

Molecular phylogeny of Indonesian *Zeuzera* (Lepidoptera: Cossidae) wood borer moths based on CO I gene sequence

Hari Sutrisno*

Laboratory of Entomology, Division of Zoology, Research Center for Biology, The Indonesian Institute of Sciences, Jl. Raya Bogor Km 46 Cibinong 16911

*Correspondent: sutrisnohari@yahoo.com

Zeuzera is one of the most important wood borer pests in South East Asia. Like of most other genera of moths, the systematic of this genus is still in dispute, especially on the monophyly and the relationship within this genus due to the fact that genus is very varied. This genus was defined based on external characters only such as cross vein Sc-Rs present, humeral plate approximately triangular in shape and anal plate moderately long to moderately short. Therefore, the monophyly of this genus need to be evaluated based on more comprehensive data. To clarify the monophyly of the genus *Zeuzera*, to reveal the phylogenetic relationships among the Indonesian species, and to establish the genetic characters of Indonesian *Zeuzera*, we analyzed seven species of Indonesian *Zeuzera* including three other species distributed around the world based on nucleotide sequence variation across a 580-bp region in the CO I gene. The results showed that the monophyly of *Zeuzera* was supported by bootstrap tests at the MP and ML tree building methods (>95%). Genus *Zeuzera* was divided into two groups (A and B) with *Z. borneana* was excluded from the two groups and occupied at the basal node. Indonesian species was distributed into two different clades. CO I gene alone was able to fully resolve the relationships among species within clade B. However, further investigations were needed by including more species and other genes that the more conserved to test the validity of the phylogenetic hypothesis proposed here.

Keywords: CO I gene, Cossidae, Lepidoptera, phylogeny, *Zeuzera*

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INTRODUCTION

Zeuzera is one of the most important wood borer pests in South East Asia. Nearly all species live as larva in plants. Most species bore in trunk or branches of trees. Larval wood tunnel the heartwood of living trees. They create large holes in the timber which degrades its value. The development from an egg to an adult can take several years during which the larvae create a J-shaped tunnel of very large diameter. The large holes usually cause smaller trees to become more susceptible to wind damage. They have a very wide host range such as Coffee (*Coffea* sp.), Tea (*Camellia sinensis*), Cotton (*Gossypium* sp.), Cocoa (*Theobroma cacao*), Kapok (*Ceiba petandra*), Teak (*Tectona grandis*), Avocado (*Persea gratissima*), Guaya (*Psidium* sp.), and Mahogany (*Swietenia* sp.) (Toxopeus, 1948; Arora, 1976). In Indonesia, *Z. coffeae* has been reported to cause serious damage to plants of economic importance. This species has been reported to cause damage in range 3.7-5.2% in the teak plantation in

Rumpin, West Java (Herdiana, 2010). This species can also cause some damage on a wide range of other hosts including *Eucalyptus deglupta*, *Terminalia brassii*, and species of *Acalypha*, *Psidium*, *Crataegus*, *Citrus*, *Theobroma* and *Casuarina* Moreover, *Z. conferta* causes damage on Cocoa (*Theobroma cacao*), Balsa, and *Berringtonia* (Arora, 1976).

Genus *Zeuzera* is a small group with wide distributional range from Holarctic, Indo-Australian to New world. It has been studied intensively by Roepke (1955; 1957) based on New Guinea and Malayan fauna and Holloway (1986) based on Bornean fauna. The latter defined this genus based on several diagnostic characters such as the thorax and abdomen are greyish white, spotted and/or banded regularly in black, the thorax usually with three pairs of spots, and the association of hindwing vein Sc with the cell. The male genitalia, especially the apical ornamentation of the aedeagus, are similar to those of *Xyleutes*. He suggested that this genus may well be merely a well-defined section of *Xyleutes* rather than the sister-genus because of the worldwide diversity of geni-

talia characters and the great variety in wing facies of the species.

