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Molecular Phylogeny of the Indo-Australian *Glyphodes* and its Allied Genera (Insecta: Lepidoptera: Crambidae: Spilomelinae) Inferred from Mitochondrial COI and COII and Nuclear EF-1 α Gene Sequences

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The phylogeny of Indo-Australian *Glyphodes* (15 species), *Talanga* (three species), *Agrioglypta* (four species), and two outgroup species, *Ostrinia nubilalis* Hübner, 1825 and *Metallarcha aureodiscalis* (Hampson, 1918), was inferred from nucleotide sequence variation in the mitochondrial COI (686 bp) and COII (687 bp) genes and the nuclear EF-1 α (973 bp) gene. While the third codon positions in COI and EF-1 α were not saturated with substitutions, those in COII were saturated when sequences were compared between genera. MP analyses based on pooled data of COI, COII, and EF-1 α resulted in two MP trees. The strict consensus tree showed that *Glyphodes*, *Talanga*, and *Agrioglypta* were all not monophyletic. Another analysis based on pooled data of all molecular and morphological data resulted in a single tree, in which monophyly of the genus *Glyphodes* was barely supported (bootstrap value <50%). *Glyphodes* species were divided into the same three clades as in an earlier published, morphology-based tree, one of these clades (the so-called Group 3) being supported by a bootstrap value of 100%. In all analyses the genus *Agrioglypta* was shown to be paraphyletic, since *A. eurytusalis* (Walker, 1859) fell within the genus *Talanga*.

Key Words: Insecta, Lepidoptera, Crambidae, *Glyphodes*, molecular phylogeny.

Introduction

Glyphodes Guenée, 1854 and its allied genera *Dysallacta* Lederer, 1863, *Talanga* Moore, 1885, and *Agrioglypta* Meyrick, 1932 are medium-sized moths (15–51 mm) belonging to the subfamily Spilomelinae (Common 1990; Shaffer *et al.* 1996). These genera are morphologically diverse and widespread in tropical regions, with some species penetrating into subtropical and warm-temperate areas (Common 1990; Robinson *et al.* 1994).

Although nothing is known of the biology of any species of *Dysallacta*, almost all species of the other mentioned genera are host-specific to latex-containing plants of the Moraceae and Apocynaceae (Kalshoven 1981; Common 1990; Robinson

et al. 1994), which occasionally suffer serious damage from larval feeding. For example, larvae of *G. caesalis* Walker, 1866 attack many species of Moraceae, such as *Artocarpus heterophyllus* Lam (jackfruit), *A. rigida* Blume (monkey jak), and *A. elastica* Reinw, in Indonesia and Thailand (Kalshoven 1981; Kuroko and Lewvanich 1993), and *T. toluentalis* (Walker, 1859) is a common pest of cultivated figs *Ficus carica* Linnaeus in Queensland and New South Wales, Australia (Common 1990).

In spite of their impact on agricultural crops, the four moth genera *Glyphodes*, *Dysallacta*, *Talanga*, and *Agrioglypta* are poorly defined. Current generic classifications of these moths are based on superficial similarities (Common 1990; Robinson *et al.* 1994) and are in need of taxonomic revision. Until now, few comprehensive efforts have been made to improve the taxonomy or phylogeny of these genera by testing the monophyly of each genus and the relationships among the genera. As the principal exception, Sutrisno (2002) performed a cladistic analysis of Australian *Glyphodes* and its allied genera, including 18 species of *Glyphodes*, two species of *Dysallacta*, three species of *Talanga*, and four species of *Agrioglypta*, based on 40 morphological characters. However, a purely morphological approach failed to demonstrate the monophyly of each genus, and the reliability of each grouping was cast in doubt by low bootstrap values. This indicates the need for a molecular-phylogenetic approach to resolve relationships within this group.

