Holloway *et al.* 1987; Chandler & Benson 1991). They have been given this common name due to their sporadic occurrence in large numbers in a manner similar to the African armyworm, *Spodoptera exempta* (Walker), which is a serious pest of many graminaceous crops (Brown 1972; Carnegie *et al.* 1974). Larvae of *Mythimna* spp. feed on pasture grasses, sugarcane, maize and rice but, unlike S. *exempta*, which is a true armyworm, they do not migrate from one feeding site to another in searching food. Both of them cause a serious damage on several crops. Compared with the genus *Spodoptera*, however, *Mythimna* larvae is more unique since they have specific-host plants, mostly on Gramineae.

The moths of *Mythimna* spp. are nocturnal and the larvae start feeding at the onset of darkness and are rarely seen during the day (Carnegie & Dick 1972). Several species have been reported as serious pests in Maurituss such as *Mythimna* (= *Leucania) loreyi* Duponchel, *M. tincta* Walker. *M. loreyi* has been reported to attack sugarcane and other graminaceous crops in Africa, Australia, Philippines, and Southern Europe (Calora 1966; Chandler & Benson 1991; Edwards 1992; Ganesha & Rajabale 1996). *M. curvula* Walker has been reported in Reunion on sugarcane and other grasses (Etienne 1976) and in Madagascar on sugarcane (Rungs 1955). Whereas *M. separata* Walker, *M. venalba* Moore, and *M. loreyi* has been reported as important pests of rice, maize, and sugarcane in Indonesia (Kalshoven 1981).

Mythimna is a large group and distributes widely in the world but the status of this genus is still in dispute, especially on their taxonomy and classification within this genus. There has been considerable taxonomic confusion regarding members of the genus *Mythimna.* Rungs (1955), Calora (1966), Edwards (1992), and Yoshimatsu (1994) have studied the species from the Phillipines, Madagascar, Australia, Japan, and Taiwan, respectively. Detailed taxonomic studies have not been carried out in Indonesia except those that have been conducted by Holloway (1989) based on materials from Borneo.

Sugi (1982) divided this *Mythimna* complex based on Japanese moths into several genera, they are: *Mythimna*, *Aletia*, *Pseudaletia*, *Dysaletia*, *Analetia*, *Leucania*, *Acantholeucania*, and *Xypholeucania*. They share two distinctive features: in the male gentitalia the valve bases are united by a bubble-like structure (Calora 1966); in the female genitalia the ovipositor lobes are strongly sclerotised, with their distal margin distinctively oblique. This character is probably assosiated with oviposition within the leaf axils or between blades of the graminaceous host in neat row. The female bursa copulatrix lack of signa.

Holloway (1989) conducted his study based on Bornean materials and divided the genus *Mythimna* into several subgenera i.e. *Aletia*, *Pseudaletia*, *Acantholeucania*, and *Leucania*. On the other hand, Edwards (1996) treated *Leucania* as a dependent genus and *Acantholeucania* placed under sub genus *Leucania* and *Pseudoaletia* under subgenus *Mythimna*. They also treated several species such as *M. decisissima* and *M.consanguis* as unknown place.

The most intensive work on classification of this *Mythimna* group was conducted by Yoshimatsu (1994) by revising the classification of the genus based on Japan and Taiwan species. He divided the genus *Mythimna* into seven subgenera, they are: *Mythimna*, *Hephilare*, *Sablia*, *Pseudaletia*, *Acantholeucania*, *Anapoma*, and *Dysaletia*. He proposed these seven subgenera based on genitalia both of male and female characters. Each subgenus has been established based on uniquely derived characters as shown in Table 1.

He treated *Aletia* and *Leucnaia* under genus *Mythimna* based on these genitalia characters. Genus *Aletia* has been been recorded from Asia and America but European authors have used the genus name *Mythimna* in which they include species *Aletia*. In fact, the structure of the genitalia characters of these two type species, *M. turca* and *A. vitellina* are similar. Moreover, previously, *Leucania* has been established based on the absence of coronal spines on the cucullus in which these characters are variable and not realiable to be used as criterion of the genus (Yoshimatsu 1990). This treatment resulted in a tremendous change on the classification of Indonesian species (Holloway 1989) (Table 2).