Schoorl (1990) made a tremendous work in reconstructing the phylogenetic relationship of Cossidae based on morphological characters. He involved the wing base and thoracic sclerites characters in his study rather than genitalia characters since the genitalia characters of Cossidae appear mostly to be of a generalized primitive apoditryasian type with few or no special characteristics. Most genera of Cossids have no diagnostic characters on color pattern or wing venation. He proved that establishing new genera basis on head appendages, color pattern or wing venation has resulted in confusion and some genera become large and heterogeneous groups. For example, he divided a large and heterogeneous *Xyleutes* into several small genera (*Xyleutes*, *Bergaris*, *Chalcidica*, *Rapdalus*, etc.); each of genera was defined based on good apomorphy characters.

Moreover, in his study, several characters thought to be apomorphies of genus *Zeuzera* was presented as well as the relationship within this genus based on his hand cladogram (Fig. 1). All the apomorphy characters of this genus were presented in Table 1. This genus was defined based on cross vein Sc-Rs present, humeral plate approximately triangular in shape and anal plate moderately

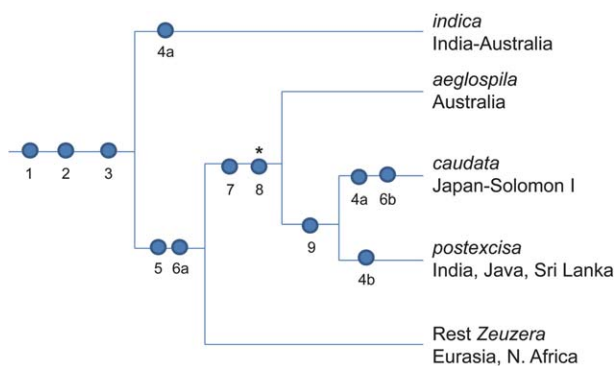


Fig. 1. Cladogram of *Zeuzera* (Schoorl, 1990) (all the numbers showed in each branch are apomorphies characters that are listed in Table 1).

long to moderately short. He also synonymized *Z. celebensis* with *Z. caudata* and proposed 14 valid species names belong to this genus (Table 2). However, this hypothetical phylogeny of this genus proposed by Schoorl (1990) is necessary to be evaluated by using a computer program with evolving more data to the test the validity of the proposed phylogeny of the genus *Zeuzera*.

There is no doubt that morphological characters are very important to recognize the identity genus but it is not always easy when deal with various species within genus. A lot of morphological characters are probably useful, such as the wing base and thoracic sclerite characters, not only to confirm identity species but also to reconstruct the phylogeny relationship among them. However, it is often difficult to score these characters due to complexity of their structure. The other problem is the objectivity of the observers, different observers will give different result when they work on the same sample specimen. It is very difficult to re-asses one of the proposed apomorphies of *Zeuzera* (anal plate moderately long to moderately short). The measurement of this character state: moderately long to moderately short is very subjective.

Molecular approach is one of the alternatives that can be applied to fill that gap. The huge number of characters resulted from a certain gene sequence is very powerful not only to differentiate among species within a large and varied genus but also to resolve the phylogenetic relationships among them, from lower to higher level. Among them, COI is more conserved and it 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%. Moreover, CO I gene is one of the most common to be considered in inferring the relationships among closely-related species in several groups of Lepidoptera, as individual gene or its combination with other genes (Sutrisno *et al.*, 2006; Yamamoto & Sota, 2007; Tsao & Yeh, 2008; Kim *et al.*, 2010).

In order to clarify the monophyly of the genus and the relationships of Indonesian *Zeuzera* within this genus, we used mitochondrial CO I gene sequence to reconstruct the relationships among seven species of *Zeuzera* which

Table 1. Apomorphies of *Zeuzera* based on Schoorl (1990).