As reported by numerous authors (reviewed in Simon *et al.* 1994; Brower and DeSalle 1998; Caterino *et al.* 2000), mitochondrial genes are useful for estimating phylogeny because of relative technical ease in processing and their maternal inheritance, haploidy, and absence of recombination events and introns (Goto and Kimura 2001). Among the mitochondrial genes, COI and COII are considered to be useful for estimating the phylogeny of closely related species (Brower 1994; Sperling and Hickey 1994; Caterino and Sperling 1999; Goto *et al.* 2000; Blum *et al.* 2003); nevertheless, mitochondrial protein-coding genes generally evolve fast and saturate rapidly (Liu and Beckenbach 1992) and, therefore, are not appropriate as phylogenetic markers in certain cases. In contrast, nuclear genes are generally more conservative and may be useful for inferring the phylogeny of distantly related taxa. Among nuclear genes, EF-1 α is the one most favored for inferring the phylogeny at different taxonomic levels in the Lepidoptera (Cho *et al.* 1995; Friedlander *et al.* 1998; Caterino *et al.* 2001; Monteiro and Pierce 2001; Rubinoff and Sperling 2002). Our purposes in the present study were: (1) to examine the monophyly of *Glyphodes*, *Agrioglypta*, and *Talanga*, and (2) to test the phylogenetic hypothesis derived from the earlier morphological analysis by analyzing the DNA sequences of the COI, COII, and EF-1 α genes.

Materials and Methods

Morphological phylogeny of *Glyphodes* and its allied genera

Sutrisno (2002) constructed a strict consensus MP tree based on 40 morphological characters (Fig. 1) and obtained the following results: first, *Glyphodes* species formed three distinct clades, referred to as Groups 1, 2, and 3; second, all species of *Agrioglypta* and *Talanga* formed an additional clade, with *Talanga* monophyletic but *Agrioglypta* paraphyletic with respect to *Talanga*; third, *Glyphodes* was not

Fig. 1. Strict consensus of 12 equal MP trees based on morphological data (from Sutrisno 2002), showing bootstrap values and division of genus *Glyphodes* into three monophyletic groups.

monophyletic, but paraphyletic with respect to both the *Agrioglypta*+*Talanga* clade and *Dysallacta*. Groups 2 and 3 of *Glyphodes* formed a clade with *Agrioglypta*+*Talanga*, not with Group 1 of *Glyphodes*. *Dysallacta* was nested within Group 1 of *Glyphodes*, making this group paraphyletic as well. The reliability of each grouping was supported only by low bootstrap values.

DNA sequencing

A total of 24 adult moths (23 species of the four genera *Glyphodes*, *Talanga*, *Agrioglypta*, and *Metallarcha*) were collected from eight localities in Indonesia and Australia (Table 1) by using a light trap and preserved in 99.5% ethanol. No specimens of *Dysallacta* were collected, and, therefore, this genus was not included in the present study. DNA was extracted from the thorax of each individual. The tis-

Table 1. Moth species from Indonesia and Australia used for the present study.