There is no doubt that morphological character is very important to recognize the identity of species but it is not always easy when we deal with a complex species. A lot of internal characters are probably useful, especially the genital characters, not only to confirm the identity species but also to reconstruct the phylogenetic relationship among them. However, it is often difficult to score these characters due the complexity of their structures. The other problem is the objectivity of the observers, different observers will give different results when they work on the same sample specimens.

Molecular approach is one of the alternatives that can be applied to fill that gap. The huge numbers of characters resulted from a certain gene sequence are very powerful not only to differentiate among species within a species complex but also in resolving their phylogenetic relationships. Among them, CO I gene has been chosen as one of the candidate genes to be applied in DNA barcoding (a novel system designed to provide rapid, accurate, and automatable species identifications by using short, standardized gene regions as internal species tags) (Hebert &Gregory 2005; Herbert *et al*. 2010). Almost all requirements that are needed in DNA barcoding can be reached by this gene. This gene can be used to distinguish species in almost all animals. The length of this gene is relatively short about 650 bp. Compared with another mitochondrial gene, CO I gene is more conserved. Therefore, this gene is very suitable to identify a species since its sequence has a low variability (in general less than 1-2%), even for the closely related species its value is less than 1%. Another benefit of using this mitochondrial gene is that it is relatively easy to sequence than nuclear genes such as Wingless, EF-1α and ITS (Sutrisno 2003,

Table 1. Seven subgenera of *Mythimna* with the uniquely derived characters (Yoshimatsu 1994)

Subgenera	Uniquely derived characters
Mythimna	1. Valvula not strongly produced
	2. Ampulla and harpe moderately large
Hyphilare	1. Tufts of black hairs on the mail basal abdominal segment
Sablia	1. Sacculus with a long process dorsoposteriorly
	2. male abdominal sternite 1+2 with a media pouch
Pseudaletia	1. Valvula strongly produced
	2. Ampulla and harpe very small
Acantholeucania	1. Valvulla with a long acute process postoventrally
Anapoma	1. Corona represented by two or three rows of spines on posterior portion of cucullus
Dysaletia	1. Corona represented by many sparse spines on cucullus

Table 2. Treatment of the member of the genus *Mythimna* by Holloway (1989) and Yoshimatsu (1994)

Species	References	
	Holloway (1989)	Yoshimatsu (1994)
albomarginata	*Mytimna* (*Aletia*) *albomarginata*	*Mythimna* (*Mythimna*) *albomarginata*
decisissima	*Mythimna* (*Aletia*) *decisissima*	Mythimna (*Hyphilare*) *decisissima*
calorai	*Mythimna* (*Aletia*) *calorai*	*Mythimna*(*Hyphilare*)*epieixilus*
radiata	*Mythimna* (*Aletia*) *radiata*	*Mythimna* (*Mythimna*) *radiata*
yu	*Mythimna* (*Acantholeucania*) *yu*	*Mythimna* (*Mythimna*) *yu*
albicosta	*Mythimna* (*Pseudaletia*) *albicosta*	*Mythimna* (*Pseudaletia*) *pallidicosta*

2006). CO I gene is one of the most common to be considered infering the relationships among closely-related species in several groups of Lepidoptera, as individual gene or to be combined with other genes (Andrew 1994; Sperling & Hickey 1994; Caterino & Sperling 1999; Landry *et al*. 1999; Blum *et al*. 2003).