No	Apomorphies
1.	Cross-vein Sc-Rs present
2.	Humeral plate approx. triangular in shape
3.	Anal plate moderately long to moderately short
4a.	Median arm moderately long to rather short
b.	Median arm rather long, with the anterior invagination up to $\frac{1}{4}$ its length
5.	Accessory plate II moderately wide to narrow or only narrow
6a.	Humeral plate at most 1.3 times size of radial bridge
b.	Humeral plate 0.8 to 1 time size of radial bridge
7.	Vestiture on ♀ antenna in longitudinal rows
8.	R1 proximal to distinctly from areole
9.	Anal region of hindwing prominently extended in the male, and in the female of <i>postexcisa</i> and <i>caudata</i>

Table 2. Treatment of the member of *Zeuzera* by Roepke (1955), Holloway (1986) and Schoorl (1990).

No	Roepke (1955; 1957)	Holloway (1986)	Schoorl (1990)
1	<i>Z. indica</i>	<i>Z. indica</i>	<i>Z. indica</i>
2	<i>Z. roricyanea</i>	<i>Z. conferta</i> <i>Z. roricyanea</i> <i>Z. neuropunctata</i>	<i>Z. conferta</i> <i>Z. roricyanea</i> <i>Z. neuropunctata</i>
3	<i>Z. coffeae</i> Nietner, 1861 <i>Z. reticulata</i> <i>Z. buergersi</i>	<i>Z. coffeae</i> Nietner, 1861 <i>Z. oblita</i>	<i>Z. coffeae</i> <i>Z. oblita</i> <i>Z. reticulata</i> <i>Z. buergersi</i>
4	<i>Z. caudata</i> <i>Z. rhabdota</i>	<i>Z. caudata</i> <i>Z. rhabdota</i>	<i>Z. caudata</i> <i>Z. rhabdota</i> <i>Z. celebensis</i>
5		<i>Z. lineata</i>	<i>Z. lineata</i>
6		<i>Z. borneana</i>	<i>Z. borneana</i>
7			<i>Z. aeglopsila</i>
8			<i>Z. potexcisa</i>
9			<i>Z. pyrina</i>
10			<i>Z. biebengeri</i>
11			<i>Z. multistrigata</i>
12			<i>Z. nepalense</i>
13			<i>Z. yuenani</i>
14			<i>Z. nuristanensis</i>

are distributed in Indonesia by involving other three species from other world. The results of the study also will give benefit to the efforts in establishing the genetic characters of Indonesian *Zeuzera*. By establishing the data of the genetic identity of these pests in Indonesia, we can predict and justify any invasive species that enter Indonesia, especially those species that are morphologically difficult to be identified such as *Zeuzera* group. Thus, by comparing their sequences, we can help the quarantine staff at the entry points to justify the status of pest species correctly.

MATERIALS AND METHODS

Moth specimens

A total of seven species of *Zeuzera* were collected from different localities in Indonesia. Adult moths were collected by using light traps and were preserved in absolute alcohol (96%). All the species used in this study were presented in Table 2.

Species identification

Species identification was conducted based on external and internal characters. The genitalia slide was prepared

by the custom method of boiling in 10% potassium hydroxide for about 10-11 minutes. Dissection of genitalia was performed under a binocular stereoscopic microscope.

DNA extraction and sequencing CO I gene

A non-destructive method which is modification from QIAGEN animal tissue protocol kit using spin column was used for DNA extraction from each moth individual (Sutrisno, 2012a). Firstly, the abdomen was removed from the body then was placed into a sterile 1.5 microtube and added 0.1 mL proteinase K (PK) 1% buffer (1% PK buffer=20 µL proteinase K solution (20 mg/mL) in 180 µL buffer ATL (QIAGEN)). This abdomen then was incubated at 55°C for 2-4 hours and was added a further 0.1 mL PK buffer and was incubated at 55°C for overnight. The next morning the abdomen was removed for morphological work. The tube containing the insect mixture was then treated by following the manual of QIAGEN animal tissue protocol kit using spin column.

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' 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

Table 3. Species of *Zeuzera* selected for molecular study.