Species	Locality	Date
<i>Agrioglypta</i>		
<i>eurytusalis</i> (Walker, 1859)	G. Pangrango NP	Sep. 2000
<i>excelsalis</i> (Walker, 1866)	Bucasia, Queensland	Apr. 2001
<i>itysalis</i> (Walker, 1859)	Halimun NP, Java	Mar. 2002
<i>naralis</i> (Felder and Rogenhofer, 1875)	Halimun NP, Java	Mar. 2002
<i>Glyphodes</i>		
<i>actorionalis</i> (Walker, 1859)	Sorong, Papua	Feb. 2002
<i>apiospila</i> (Turner, 1922)	Sorong, Papua	Feb. 2002
<i>bicolor</i> (Swainson, 1821)	Sorong, Papua	Feb. 2002
<i>bivitalis</i> Guenée, 1854	Patinuang, Sulawesi	Apr. 2001
<i>caesalis</i> (Walker, 1866)	Manado, Sulawesi	Mar. 2001
<i>conjunctalis</i> Walker, 1866	Sorong, Papua	Feb. 2002
<i>cosmarcha</i> Meyrick, 1887	Patinuang, Sulawesi	Apr. 2001
<i>doleschalii</i> Lederer, 1863	Sorong, Papua	Jan. 2002
<i>flavizonalis</i> Hampson, 1898	Sorong, Papua	Jan. 2002
<i>margaritaria</i> (Clerck, 1754) 1	Patinuang, Sulawesi	Apr. 2001
<i>margaritaria</i> (Clerck, 1754) 2	Patinuang, Sulawesi	Apr. 2001
<i>multilinealis</i> Kenrick, 1907	Bantimurung, Sulawesi	Apr. 2001
<i>onychinalis</i> (Guenée, 1854)	Bucasia, Queensland	Apr. 2001
<i>pulverulentalis</i> Hampson, 1896	Sukabumi, Java	Feb. 2002
<i>pseudocaesalis</i> Rotschild, 1915	Sorong, Papua	Feb. 2002
<i>stolalis</i> Guenée, 1854	Halimun NP, Java	Mar. 2002
<i>Metallarcha</i>		
<i>aureodiscalis</i> (Hampson, 1918)	Bucasia, Queensland	Apr. 2001
<i>Talanga</i>		
<i>sabacusalis</i> (Walker, 1859)	Patinuang, Sulawesi	Apr. 2001
<i>sexpunctalis</i> Moore, 1885	Patinuang, Sulawesi	Apr. 2001
<i>tolumnialis</i> (Walker, 1859)	Bucasia, Queensland	Apr. 2001

sue was homogenized in a 1.5 ml microcentrifuge tube containing 600 μ l of CTAB buffer with 4% polyvinyl pyrrolidone, and incubated at 55°C for two hours. The solution was extracted using phenol saturated with TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA), then washed once with one volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and twice with chloroform:iso-amyl alcohol. The aqueous phase was transferred to a new tube and then 1.5 volumes of isopropanol were added to precipitate the DNA. This tube was then left at -20°C for more than one hour. The DNA precipitant was pelleted by centrifugation at 13,000 rpm for 20 min, then washed with 70% ethanol, air dried, and re-dissolved in 50 μ l of TE buffer.

For PCR amplification and DNA sequencing we used Mt D-4 and Mt D-9 primers (<http://www.biotech.ubc.ca>) to amplify the COI gene for a total of 686 bp, and A-tLEU and B-tLYS primers (Liu and Beckenbach 1992) to amplify the COII gene for a total of 687 bp. The complete sequences of the primers used were Mt D-4: 5'-TACA ATTTATCGCCTAAACTTCAG CC-3', Mt D-9: 5'-CCCGGTAAAATTTAAA ATATA AACTTC-3', A-tLEU: 5'- ATGGCAGATTAGTGCAATGG-3', and B-tLYS: 5'-GTTTA AGAGACCAGTACTTG-3'. The amplification of COI and COII was conducted as follows: one cycle of denaturation at 94°C for 10 min, followed by 30 cycles each consisting of 30 s of denaturation at 94°C, 60 s of annealing at 47°C, and

120 s of extension at 72°C.

For amplifying 973 bp of the EF-1 α gene, we used two pairs of primers, EF 44: 5'-GCTGARCAGYARCGTGGTATYAC-3' (2277) and EF Mid: 5'-CA ATACCRCCRA TTTTGT-3' (2717) for 493 bp and EF M51.9: 5'-CARGACGTATAC AAAATCGG-3' (2832) and EF rcM44: 5'-ACAGCVACKGTYTGYCTCATRTC-3' (3344) for 480 bp (Cho *et al.* 1995). Numbers in brackets at the 3'-end of each primer refer to nucleotide positions in the *Drosophila melanogaster* DNA sequence (Cho *et al.* 1995). All PCR reactions were performed in a 50 μ l volume containing 5 pM of each primer, 2 mM dNTPs, 2.5 mM MgCl₂, 1 $\times$ buffer, and 0.25 U of Taq polymerase, using a Takara Thermal Cycler MP (Takara) with the following PCR protocol: one cycle of denaturation at 94°C for 10 min, followed by 35 cycles each consisting of denaturation at 92°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 1 min 30 s. These cycles were completed by final extension at 72°C for 10 min and the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA).

