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Molecular Phylogeny of *Agrioglypta* Meyrick and *Talanga* Moore (Lepidoptera: Crambidae; Spilomelinae) Inferred from Nuclear *EF-1α* Gene

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The phylogeny of the two closely- related genera, *Agrioglypta* Meyrick and *Talanga* Moore, was inferred from nucleotide sequence variation across a 973-bp region in the nuclear *elongation factor-1α* (*EF-1α*) gene. Seven species representing the two genera and two outgroup species (*Feltia jaculifera* Guenée and *Metallarcha aureodiscalis* Meyrick) were analyzed. The results showed the averages of the *p*-distances in the comparisons between species within genus and between species belonging to other different genera were 3.5% and 4.9%, respectively. *EF-1α* gene had almost reached saturation at the level of the divergence of these two genera. The phylogenetic analysis using MP and NJ methods showed that each genus was found to be a monophyletic group and the species relationships within each genus were almost consistent as well. *A. eurytusalis* is the basal species in the genus *Agrioglypta*. In the genus *Talanga, T. sabacusalis* lied in the basal node and *T. tolumnialis* was found to be sister group of *T. sexpunctalis*.

INTRODUCTION

It has been suggested that the two moth genera, *Agrioglypta* Meyrick, and *Talanga* Moore are poorly defined. The previous taxonomic studies, largely from early this century (Hampson 1896), were based only on external characters (Common 1990). As other Spilomelinae, these two genera are less studied and no comprehensive efforts have been made on their taxonomy and their phylogeny except for a preliminary study on the relationships of these two genera based on morphological characters and nucleotide sequence variation in mitochondrial *cytochrome oxidase II* (*COII*) (Sutrisno 2004).

The preliminary study showed that the analysis based on morphological character and *COII* gene failed to resolve the relationships among these two genera; *Agrioglypta* was shown to be paraphyletic in term of *Talang* (Sutrisno 2004). The genitalia structures of these two genera were very complex and difficult to be scored as informative characters and the mitochondrial *COII* gene might attain saturation at the level of divergence of these two genera. Therefore, a more conserved gene such as nuclear genes should be used to resolve the relationships among these genera.

Among nuclear genes, *EF-1α* is the most favored to infer the phylogeny in Lepidoptera at different level of taxa. Those ranging from closely-related species (Monteiro & Pierce 2001; Morinaka *et al*. 2002; Rubinoff & Sperling 2002) to relationships among genera or tribes (Cho *et al*. 1995; Friedleander *et al*. 1998) and even at family level (Caterino *et al*. 2001).

In the present study, I used the exon region of *EF-1α* gene to infer the phylogeny of the two closely-related genera, *Agrioglypta* and *Talanga*. This is due to the rate of substitutions in *EF-1α* had been estimated slower than in mitochondrial genes in another group of Lepidoptera (Reed & Sperling 1999). Applying this approach, I expect that a similar rate of the gene in these two genera would give better resolution at the basal level on the relationships between these two genera than mitochondrial genes do.

MATERIAL AND METHODS

Moth Specimens. A total of seven species representing the two genera and one species outgroup were collected from various localities in Indonesia and Australia (Table 1). Adult moths were collected by using light traps and were preserved in absolute alcohol.

DNA Extraction and Sequencing of *EF-1α* Gene. For DNA extraction from each moth individual, a thorax was ground in a 1.5 ml microcentrifuge tube containing 600 µl CTAB buffer with 4% polyvinyl pyrrolidone and incubated at 55 °C for 2 hours. The solution was extracted several 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

Table 1. Moth species selected for molecular study

Genera	Species	Locality	Accession numbers
Agrioglypta	*excelsalis* Walker	Menado, Sulawesi	AB 158396
	eurytusalis Walker	Pangrango NP, West Java	AB 158394
	itysalis Walker	Halimun NP, West Java	AB 158397
	naralis Felder & Rogenhofer	Halimun NP, West Java	AB 158395
Talanga	*sabacusalis* Walker	Patunuang, Sulawesi	AB 158400
	sexpunctalis Moore	Bantimurung, Sulawesi	AB 158399
	tolumnialis Walker	Bucasia, Queensland	AB 158398
Metallarcha	*aureodiscalis* Meyrick	Bucasia, Queensland	AB 158377

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 15 300 g for 20 minutes. The DNA pellet was washed with 70% ethanol, air dried, and dissolved in 50 µl l of TE buffer.