No	Species	Collector	Voucher specimen no	No.acc genbak
1	<i>Z. coffeae</i>	Cholik & Momoh	MZB.Lepi.121	AB935215
2	<i>Z. conferta</i>	Darmawan	MZB.Lepi.122	AB935216
3	<i>Z. indica</i> 1	H. Sutrisno & Sarino	MZB.LIPI.123	AB935217
4	<i>Z. borneana</i>	Cholik	MZB.Lepi.124	AB935218
5	<i>Z. lineata</i>	H. Sutrisno	MZB.Lepi.125	AB935219
6	<i>Z. caudata</i>	H. Sutrisno	MZB.Lepi.126	AB935220
7	<i>Z. indica</i> 2	Darmawan	MZB.Lepi.128	AB935221
8	<i>Z. aeglopsila</i>	E.D. Edwards, H. Sutrisno	10ANIC-09445	HQ952096.1
9	<i>Z. pyrina</i>	A. Hausmann	BC ZSM Lep 22415	HM393503.1 GI:300203742
10	<i>Z. queita</i>	Nielsen, Edwards, Horak	10ANIC-09449	HQ952098
11	<i>M. pyracmon</i>	R. Franco & F. Quesada	–	JQ577804
12	<i>M. ramosa</i>	R. Franco & H. Cambroner	–	JQ571050

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 (Hebert *et al.*, 2010; Sutrisno, 2008; 2011; 2012a; 2012b).

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 was 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 homogeneity of the base frequency across taxa (Swofford, 2001). For the sequence divergence we chose K2P distance model.

Phylogeny reconstruction

Phylogeny reconstructions were performed with MEGA version 5.10 for Maximum Likelihood (ML) tree building method (Tamura *et al.*, 2011) and with PAUP* version 4.0b.10 for 32-bit Microsoft Windows for MP tree building method (Swofford, 2001). The statistical confidence of a particular clade in all the tree building methods was evaluated by using bootstrap test with 1000 replications.

RESULTS

Base composition

Sequences of 10 species of *Zeuzera* and two species outgroups, *Morpheis ramosa* and *M. pyracmon* were aligned (580-bp) with no evidence of insertion and dele-

tion. The conserved regions were found at position: 126 ATAATTGGAGGATT 139; 261 GAAAATGGAGC 271; 393 AATTTTATTAC 403; 408 ATTATTAATATACG 421. Aligned sequences have been submitted to the genbank with accession numbers presented in the Table 2.

Table 3 shows the 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: $C = (2/3) \sum_{i=1}^4 |c_i - 0.25|$, where c_i is

base frequency i . The results shows that the base composition was A+T biased (C: 0.06564) 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 indicates that there was no significant difference in the frequency of bases between taxa (Chi-square=8.217388 (df=33), P=0.99999666).

Sequence divergence

The mean of pairwise sequence divergences of CO I gene based on K2P distance model within Group A and B were 10.20% and 16.47%, respectively. The closest relationships within group A was a pairwise between species *Z. conferta* and *Z. lineata* (1.04 %), while in the Group B was between species *Z. caudata* and *Z. aeglopsila* (5.7%). The mean of sequence divergence within this genus was very high (20.09%).

Fig. 2 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 and its linear regression was $Y = 0.5831x + 0.0272$; $R^2 = 0.9209$.

Fig. 3 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 was low

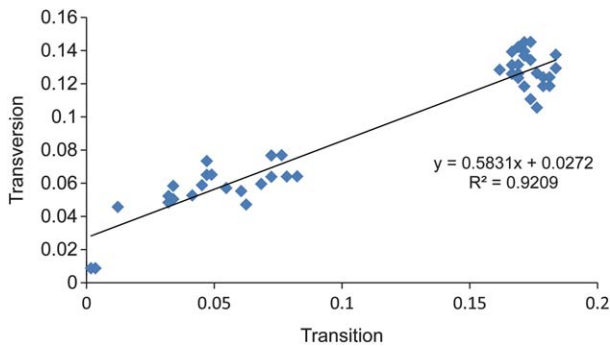


Fig. 2. Scatter plots of K2P model distance for Transition (Ts) versus Transversion (Tv).