In order to clarify the relationships among subgenera, we used mitochondrial CO I gene sequence to reconstruct the relationships among 9 species of 3 subgenera of *Mythimna* which are distributed in Indonesia. Due to only fresh materials from G. Salak that was available for DNA extraction (all voucher specimens were deposited in Museum Zoologicum Bogoriense), no other material specimens included in this study except that those sequence available in Genbank. The results of the study also will give benefit in populating the genetic characters of Indonesian *Mythimna*. By establishing the data of the genetic identity of these pests in Indonesia, we can predict and justify any invasive species that enter to Indonesia, especially for those species that are morphologically difficult to be identified such as *Mythimna* group. Thus, by comparing their sequences, we can assist the quarantine staff at the entry points to justify the status species of pests correctly.

MATERIALS AND METHODS

Moth Specimens. A total of 9 species of *Mythimna* (6 described species and 3 undescribed species) of three subgenera were collected from Gunung Salak, West Java, Indonesia at 900 m above sea level (Table 3). Adult moths were collected by using light traps and were preserved in absolute alcohol (96%).

Species Identification. Species identification was conducted based on external and internal characters. The genitalia slide was prepared by the customary method of boiling in 10% potassium hydroxide for about 10-11 minutes. Dissection of genitalia was performed under a binocular stereoscopic microscope.

DNA Eextraction and Sequencing CO I Gene. For DNA extraction from each moth individual, a thorax was ground in a 1.5 ml microcentrifuge tube containing 600 ul CTAB buffer with 4% polyvinyl pyrrolidone and incubated at 55 °C for 2 hours. This solution was extracted three times using phenol saturated with TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA); firstly with one volume of phenol: Chloroform: iso-amyl alcohol (25:24:1). The solution was again extracted twice with chloroform: iso-amyl alcohol. The aqueous phase was transferred to a new tube, and then 1.5 volume of isopropanol was added to precipitate DNA and left at -20 °C for more than 1 hour. The DNA precipitant was pelleted by centrifugation at 13.000 rpm for 20 minutes. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 50 Ul of TE buffer.

The complete sequence primers used were LepF1: 5' ATT CAA CCA ATC ATA AAG ATA TTG G 3', and LepR1: 5' TAA ACT TCT GGA TGT CCA AAA AATCA 3' (Hebert *et al*. 2010). The amplification was conducted in the following PCR conditions: one cycle of denaturation at 94 °C for 10 min, followed by 35 cycles, with each cycle consisting of denaturation at 92 °C for 30 sec., annealing at 47 °C for 30 sec, and extension at 72 °C for 1 min. 30 sec. These cycles were completed by final extension at 72 °C for 10 min (Sutrisno 2008).

The PCR products were purified using Qiaquick PCR purification Kit (Qiagen. USA). Sequencing was performed using ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit (Perkin-Elmer) on ABI PRISM model 310 Genetic analyzer (PE Applied Biosystems). The sequence were alignment using BioEdit sequence alignment Editor (Hall 1999)

Base Composition Analysis. We used the base frequency's option in PAUP* version 4.0b. 10 for 32-bit Microsoft Windows to evaluate the base composition of each sequence and the homogenity of the base frequency across taxa. For the sequence divergence we chosen K2P distance model.

Phylogenetic Analysis. Phylogenetic analysis were performed with PAUP* version 4.0b. 10 for 32-bit Microsoft Windows based on CO I gene sequences by using maximum parsimony (MP), neighbor joining (NJ), and maximum-likelihood (ML) approaches (Swofford 2001). The statistical confidence of a particular clade in the ML was evaluated by puzzling score with 1000 replications while the other first two methods by using bootstrap test with 1000 replications.

Table 3. Moth species selected for molecular study (Classification based on Yoshimatsu 1994)

Species	Date of collection	Collector	Voucher specimen No.*	No. Acc genbank DDBJ
Mythimna (*Mythimna*) *albomarginata* Wileman & South	2.vii.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 0001	AB617655
Mythimna (*Mythimna*) *yu* Guenee	2.vii.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 0003	AB617656
Mythimna(Mythimna) *radiata* (Bremer)	26.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 0009.	AB617659
Mythimna (Mythimna) sp. A	26.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 00013	available on request
Mythimna (Mythimna) sp. B	26.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 00015	available on request
Mythimna (*Mythimna*) sp. C	27.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 00017	available on request
Mythimna (*Hyphilare*) *decisissima* (Walker)	27.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 0005	AB617657
Mythimna (*Hyphilare*) *epieixilus* Rothschild	28.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 0007	AB617658
Mythimna (Pseudaletia) pallidicosta Hampson	29.vi.2009	Sutrisno H & Darmmawan	Slide MZB. Lepi. 00011	AB617660

*all voucher specimens were deposited in Museum Zoologicum Bogoriense.