Due to difficulties to amplify *EF-1α* gene for a total of 973 bp, two pairs of primers, namely, EF 44 and EF Mid, and EF M51.9 and EF rcM44 (Cho *et al*. 1995) were used. The first pair was successfully amplified approximately 493 bp whereas 480 bp were amplified by the second pair of primer. The complete sequence primers used were EF 44: 5'-GCYGARCGYARCGTGGTATYAC-3' (2277), EF Mid: 5'-CAATACCRCCRATTTTGT-3' (2717), EF 51: 5'-CARGA CGTATACAAAATCGG-3' (2832), and EF rcM 44: 5'-ACAGCVACKGTYTGYC TCATRTC-3' (3344). Numbers in brackets at the 3'end of each primer refer to nucleotide position relative to the *Drosophila melanogaster* DNA sequence.

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.

The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, USA). Sequencing was performed using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) on ABI PRISM model 310 Genetic Analyzer (PE Applied Biosystems). The sequences were alignment using BioEdit Sequences Alignment Editor (Hall 1999).

Base Composition Analysis. I 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.

Transition/Transversion Analysis. Transitional and transversional substitutions and transition/transversion ratio were analyzed by using DNA Sequence Analyzer Version 1.00 (Kyukov 1997).

Phylogenetic Analysis. Phylogenetic analyses were performed with PAUP* version 4.0b.10 for 32-bit Microsoft Windows based on *EF-1α* gene by using Maximum-Parsimony and Neighbor-Joining approaches (Swofford 2001). For comparison, I also analyzed the combination of *EF-1α* and *COII* data sets either using all substitutions or transversion only partly based on data of my previous study (Sutrisno 2004). Most parsimonious trees were constructed by exhaustive searches. For the NJ analyses, I used the K80 (Kimura 1980) models. The statistical confidence of a particular clade in the MP and NJ trees was evaluated by Bootstrap test with 1000 replications (Felsenstein 1985).

The outgroup species *Sameodes cancellalis* Zeller, which had been used as outgroup in the preliminary study was not able to be collected. Therefore, I included *M. aureodiscalis* Meyrick and *Feltia jaculifera* Guenée (Noctuidae) in the present analysis. The first species had been used as outgroup in my preliminary study (Sutrisno 2004). The second species was chosen as outgroup comparison since this species has been reported as one of the most possible outgroups for Crambidae (Regier *et al*. 1998) and its sequence of *EF-1α* gene is also available from the gene bank Accession Numbers AF 173390.

RESULTS

Base Composition and Sequence Divergence. Sequences of seven species of *Agrioglypta* and *Talanga* and two species outgroups were aligned with no evidence of insertion and deletion. Aligned sequences have been submitted to the gene bank with the accession numbers are as presented in the Table 1. Over the entire 973-bp region, 82.9% (807) of the nucleotide positions were constant, 9.76% (95) were uninformative (i.e., any variants were found in single sequences), and 7.29% (71) were informative (Table 2). The most informative sites were found in the third-codon positions and the least were in the second-codon positions. The bias in base composition was calculated following Irwin *et al*. (1991). The result showed that the base composition was slightly A+T biased (*C*: 0.016) with the average of A+T contents was 0.45.6% (Table 3).

Moreover, interspecific variations in the base compositions were 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 in *EF-1α* ($\chi^2 = 2.922$, df= 24, $P = 0.9995$).

Nucleotide transitions were higher than transversions (Ts/Tv ratio >1) (Figure 1a). This occurred in the comparison between species within genus and between species belonging to different genera as well.

Nucleotide transitions gradually increased and reached its peak just below the saturation level of the divergence of these two genera (Figure 1b). The range of *p*-distance in the comparison species within genus slightly overlapped with the range of the *p*-distance between species belonging to different genera. The averages of estimated sequence divergence in the comparisons between species within genus and between species belonging to other different genera were 3.5% and 4.9%, respectively.

Table 2. Variable site percentages by codon positions of *EF-1α* gene

	1st-codon	2nd-codon	3rd-codon
Constant (%)	32.00 (312)	32.16 (313)	18.70 (182)
Uninformative (%)	0.82 (8)	0.82 (8)	8.11 (79)
Informative (%)	0.51 (5)	0.30 (3)	6.47 (63)

Table 3. Proportion of each nucleotide and its bias in *EF-1α* gene

	Codon position			
	1st-codon	2nd-codon	3rd-codon	Mean
A	0.284	0.324	0.119	0.242
C	0.183	0.265	0.451	0.299
G	0.322	0.140	0.204	0.222
T	0.149	0.268	0.224	0.213
Bias				0.016

