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PHYLOGENY OF *Glyphodes* Guenée (Lepidoptera: Crambidae: Spilomelinae) BASED ON NUCLEOTIDE SEQUENCE VARIATION IN A MITOCHONDRIAL *CO I* GENE: CONGRUENCE WITH MORPHOLOGICAL DATA

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Abstract

The phylogeny of Glyphodes *Guenée (14 species) and four outgroup species (*Feltia jaculifera, Metallarcha aureodiscalis, Talanga sexpunctalis *and* Agrioghrypta eurytusalis*) was inferred from nucleotide sequence variation across a 686-bp region in the CO I gene. Over the entire 686-bp region, 19.9% sites were informative (3.35% in the 1st-, 0.29% in the 2nd- and 16.32% in 3rd-codon position). The results also showed that the base composition of this region was high A+T biased (C= 0.258) and the averages of estimated sequence divergence in the comparisons between species within and between groups were 7.1% and 9.0%, respectively. In general, the phylogeny based on* CO I *gene by including all substitutions or any partial data set used in this study was not only able to recover almost the three monophyletic groups within* Glyphodes *as previously recovered by morphological phylogeny but also showed more clearly the relationships among them:* Glyphodes *group 2 was branched off first then followed by group 3 and 1.*

Key words: CO I, *Glyphodes*, morphology, phylogeny.

Introduction

The *Glyphodes* Guenée, 1854, is medium-sized moth (15-51 mm) belongs to the subfamily Spilomelinae (Common, 1990; Shaffer *et al.*, 1996). This genus is diverse and widespread in tropical regions with some species penetrating into subtropical and warm temperate areas (Common, 1990; Robinson *et al.*, 1994). The genus consists of 120 species throughout the world, and about 25 and 17 species have been recorded in the Southeast Asia and Australia, respectively (Robinson *et al.*, 1994; Shaffer *et al.*, 1996).

As other pyraloids in the Indo-Australian regions, this genus is also poorly defined and has been suggested that it is seriously in need of revision since the current generic classification of this genus was based on superficial similarities only (Robinson *et al.*, 1994; Sutrisno & Horak, 2003). However, the comprehensive study on the taxonomy or phylogeny to assess the monophyly and the relationships among them has never been conducted except for a cladistic analysis based on the Australian *Glyphodes* and its allied genera using morphological data (Sutrisno, 2002).

The cladistic analysis showed that the genus *Glyphodes* falls into three monophyletic groups (*Glyphodes* group 1, 2 and 3); each of them can be diagnosed by the adult morphology. In general, the result showed that morphological characters used

in the previous study were able to infer the phylogenetic relationships of this genus. However, the evolutionary change of morphological characters in this genus is extremely complicated that this approach did not produce a clear-cut picture of evolutionary history; i.e., for the relationships among groups within *Glyphodes*. In addition, the relationships among closely-related species within *Glyphodes* group 3 seem very difficult to be resolved. Therefore, another possible approach such as molecular phylogeny that has been proved to give more powerful resolution is really needed.

Mitochondrial genes have been used intensively to infer the phylogenetic relationships among groups of insects as have been repeatedly reported by numerous authors (reviewed in Simon *et al.*, 1994). *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; and Blum *et al.*, 2003). Therefore, in this issue I used mitochondrial *CO I* gene to reassess the monophyly of *Glyphodes* and to estimate the relationships among species within this genus.

Material and Methods

DNA extraction and Sequencing

A total of 17 species representing the four genera (*Glyphodes, Talanga, Agrioglypta* and *Metallarcha*) from various localities in Indonesia and Australia were collected using a light trap and preserved in absolute alcohol (Table 1). The DNA extraction method and the PCR amplification conditions used in this study were similar with the procedures described in my previous study (Sutrisno, 2003). I used Mt D-4 and Mt D-9 primers to amplify *CO I* gene for a total of 686-bp and the complete sequences were Mt D-4: 5'-TACAATTTATCGCCTAAACT TCAG CC-3', and Mt D-9: 5'-CCCGGTAAAATT AAAATATAAACTTC-3' (http://www.biotech.ubc.ca/services/naps/primers.html). 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).