Cycle sequencing was done using 35 cycles of denaturation at 96°C for 10 min, annealing at 50°C for 5 min, and extension at 60°C for 4 min; thereafter, the products were purified using phenol-chloroform. Sequencing of the purified PCR product was performed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) on an ABI PRISM model 310 Genetic Analyzer (PE Applied Biosystems). The sequences were aligned using BioEdit Sequence Alignment Editor (Hall 1999).

The remaining parts of the specimens used for the present study were deposited at the Museum Zoologicum Bogoriense, Bogor, Indonesia.

Substitutions and transition/transversion ratio

Transitional and transversional substitutions and the transition/transversion ratio were calculated using the DSET option in PAUP 4.0b4a (Swofford 2001) and then were transferred into Microsoft Excel for plotting on graphs.

Phylogenetic analysis

In the phylogenetic analysis with PAUP 4.0b4a (Swofford 2001), we adopted the Maximum-Parsimony (MP) method with heuristic searches using the TBR branch-swapping option and 1000 random addition sequences (Swofford and Olsen 1990), and statistical confidence was evaluated by bootstrapping tests with 1000 replications (Felsenstein 1985).

Metallarcha aureodiscalis (Hampson, 1918) was used as an outgroup in the present analysis because *Metallarcha* is closely-related to *Glyphodes* and its allied genera (Sutrisno 2002). As another outgroup, *Ostrinia nubilalis* Hübner, 1825 (Pyralidae) was used because sequences of its COI-COII and EF-1 α genes were available from GenBank (accession numbers U60990 and AF 173390, respectively).

Results

Base composition

Sequences of 22 species of *Glyphodes*, *Talanga*, and *Agrioglypta*, as well as those of the outgroup species, *Metallarcha aureodiscalis*, were aligned with no evidence of insertion or deletion. The sequences of all 23 species have been submitted

Table 2. Percentages of variable and informative sites of COI, COII, and EF-1 α , with the number of positions in parentheses.

	COI	COII	EF-1 α
Total positions	686	687	973
Variable (each codon position)	36.1 (248/686)	32.3 (222/687)	24.5 (239/973)
First codon position	21.3 (49/229)	18.7 (43/229)	6.4 (21/325)
Second codon position	8.7 (20/229)	6.5 (15/229)	4.9 (16/324)
Third codon position	78.5 (179/228)	67.2 (154/229)	62.2 (202/324)
Informative (each codon position)	22.7 (156/686)	19.9 (137/687)	16.1 (157/973)
First codon position	10.9 (25/229)	12.2 (28/229)	3.4 (11/325)
Second codon position	1.3 (3/229)	3.5 (8/229)	0.9 (3/324)
Third codon position	56.14 (128/228)	44.1 (101/229)	44.1 (143/324)

Table 3. Base composition of COI, COII, and EF-1 α .

	Codon position			Mean	SD
	First	Second	Third		
	COI				
A	0.315	0.170	0.479	0.322	0.009
C	0.140	0.266	0.277	0.145	0.006
G	0.266	0.169	0.011	0.149	0.002
T	0.277	0.411	0.460	0.382	0.01
Bias (C)				0.273	
	COII				
A	0.376	0.294	0.415	0.362	0.006
C	0.140	0.166	0.034	0.112	0.006
G	0.183	0.127	0.012	0.107	0.004
T	0.298	0.41	0.537	0.416	0.008
Bias (C)				0.372	
	EF-1 α				
A	0.282	0.321	0.134	0.253	0.004
C	0.184	0.268	0.439	0.298	0.004
G	0.380	0.141	0.206	0.243	0.002
T	0.151	0.268	0.219	0.212	0.003
Bias (C)				0.064	

to the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>) under the accession numbers AB158228-158251, AB158321-158344, and AB158377-158400.