Maximum Parsimoy (MP). A band and bound search option or an exact search was applied in this MP analysis since our number of taxa was only 16. We evaluated several options based on different included characters in order to get the most reliable parsimonious tree. We tried also to apply weighting transversion substitutions in the MP analysis.

Neighbor Joining (NJ). To reconstruct Neighbor joining tree, we applied distances based on Kimura-two parameter (K2P) and HKY 85 models (Kimura 1980; Hasegawa *et al*. 1985).

Maximum-Likelihood (ML). In reconstructing ML tree, we applied a settings as follow: number of substitution types= 2 (HKY85 variant); Branch-length optimization = one-dimensional Newton-Raphson with pass limit = 20, delta=1e-00; -ln L (unconstrained) = 1898.51954; Starting tree(s) obtained via stepwise addition; Addition sequence: as-is.

For outgroup comparison, *Spodoptera litura* (No. Accession genbank: FN908025.1 GI:313185802) and *Spodoptera exigua* (No. FN908024.1 GI:313185800) from family Acronictinae were used. All species of the genus *Mythimna* which have been submitted to the Genbank whether present or absent in Indonesia were included in the analysis. They were *M*. (*Pseudoaletia*) *unipunctata* (No. GU574797.1 GI: 289166905), *M*. (*Mythimna*) *oxygala* (No. GU094655.1), *M*. (*Mythimna*) *curvula* (No. GU073226.1 GI: 261864025), *M*. (*Mythimna*) *phaea* (GQ353295.1 GI: 256857669), and *M*. *(Mythimna*) *pseudoloreyi* (GQ353296.1)

RESULTS

Base Composition. Sequences of 14 species of *Mythimna* and two species outgroups, *Spodoptera litura* and *S. exigua*, were aligned with no evidence of insertion and deletion. The conserved region was found at position between 148 and 170 (23 bp: CCTATTATAATTGGAGGA TTTGG). Aligned sequences have been submitted to the Genbank with accession numbers are as presented in the Table 3.

Table 4 shows that over entire 649 bp region 72% (470) of the nucleotide positions were constant, 10.6% (69) were uninformative (i.e. any variants were found in a single sequence and 16.9% (110) were parsimony informative. The informative site constituted in the 3rd codon position was the highest (14.02%), whereas in 2nd codon position was the lowest (0.15%).

Table 5 shows proportion of A-C-G-T of CO I gene, and its bias (*C*). The bias was calculated following Irwin *et al*. (1991) as follow:

$$C = \left(\tfrac{2}{3}\right)\sum_{i=1}^{4}\left|c_i - 0.25\right|$$

Table 4. Variable site percentages by codon position of CO I gene (values within bracket indicate the site numbers)

	1st codon	2nd codon	3rd codon
Constant (%)	25.94 (179)	32.81 (213)	12.01 (78)
Uninformative (%)	3.0 (20)	0.3 (2)	7.24 (47)
Informative (%)	2.77 (18)	0.15 (1)	14.02 (91)

where c_i is base frequency *i*. The results showed that the base composition was slightly A + T biased (*C*: 0.0052) with the average of A + T contents was 69%.

Interspecific variation in the base composition in CO I was very low for the total nucleotides. The chi-square test of homogeneity of base frequencies across taxa indicated that there was no significant difference in the frequency of bases between taxa (X^2 = 12.276, *df* = 45, *P* = 0.99).