Phylogenetic Analysis

For the phylogenetic analysis, I used Maximum-Parsimony method using an exhaustive search with different data sets: all substitutions; transversions only; weighting scheme 2:1; nt1 + nt3; and nt3 only and Neighbor Joining method using K80 model (Kimura, 1980) in PAUP 4.0b4a (Swofford, 2001); and Bootstrap test (Felsenstein, 1985) to evaluate the statistical confidence of the MP and NJ trees.

Four species, *Agrioglypta eurytusalis* (Walker), *Talanga sexpunctalis* Moore, *Metallarcha aureodiscalis* Meyrick and *Feltia jaculifera* Meyrick (Noctuidae) were included as outgroup taxa in the present analysis. The sequence of *CO I-CO II* of the last species is available from the gene bank with the accession number U60990.

Table 1. Species examined for the molecular study

Species	Collection locality	Date of Collection
Agrioglypta eurytusalis (Walker)	G. Pangrango, Java	September 2000
Glyphodes		
G. actorionalis (Walker)	Sorong, Papua	February, 2002
G. apiospila (Turner)	Sorong, Papua	February, 2002
G. bicolor (Swainson)	Sorong, Papua	February, 2002
G. bivitralis Guenée	Patunuang, Sulawesi	April, 2001
G. caesalis (Walker)	Menado, Sulawesi	March, 2001
G. conjunctalis Walker	Sorong, Papua	February, 2002
G. cosmarcha Meyrick	Patunuang, Sulawesi	April, 2001
G. doleschalii Lederer	Sorong, Papua	January, 2002
G. flavizonalis Hampson	Sorong, Papua	January, 2002
G. margaritaria (Clerck)	Patunuang, Sulawesi	April, 2001
G. multilinealis Kenrick	Bantimurung, Sulawesi	April, 2001
G. onychinalis Guenée	Bucasia, Queensland	April, 2001
G. pulverulentalis Hampson	Sukabumi, Java	February, 2002
G. stolalis Guenée	G. Halimun NP, Java	March, 2002
Metallarcha aureodiscalis Meyrick	Bucasia, Queensland	April, 2001
Talanga sexpunctalis Moore	Patunuang, Sulawesi	April, 2001

Table 2. Percent variable sites across 14 species of *Glyphodes* and four outgroup species

	Total	1st-codon	2nd-codon	3rd-codon
Constant (%)	67.9	27.25	31.77	8.89
Uninformative (%)	12.1	2.76	1.31	8.01
Informative (%)	19.9	3.35	0.29	16.32
Number of sites	686	229	229	228

Results

Base compositions

Sequences of 14 species of the *Glyphodes*, and four species outgroup were aligned with no evidence of insertion and deletion (aligned sequences are available from the author on request). Over the entire 686-bp region, 67.9 % of the nucleotide positions were constant, 12.1 % were uninformative (i.e., any variants were found in single sequence), and 19.9 % were informative (Table 2). In addition, the most informative

sites for maximum parsimony analysis lied in the third-codon positions and the least informative site was in the second-codon positions.

Table 3 shows the proportion of nucleic acids and bias in *CO I*. The results showed that the base compositions were strongly biased (C= 0.258) with the A+T proportions ranged from 69.82% to 72.01%. Interspecific variations in base compositions *CO I* were very low for a total of nucleotides (the Chi-square test, X^2= 10.684, df= 51, P=1.00). In addition, the averages of estimated sequence divergence in the comparisons between species within and between groups were 7.1% and 9.0%, respectively.

Table 3. Proportion of each nucleotide and bias in *CO I*

	Codon position			
	1st-codon	2nd-codon	3rd-codon	Mean
A	0.316	0.170	0.485	0.324
C	0.139	0.249	0.042	0.142
G	0.264	0.169	0.097	0.175
T	0.279	0.410	0.462	0.384
Bias				0.258