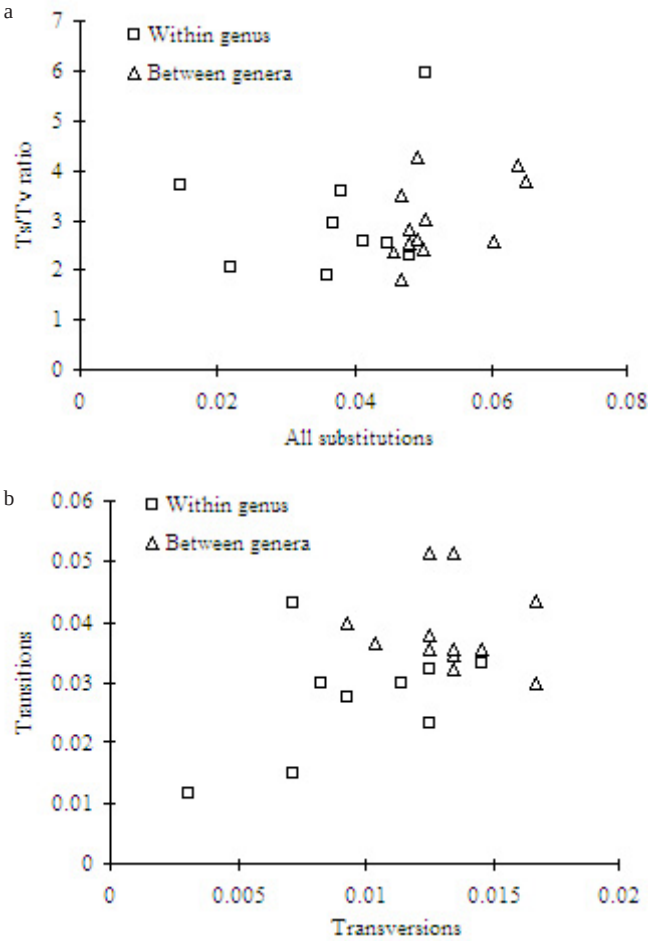

Figure 1. Scatter plots of p-distance, a. Ts/Tv ratios versus All substitutions; b. Transitions versus Transversions.

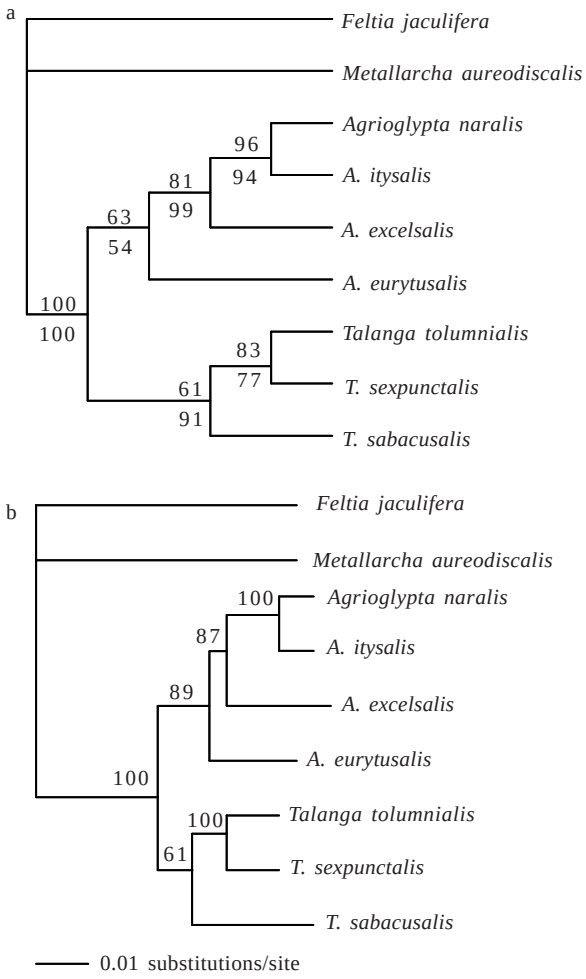

Figure 2. Phylogenetic trees, a. MP tree based on *EF-1α* and MP tree based on concatenated of *EF-1α* and *COII* using all substitutions; b. NJ tree based on *EF-1α*. Bootstrap values of 1000 replicates are shown at each node of the MP and NJ trees. Above node is based on *EF-1α*, below node is based on concatenated of *EF-1α* and *COII* using all substitutions.

Phylogeny. An exhaustive search based on equal weighting of all nucleotides substitutions in *EF-1α* gene resulted in single MP tree (length = 241, CI = 0.801, and RI = 0.634). The NJ tree also showed similar topology, each genus was found to be a monophyletic group and the relationships among species within each genus were agreed with those found in MP tree (Figures 2a, b).