Sequence Divergence. The mean of pairwise sequence divergences of CO I gene based on K2P distance model within subgenera *Mythimna*, *Pseudaletia*, and *Hyphilare* were 8.18, 8.82, and 5.32%, respectively. While that of between subgenera was slightly higher than that of within subgenera *Pseudaletia* (9.04%). The most close of it within subgenera was a pairwise between species *M*. (*Mythimna*) *radiata* with *M*. (*Mythimna*) sp. B (5.11%) while the most distant was between *M*. (*Mythimna*) *albomarginata* and *M*. (*Mythimna*) *yu*. A pairwise sequence between *M. curvula* and *M. pseudoloreyi* has no value and it indicates that these two species have the same sequence.

Figure 1 shows the relationship between pairwise distance for Transition (Ts) and Transversion (Tv) based on K2P distance model. Ts almost linearly increased with respect to Tv and exceed Tv in all pairwise species comparisons [except on pairwise *M*. (*Mythimna*) *curvula* and *M*. (*Mythimna*) *psudaloreyi*] and its linear regression was Y = 0.2197X + 0.0392; R^2 = 0.0727.

Figure 2 Shows the scatter plot of K2P distance between Transition/Transversion (Ts/Tv) and all substitutions in CO I gene. The means of Ts/Tv ratio in CO I gene was moderately high (1.26) for insect mitochondrial gene. The highest ratio was found on the pairwaise *M*. (*Hyphilare*) *epieixilus* and *M*. (*Hyphilare*) *decississima* (5.85) while the lowest on those *M*.

Table 5. Proportion of each nucleotide and the bias in CO I gene

	1st codon	2nd codon	3rd codon	Mean
A	0.299	0.146	0.442	0.2942
C	0.157	0.254	0.068	0.1589
G	0.267	0.173	0.012	0.1511
T	0.275	0.425	0.476	0.3957
A + T Bias				0.0052

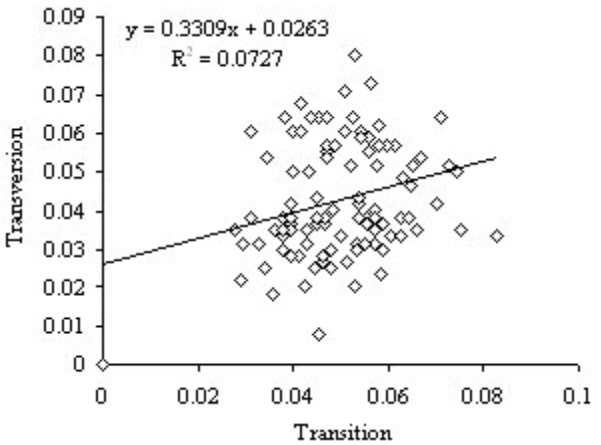

Figure 1. Scatter plots of K2P model distance for Transition (Ts) versus Transversion (Tv).

(*Pseudaletia*) *pallidicosta* and *M.* (*Pseudaletia*) *unipunctata*.

Phylogeny Maximum Parsimoy (MP). The results of MP analysis (Table 6) showed that there was no single topology tree that was able to resolve the relationships either among species within subgenera or between genera with a confident strong bootstrap support. Almost all strict concensus MP trees given agreed that there is no doubt about the monophyly of this genus (95% of bootstrap support) but they failed to show the relationships among species within genera or between subgenera except for the clade subgenera *Hyphilare* that always get strong bootstrap support (95%) at almost any analysis methods. One of the four MP trees resulted from all data substitutions of CO I gene are presented on Figure 3.

Neighbor Joining (NJ). There was no different in the topology, therefore only the NJ tree based on K2P model was presented in Figure 4. Based on the tree it seem that three subgenera of *Mythimna* based on morphological characters (Yoshimatsu 1994) was recovered but only subgenus *Hyphilare* that has a strong bootstrap support.

Maximum-Likelihood (ML). The single tree resulted from the analysis was exactly the same as one of the four MP trees resulted in MP analysis presented in (Figure 3).