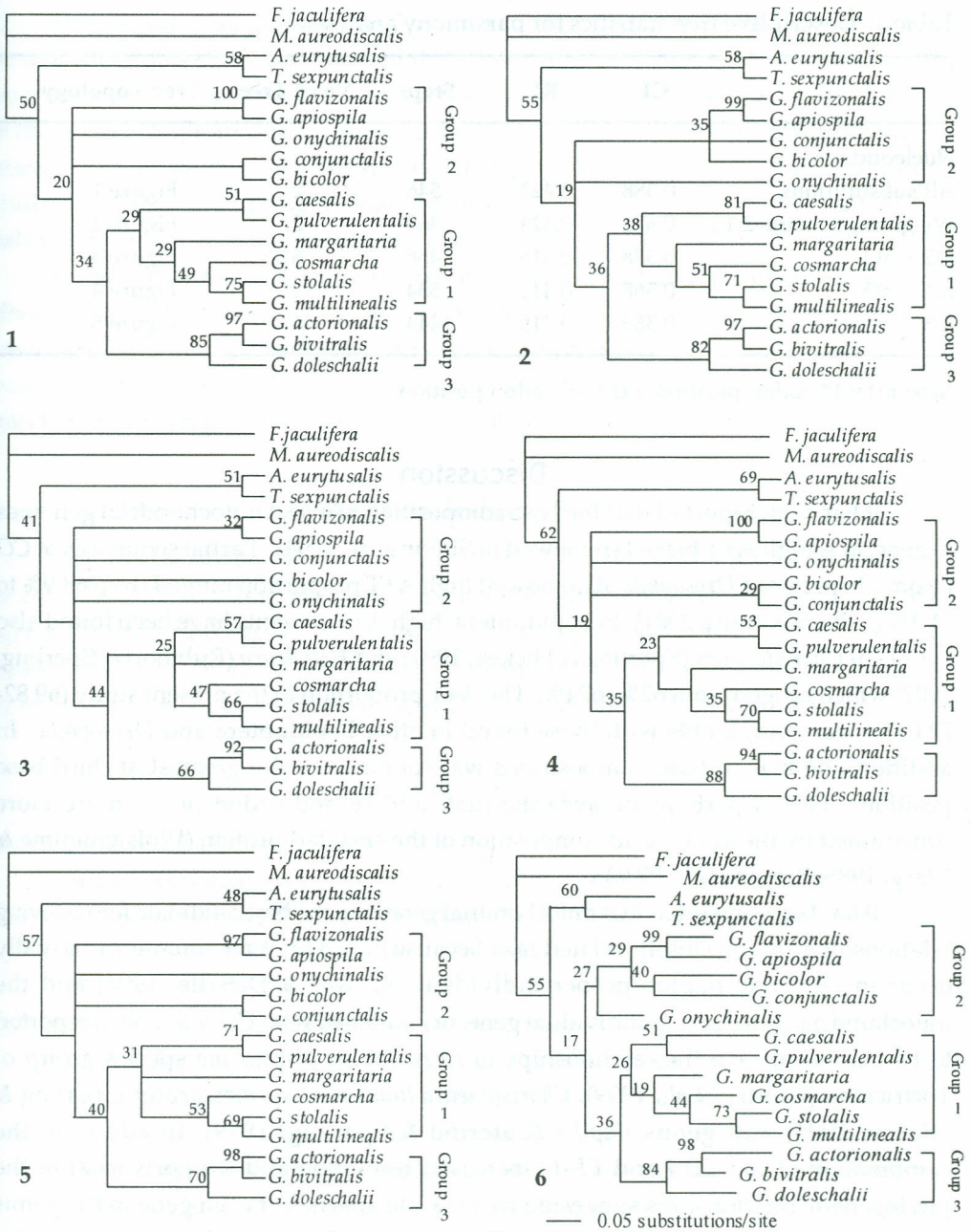
Bias in base composition is calculated as

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

Where C is the composition bias and c_i is the frequency of its base.