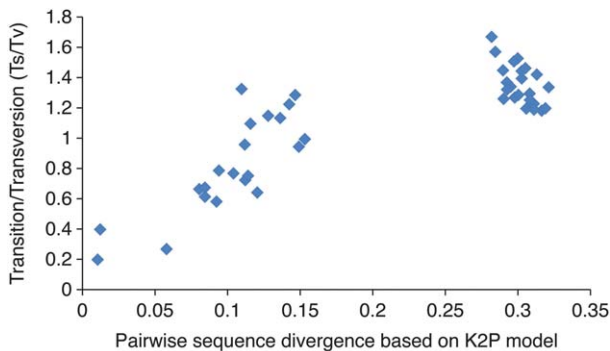


Fig. 3. Scatter plots of pairwise sequence divergence based on K2P model versus Transition/Transversion (Ts/Tv).

(1.10) for insect mitochondrial gene. The lowest ratio was found on the pairwise of species within Group B: between *Z. aeglopsila* and *Z. caudata* (0.267834).

Maximum parsimony (MP)

The results of MP analysis (Table 6) showed that almost all strict consensus MP trees given agreed that there is no doubt about the monophyly of this genus (>95% of bootstrap support). *Zeuzera* was divided into three groups with *Z. borneana* was excluded from them and occupied at the basal node. The three clades were: A1 (*cofeae*+*queita*+*pyrina*), A2 (*conferta*+*lineate*) and B (*indica* 1 +*indica* 2)+(*aeglopsila*+*caudata*). The relationships among species within this genus with a confident strong bootstrap support were found within clade B. The 50% majority-rule consensus tree resulted from all substitution of CO I gene are presented in Fig. 4.

Maximum-Likelihood (ML)

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985). Initial trees for the heuristic search were obtained automatically by

Table 4. Variable site percentages by codon position of CO I gene.

	1 st -codon	2 nd -codon	3 rd -codon	Total
Constant (%)	125	152	51	328
Uninformative (%)	11	5	39	55
Informative (%)	57	36	103	196

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

	1 st codon	2 nd codon	3 rd codon	Mean
A	0.33420	0.14663	0.42850	0.30311
C	0.16839	0.26632	0.07254	0.16908
G	0.24611	0.15596	0.01088	0.13765
T	0.25130	0.43109	0.48808	0.39016
A+T Bias				0.06564

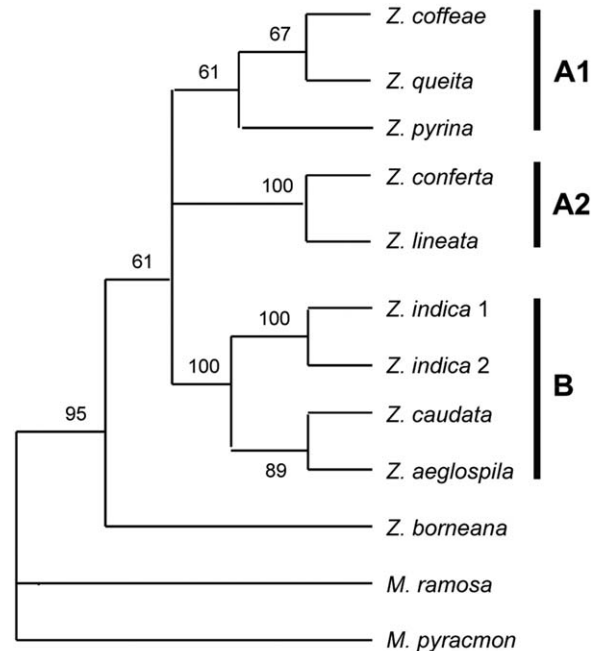


Fig. 4. A 50% majority-rule consensus tree based on all substitutions of CO I gene (Bootstrap support are shown only for the nodes which have value > 50%).

applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter=0.3386)). The tree with the highest log likelihood (-2679.7040) (Fig. 5) was almost the same as the 50% majority-rule consensus tree resulted from all substitution of CO I gene in the MP tree building method except for the position the group A1 and A2 which forming a single group A.