The concatenated of the *EF-1α* and *COII* genes were analyzed by using all substitutions and resulted a single MP tree (length = 501, CI = 0.784, and RI = 0.561). Both MP and NJ tree topology based on this combination agreed with those resulted from the *EF-1α* data alone (Figures 2a, b and 3a).

By using only the transversal substitutions of the pooled data (*EF-1α* and *COII*) in the analysis, an exhaustive search resulted two MP trees (length = 210, CI 0.776, and RI = 0.490). These two MP trees differ only on the position of *A. naralis*. The strict consensus of the two MP trees and the NJ trees with their bootstrap supports at each node are presented in Figures 3b, c.

The MP and NJ trees based on *EF-1α* or combination of *EF-1α* and *COII* either using all substitutions or transversions only showed that each genus was found to be a monophyletic group even though the bootstrap supports for the genus *Agrioglypta* were not high (54-70%) in any tree building methods. In the genus *Agrioglypta*, *A. eurytusalis* located as the basal species and then followed by *A. excelsalis*. In the genus *Talanga*, *T. sabacusalis* lied in the basal node and *T. tolumnialis* was found to be sister group of *T. sexpunctalis*.

DISCUSSION

It has been reported that the base composition in insect genomes was biased, which is less in nuclear genes and the more A+T biased was in mitochondrial genes (Reviewed in Simon *et al*. 1994; Moriyama & Powell 1997; Goto & Kimura 2001). More over, the nuclear genes had lower A+T contents than mitochondrial genes as had been shown in the studies of several kinds of nuclear genes in different groups of Lepidoptera such as *EF-1α* in noctuid and saturniid moths (Cho *et al*. 1995; Rubinoff & Sperling 2002), *period* in lepidopterans (Regier *et al*. 1998), and *wingless* in nymphalid and papilionid butterflies (Brower & DeSalle 1998; Campbell *et al*. 2000). The averages of the A+T contents in those genes ranges from 46.7% to 55%. The bias in the base compositions of *EF-1α* found in this study is consistent with the general

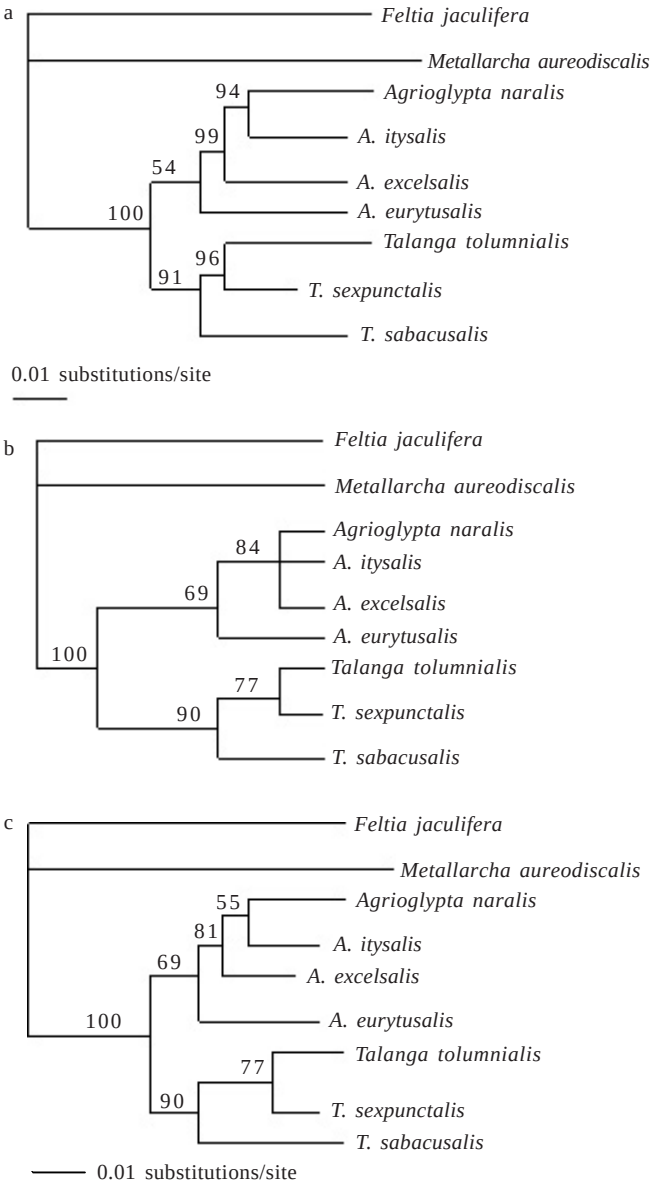

Figure 3. Phylogenetic trees, a. NJ trees based on concatenated of *EF-1α* and *COII* using all substitutions; b. Strict consensus of the two MP trees based on concatenated of *EF-1α* and *COII* using transversions only; c. NJ tree based on concatenated of *EF-1α* and *COII* using transversions only. Bootstrap values of 1000 replicates are shown at each node of the MP and NJ trees.

pattern of insect genomes but the proportion of the A+T contents (45.6%) was slightly lower with those found in other groups of Lepidoptera.