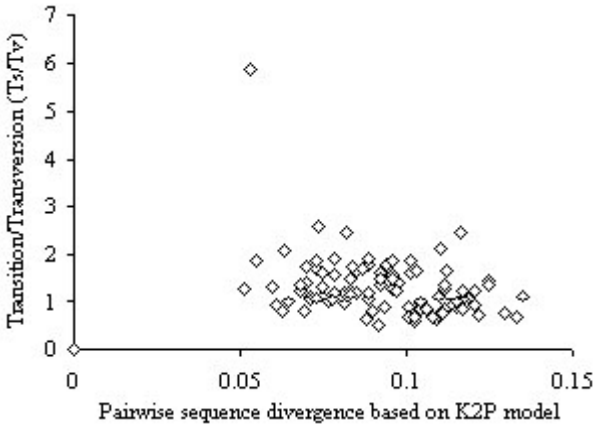

Figure 2. Scatter plots of pairwise sequence devergence based on K2P model versus Transition/Transversion (Ts/Tv).

DISCUSSION

The results of our study showed that CO I genes from 14 species of *Mythimna* was high A + T biased. It is consistent with mitochondrial genomes of other Lepidoptera previously reported by many authors (Reviewed in Simon *et al*. 1994). In other genera of Lepidoptera, high A + T contents have been found in CO I of *Choristoneura* (Sperling & Hickey 1994), *Hemileuca* (Rubinoff & Sperling 2002), *Glyphodes* (Sutrisno 2003; Sutrisno *et al*. 2006) which ranged from 62 to74%. The average of A + T proportion in the present study (69%) was comparable with those found in other genera of Lepidoptera. In addition, the bias in base compositions

Table 6. Results of evaluation of several options on characters in parsimonious tree building method

Character included	Parsimonious informative	MP tree	Tree length	CI	RI	HI	Robustnessof topology
All codon	110	4	428	0.557	0.423	0.446	Low
1st pos only	18	1591	63	0.634	0.365	0.520	Worst
2nd pos only	1	1	3	1.000	1.000	0.000	Worst
3rd pos only	91	3	351	0.552	0.443	0.447	Worst
1st + 2nd pos	19	1	68	0.632	0.537	0.367	Worst
2st + 3rd pos	92	3	358	0.550	0.431	0.449	Worst
1st + 3rd pos	109	1	421	0.555	0.424	0.444	Worst
Transversion only	107	3	184	0.505	0.480	0.494	Low
TI:TV = 1:2	114	3	616	0.535	0.435	0.464	Low
TI:TV = 1:3	114	1	802	0.527	0.444	0.472	Worst

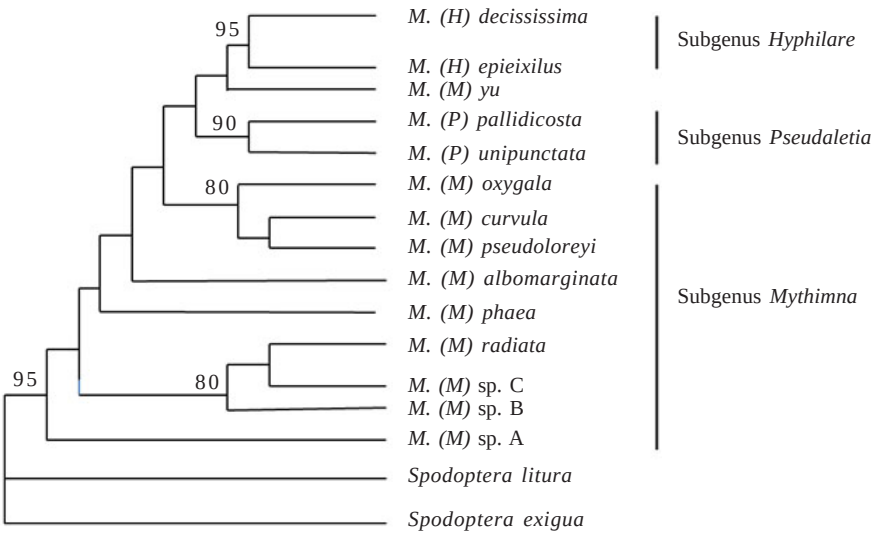

Figure 3. One of 4 MP trees resulted from all substitutions of CO I gene (Bootstrap supports are shown only for the nodes which have value > 50%).