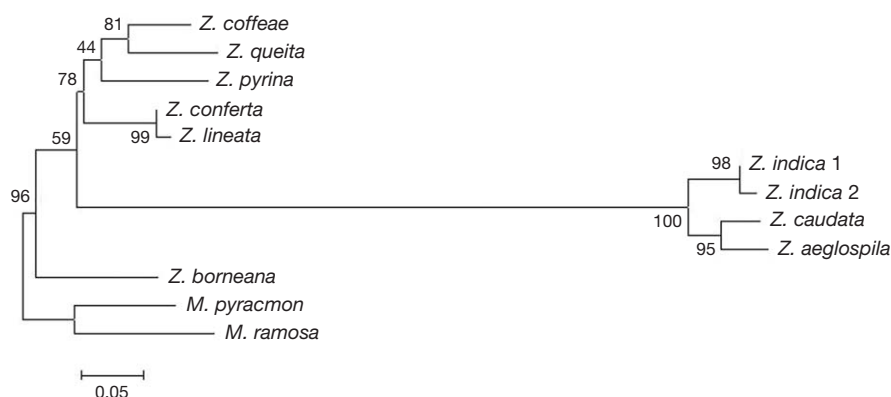


Fig. 5. Maximum likelihood tree for 10 species of *Zeuzera* based on all substitution of CO I gene (Bootstrap support are shown at the nodes).

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	Robustness of topology
All codon	196	2	451	0.6807	0.7382	0.3193	Moderate
1 st pos only	57	1	106	0.7170	0.8171	0.2830	Low
2 nd pos only	36	6	48	0.9375	0.9741	0.0625	Worst
3 rd pos only	103	3	294	0.6327	0.6000	0.3673	Moderate
1 st +3 rd pos	160	1	401	0.6534	0.6797	0.3466	Moderate
3 rd +2 nd pos	139	1	344	0.6715	0.7073	0.3285	Moderate
1 st +2 nd pos	93	2	155	0.7806	0.8786	0.2194	Low
TI:TV=1:2	182	1	669	0.6547	0.7227	0.3453	Moderate
TI:TV=1:3	182	1	895	0.6492	0.7255	0.3508	Moderate

DISCUSSION

The result of our study showed that CO I genes from 10 species of *Zeuzera* was a 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 *Helicoverpa*; *Glyphodes*, *Mythimna*, *Lymantria* (Kranthi *et al.*, 2006; Sutrisno *et al.*, 2006; Sutrisno, 2012b; 2014) which ranged from 62% to 74%. 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 was found to be the greatest at the third-base position. This perhaps because first- and second-codon position were more constrained by the amino acid composition of the encoded protein (Dorp, 2004; Zhang *et al.*, 2011).

The sequence divergence of CO I gene within each group was relatively high (10.20%-16.47%). It indicates that two groups within genus *Zeuzera* was very diverse, especially within Group A. These values were higher with those found within group of *Glyphodes* (5.92-7.55%) and subgenera within *Mythimna* (5.32-8.82%) (Sutrisno *et al.*, 2006; Sutrisno, 2012b). Even the mean of pairwise

sequence divergence between the two groups (A and B) was very high (20.09). It is very rare that the sequence divergence of CO I gene within genus in Lepidoptera was more than 15% except in very large and varied genus *Lymantria* (Sutrisno, 2014). It assumes that the group A and B within *Zeuzera* has split and evolve for a long time.