In comparison with the same gene in other groups of Lepidoptera, the mean of *p*-distance within genus and between species belonging to other different genera of this present study are 3.5% and 4.9%, respectively. These values are much higher than those found in heliothine moths which range from 0.1% to 1.0% and from 1.4% to 4%, respectively (Cho *et al*. 1995). However, those mean values lie within the ranges found in *Eichorni* group of *Delias* Hübner which range from 0.1% to 12.7% and from 0.1% to 17.8%, respectively (Morinaka *et al*. 2002).

The present study reveals that in *EF-1α*, the higher transitional substitutions (Ts/Tv ratios > 1) were found not only in the comparisons between closely-related species but also between distantly-related species. This finding is almost congruence with the study of *Gdph* gene in *Drosophila melanogaster* species group. It showed that transitions in this gene were generally higher than transversions in the comparison between species belonging to the same subgroup and between those belonging to different subgroups (Ts/Tv ratios > 1) (Goto & Kimura 2001). Moreover, the comparison of the third domain of the *12S rRNA* gene in *Cicada*, *Drosophila*, and human also showed that in the conserve regions among the three distantly-related taxa, transitions were nine times more common than transversions (Simon *et al*. 1990). It is clear that the observed transitions exceed transversions in the recently diverged species or slowly evolving gene (Irwin *et al*. 1991; Beckenbach *et al*. 1993; Simon *et al*. 1994).

Tree construction based on *EF-1α* showed that the relationships between the two genera, *Agrioglypta* and *Talanga*, were well resolved even though with a low bootstrap support for the monophyly of genus *Agrioglypta*. This result was incongruent with the MP tree based on the morphological data or *COII* in my previous study (Sutrisno 2004). The main problem of using the morphological character in moth is the difficulties to score some characters on *A. eurytuslais*. Several genitalia characters of *A. eurytusalis* (i.e. character numbers 7, 10, 12, 14, and 17) could not be scored as informative characters, thus, this genus was shown artificially to be paraphyletic (Sutrisno 2004). In this study, it is clear that *EF-1α* gene obviously contributes great phylogenetic signals to resolve the relationships at generic level as the result of the substitutions of this gene has not reached saturation at those levels of divergence (Figure 1b). A nearly fully resolved tree also found in the study of the relationships among genera within tribe Attacini by using the *EF-1α* gene (Friedleander *et al*. 1998). Even for low-level phylogenetics; i.e. at subfamily level, *EF-1α* found very useful to infer the phylogeny as has been shown in the study of heliothine moths (Cho *et al*. 1995).

In contrast, the *COII* usually evolve rapidly and reach a saturation level faster than nuclear genes. Thus it is only sensible to be used for inferring relationships among species within genus as has been reported in the study of genus *Ostrinia* moths (Pyralidae) (Kim *et al*. 1999) and *Greya* moths (Prodoxidae) (Brown *et al*. 1994). In my previous study, the *COII* failed to show that each genus is a monophyletic group (Sutrisno 2004). One possibility is the saturation occurs not only for transitions but also for transversions or the number of the phylogenetic informative from the transversions is very low as well.

There is no doubt that the concatenated of data sets between nuclear and mitochondrial genes will give a better resolution or a robust topology than a single data set since each of them contribute to the resolution at different level of taxa. Nuclear genes which are conserved will contribute to the resolution at basal node or deeper node. On the other hand the mitochondrial genes will support at the specific level. In this study, the concatenated of *EF-1α* and *COII* either using

all substitutions or transversions only agreed with those found in *EF-1α* that each of the genus is a monophyletic group although bootstrap support for the monophyly of *Agrioglypta* is low (54-69%).

All the findings in the present study agreed with Shaffer *et al*. (1996) who treated each of these two genera as a monophyletic group based on morphology. Though only few species were included in the analysis, adding more species within this genus, re-evaluate morphological characters, and including all possible molecular data in the future analysis might be resulted a robust topology.

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