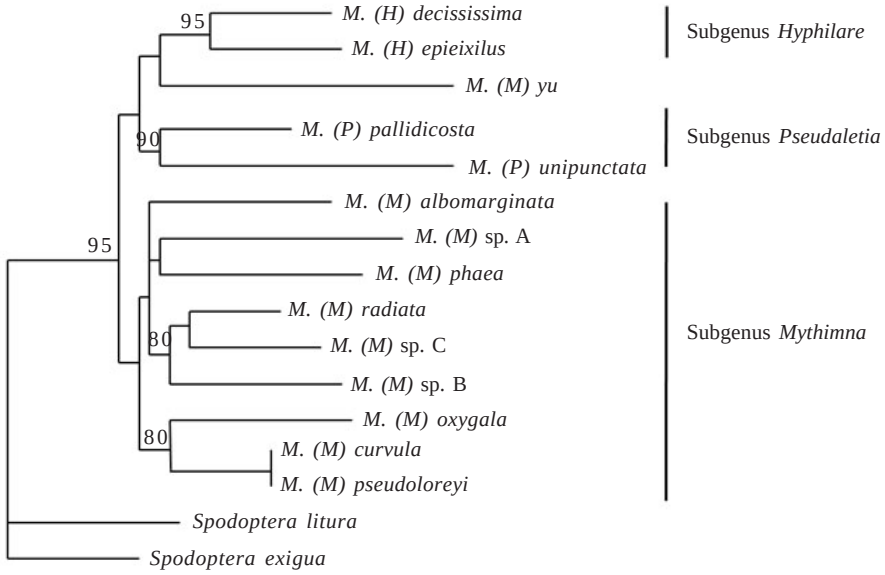

Figure 4. Neighbor joining tree based on K2P distance model of all substitutions of CO I gene (Bootstrap supports are shown only for the nodes which have value > 50%).

was found to be the greatest at third-base position. This perhaps because first- and second-codon position are more constrained by the amino acid composition of the encoded protein (Brown *et al*. 1994).

Moreover, based on the pairwise sequence identity, it is clear that *Mythimna* (*Mythimna*) *curvula* and *Mythimna* (*Mythimna*) *psedoloreyi* having exactly the same sequence. This study supports Holloway's idea (1989) that synonimise *pseudoloreyi* as *curvula*. However, in the genbank these two species are treated as different species. In general view, two individual specimens will be treated as difference species when their sequence of CO I gene have similarity <96%. Thus, there is no reason to treat them into different species since they have the same sequence.

The sequence divergence of CO I gene within subgenera was relatively high (5.32-8.82%). It indicates that each subgenera within genus *Mythimna* consists of a large number species and very diverse, especially within subgenus *Mythimna* (*Mythimna*). These values, however, comparable with those found within group of *Glyphodes* (5.92-7.55%) (Sutrisno 2006).

The present study revealed that the transition/ transversion ratio of CO I within *Mythimna* was moderate (1.26). It indicates that Transitions (Ts) occur more frequently than Transversions (Tv), and Ts values are usually expected to exceed Tv values (DeSalle *et al*. 1987; Kondo *et al*. 1993); however, it has been reported for some mitochondrial DNA that Tv values exceed Ts values (Xiong & Kocher 1993; Simon *et al*. 1994; Goto & Kimura 2001). This is primarily because transversions erase the record of transitions after genes are saturated with the latter (Irwin *et al*. 1991; Beckenbach *et al*. 1993; Simon *et al*. 1994). By contrast, the CO I in this study indicated that this gene was not yet saturated with transitions (Figure 3). This finding also supports the general view that observed transition exceed transversion only when recently diverged species or slowly evolving gene are compared (Irwin *et al*. 1991; Simon *et al*. 1994).