Phylogenetic analyses

An exhaustive search in maximum-parsimony analysis using all nucleotides substitutions resulted in two equally MP trees (Fig.1). The MP trees showed that the three groups within the *Glyphodes* as previously recognized in the morphological study (Sutrisno, 2002) were recovered. However, the relationships among species within *Glyphodes* group 2 were less-resolved: *G. onychinalis* was evolved independently. When the weighting scheme 2:1, or transversional substitutions only or the combination between 1st- and 3rd-codon (nt1+nt3) or 3rd-codon (nt3) only were included in the analysis, the relationships among five species of *Glyphodes* group 2 was also poorly-resolved, except for the relationships between *G. flavizonalis* and *G. apiospila* (Figs. 2-5). The descriptive tree statistics for parsimony analyses are presented in Table 4. The NJ tree based on all substitutions revealed that each group was found to be monophyletic (Fig. 6) and the relationships among them were well-resolved: *Glyphodes* group 2 was branched off first then followed by group 3 and 1. In addition, the relationships among the three species of *Glyphodes* group 3 were also well-resolved in both MP and NJ analyses: *G. doleschalii* lied in the basal group, *G. actorionalis* was the sister group of *G. bivitralis*.



Figs. 1-6. MP and NJ trees, 1, Strict consensus of the two MP trees based on all substitutions; 2, Strict consensus of the two MP trees based on weighting scheme 2:1; 3, Strict consensus of the six MP trees based on nt2+nt3; 4, Strict consensus of the two MP trees based on nt1+nt3; 5,Strict consensus of the six MP trees based on nt3 only; 6, NJ tree based on all substitutions.

Table 4. Descriptive tree statistics for parsimony analyses

	CI	RI	Steps	No of Trees	Tree Topology
Nucleotide					
All substitutions	0.388	0.423	546	2	Figure 1
Weighting scheme 2:1	0.378	0.424	869	2	Figure 2
nt2 + nt3	0.388	0.415	456	6	Figure 3
nt1 + nt3	0.368	0.412	534	2	Figure 4
nt3	0.385	0.415	444	6	Figure 5

Note: nt1= 1st-codon position; nt3= 3rd-codon position

Discussion

It has been reported that the base composition in insect mitochondrial genomes in general is high A+T biased (reviewed in Simon *et al.*, 1994). Partial sequences of *CO I* from 21 species of *Drosophila* also showed high A+T proportion ranged from 68.9% to 71.4% (Goto & Kimura 2001). In Lepidoptera, high A+T contents have been found also in *CO I* of *Choristoneura* (Sperling & Hickey, 1994) and *Hemileuca* (Rubinoff & Sperling, 2002) which ranged from 62% to74%. The A+T proportion in the present study (69.82-72.01%) was comparable with those found in other Lepidoptera and *Drosophila*. In addition, the bias in base compositions was found to be the greatest at third-base position. This is perhaps because the first- and second-codon position are more constrained by the amino acid composition of the encoded protein (Wolstenholme & Clary, 1985; Brown *et al.*, 1994).

It has been suggested that mitochondrial genes are the best candidate for resolving relationships among closely-related taxa because they do not recombine and usually occur in only one haplotype per individual (Brower & DeSalle, 1994) and the mitochondrial gene *CO I* as individual gene, or combined with *CO II* has been reported to be able to resolve the relationships in *Argyrotaenia franciscana* species group of 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-1á* increased resolution and supports most of the phylogenetic relationships suggested by separate analysis of each gene in the genus *Hemileuca* (Rubinoff & Sperling, 2002). The results of this study also showed that the MP and NJ analyses based on *CO I* resolved the relationships among species within *Glyphodes* eventhough with low bootstrap supports for each group except for the group 3. There is no doubt that including more characters by adding a longer sequence of *CO I* or other mitochondrial genes may increase the resolution and supports for the relationships suggested in the present study.

It is not surprising that group 3 is always has a strong bootstrap support since the morphological study also showed that this group was supported by several good synapomorphies. In addition, the analysis based on *CO I* also resulted better resolutions on the relationships among the three species of this group, which hardly possible to assess using morphological analysis since their male and female genitalia were quitle similar and the evolutionary changes of those characters were difficult to be distinguished and scored as informative characters (Sutrisno, 2002).

In general, all the findings in the present study suggest that phylogeny of *Glyphodes* inferred from a mitochondrial *CO I* gene was congruent with the morphological data: *Glyphodes* was divided into three groups; *Glyphodes* group 1 was branched off first then followed by group 3 and 1. Further studies are needed to be done by including more species and other mitochondrial genes in order to test the validity of the relationships proposed here.

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