The present study revealed that the transition/transversion ratio of CO I within *Zeuzera* was low for mitochondrial insect gene (1.10). It was indicated by Transitions (Ts) occur slightly higher than Transversions (Tv). In some mitochondrial DNA of insect, Tv values exceed Ts values when transversions erase the record of transitions after genes are saturated with the latter (Dorp, 2004; Roe & Sperling, 2007). In contrast, the CO I in this study indicated that this gene was not yet saturated with transitions (Fig. 3). This finding also supports the general view that observed transition exceed transversion only when recently diverged species or slowly evolving gene are compared (Dorp, 2004; Sutrisno *et al.*, 2006).

The topology resulted in the 50% majority-rule consensus tree based on all substitution of CO I gene in the MP tree building in this study was almost similar with those found in a ML tree except of the position Group A. In the MP and ML analysis, species *indica* was placed into clade B. Within this clade B, the relationship among species was well resolved with strong bootstrap supports.

Species *indica* branched off first then followed by *Z. caudata*+*Z. aeglopsila*. On the other hand, the relationship among species within clade A is not clear, especially in the MP tree building method, except for the relation closely-related species *Z. lineata*+*Z. conferta* and *Z. coffeae*+*Z. queita*. Some of these findings were not agreed with the hand cladogram based on morphological characters that was conducted by Schoorl (1990). He established monophyly of *Zeuzera* by placing species *indica* which has a wide distribution, from India to Australia, at the basal node based on a single automorphy: median arm moderately long to rather short (Table 1: 4a). On the other hand, the rest of *Zeuzera* (*borneana*, *pyrina*, *queita*, *coffaeae*, and *lineata*) was separated from the *indica* group in this study.

Moreover, the results of the study showed that the seven Indonesian species were evolved independently and distributed into two clades. Those species are only a half part of the *Zeuzera* (50%) that have been distributed world-widely for a long time.

There are many possible reasons for way that this COI gene resulted in inconsistency tree topologies in different tree building methods and given low support bootstrap for their relationships for clade A. Only very clean data will result in a similar topology tree with consistent strong bootstrap supports in different tree building methods. This study showed that CO I gene alone was able to produce synapomorphies to support the monophyly of *Zeuzera* and also was able to show a good support for a certain closely-related species in clade B: (*Z. indica*+*Z. ecelebnis*)+(*Z. caudata*+*Z. aeglopsila*). Previous study showed the mitochondrial gene *CO I* was very useful when combined with *Cytochrome b* to resolve the relationships in the genus *Morpho* (Nymphalidae) (Cassildé *et al.*, 2012), or when combined with *CO II* in the genus *Papilio* (Caterino and 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 *Ectoedemia* s. str. (Lepidoptera: Nepticulidae) (van Nieukerken *et al.*, 2012).

The low bootstrap test on each node in group A was also possibly caused by many conflicts among the sequence in CO I due to the lack of species sampling in the analysis. We believed that *Zeuzera* included in this analysis is only a part of the whole *Zeuzera* in the world or about 75% (10 of 14 species). These problems can be resolved only by increasing the number of sample species in the analysis to reduce the distance sequences and also by involving gene having slow evolutionary rate (Nei & Kumar, 2000; Yang, 2008). The closely-related distances among sequences will produce a robust phylogenetic relationship as indicated in the closely-related species within clade B or between *Z. lineata* and *Z. conferta* within clade A which always has a consistent strong

bootstrap support in any tree building methods.

In general, all the findings in the present study suggest that monophyly of *Zeuzera* supported by bootstrap support and there were apomorphy characters (cross-vein Sc-Rs present, humeral plate approx. triangular in shape, and anal plate moderately long to moderately short to support this genus. Phylogenetic analysis of 10 species of *Zeuzera* based on mitochondrial *CO I* gene was able to resolve the relationships among species within *Zeuzera* especially for the clade B. Moreover, the Indonesian *Zeuzera* species distributed into two clades: A and B. However, all internal nodes gained least supports except for the monophyly of clade B. 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 more conserved genes in order to test the validity of the relationships proposed here.

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