The results of this study showed that the MP, NJ, and ML analyses based on CO I recovered the three subgenera: *Mythimna* (*Mythimna*), *Mythimna* (*Hyphilarea*), and *Mythimna* (*Pseudaletia*). The monophyly of this genus was consistently found in any tree building methods and was supported by 95% of bootstrap test. However, subgenus *Mythimna* (*Mythimna*) was shown to be paraphyletic group in term of *Mythimna* (*Hyphilare*) and *Mythimna* (*Pseudaletia*). The relationships among subgenera within *Mythimna* were not consistently found within MP, NJ, and ML tree building methods and they were also supported by low bootstrap values for each internal nodes except for the clade *Mythimna* (*Hyphilare*).

There are many possibilities for way that this CO I gene resulted in inconsistency tree topologies in different tree building methods and given low support bootstrap for their relationships. There is no doubt that MP, NJ, and ML tree building methods will produce different topology since they have different in algorithms. MP tree analysis usually produces more than one MP trees while NJ as well as ML always produce in a single tree. Only very clean data will resulted in a similar topology tree with consistent strong bootstrap supports in different tree building methods. This study showed that CO I gene alone was not enough to produce synapomorphies on each node. Even though the number of informative site was high (16%), it may be a lot of conflict data which resulted in inconsistency tree in the MP analysis. So, it is for why when using transversions only or weighting transversions 2:1 or 3:1 in the MP analysis, the resolution was not improving. Previous study showed the mitochondrial gene CO I was very useful when combined with CO II to resolve the relationships in *Argyrotaenia franciscana* species group (Tortricidae) (Landry *et al*. 1999), *Choristoneura fumiferana* species group (Sperling & Hickey 1994), and genus *Papilio* (Caterino & Sperling 1999). In addition, the combination of the CO I and *EF-1*α increased resolution and supports most of the phylogenetic relationships

suggested by separate analysis of each gene in the genus *Hemileuca* and *Glyphodes* in any different tree building methods (Rubinoff & Sperling 2002; Sutrisno *et al*. 2006). The low bootstrap test on each node in any tree building methods was also possibly caused many conflicts among the sequence in CO I due to the lack of species sampling in the analysis. There is not enough information how many species of *Mythimna* in Indonesia but we beleive that Indonesian *Mythimna* is only a small part of the whole *Mythimna* in the world. This is a common problem in MP tree building method, that the lack of species sampling will resulted in inconsistency tree topology and other problems such as a branch length attraction. These problems can be resolved only by increasing the number of sample species in the analysis to reduce the distance sequences (Nei & Kumar 2000; Yang 2008). The closely-related distances among sequences will produce a robust phylogenetic relationship as indicated in the subgenus *Mythimna* (*Hyphilare*) which always has a consistent strong bootstrap support in any tree building methods. Morphologically, its closest-relatedness was supported by a good synapomorphy (tufts of black hairs on the male basal abdominal segment) (Yoshimatsu 1994).

In general, all the findings in the present study suggest that phylogeny of Indonesian *Mythimna* based on mitochondrial CO I gene recovered the three subgenera based on morphology (Yoshimatsu 1994). *M*. (*Mythimna*) was shown to be a paraphyletic group in term of *M*. (*Hyphilare*) and *M*. (*Pseudalteia*); *M*. (*Mythimna*) was branched off first then followed by *M* (*Pseudaletia*) and *M*. (*Hyphilare*). However, all internal nodes gained least supports except for the monophyly of subgenus *M*. (*Hyphilare*). It indicates that the relationships among internal nodes proposed here were least valid due to the number of species included in the analysis which may not be enough to represent the real number of species in the nature. Further studies are needed to be done by including more other species and other mitochondrial genes in order to test the validity of the relationships proposed here.

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