As shown in Table 2, the proportion of informative sites varied among genes, constituting 22.7% of 686 bp for COI, 19.9% of 687 bp for COII, and 16.1% of 973 bp for EF-1 α . The third-codon positions were most variable, and the second-codon positions least variable, in all genes. The proportion of informative sites in the first, second, and third positions of a codon were 3.4–12.2%, 0.9–3.5%, and 44.1–56.14%, respectively.

Fig. 2 shows the relationships between un-corrected pairwise distances for transitions (Ts) and those for transversions (Tv) at the third codon position. In COI and EF-1 α , Ts almost linearly increased with respect to Tv and exceeded Tv in all

Fig. 2. Transitions (Ts) versus transversions (Tv) of third codon positions in *COI*, *COII*, and *EF-1α*.

Fig. 3. Strict consensus of eight MP trees based on COI and EF-1 α , showing bootstrap values and division of genus *Glyphodes* into three monophyletic groups.

pairwise species comparisons, indicating no saturation of Ts and Tv. The values of linear regressions in COI and EF-1 α were $Y=0.968x$, $R^2=0.951$, and $Y=2.719x$, $R^2=0.1882$, respectively. In contrast, COII appeared saturated with transitions when compared between genera and its linear regression was $Y=0.497x$, $R^2=-0.496$.

Phylogeny based on pooled data of COI, COII, and EF-1 α

With all base substitutions in COI and EF-1 α accorded equal weighting, an heuristic search resulted in eight MP trees (TL=1264, CI=0.471, RI=0.515), the strict consensus of which is presented in Fig. 3. The genus *Glyphodes* appears as a monophyletic group divided into three clades, but the reliability of the relationship among the latter is scarcely supported by the bootstrap values. The genera *Agrioglypta* and *Talanga* together form one clade with very strong bootstrap support.

Using all the molecular data of COI, COII, and EF-1 α , an heuristic search resulted in two MP trees (TL=1880, CI=0.4649, RI=0.502), the consensus of which is presented in Fig. 4. These two trees differ in the position of *Glyphodes* group 2. In one tree, this group branches off first, followed by the *Agrioglypta*+*Talanga* clade, while in the other tree, the *Agrioglypta*+*Talanga* clade occupies the basal node. In

Fig. 4. Strict consensus of two MP trees based on COI, COII, and EF-1 α , showing bootstrap values and division of genus *Glyphodes* into three monophyletic groups.

both trees the genus *Glyphodes* is divided into three clades and the genus *Agrioglypta* is not monophyletic, since *A. eurytusalis* (Walker, 1859) falls out with the genus *Talanga*, occupying the basal node of this latter genus.

Phylogeny based on a combination of molecular and morphological data

Another phylogenetic analysis was conducted using all substitutions in COI, COII, and EF-1 α as well as 40 morphological characters from Sutrisno (2002). An heuristic search discovered a single MP tree (TL=2014, CI=0.478, RI=0.541) (Fig. 5). The topology of this tree is very similar to that of the second tree in the previous analysis based on molecular data (compare Figs 4 and 5). The three *Glyphodes* groups were again recognized, with Group 2 branching off first, followed by Groups 3 and 1. However, the monophyly of this genus was only supported by a low bootstrap value (<50%). The best support was found for the monophyly of Group 3 (100%).

Fig. 5. MP tree based on a combination of molecular and morphological data, showing bootstrap values and division of genus *Glyptodes* into three monophyletic groups.

Discussion

In insect genomes, the base composition is generally A+T-biased in mitochondrial genes but not in nuclear genes (Clary and Wolstenholme 1985; DeSalle *et al.* 1987; Goto and Kimura 2001). The present study also revealed an A+T bias in COI (70.6%) and COII (77.8%), but not in EF-1 α (46.5%). In COI and COII, the highest bias is observed at the third-base position. In general, A+T-rich compositions show a higher substitution rate than G+C-rich or non-biased compositions (Moriyama and Powell 1997).

Transitions occur more frequently than Transversions, 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 and Kocher 1993; Simon *et al.* 1994; Goto and 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). The substitutions at the third codon position of COII revealed in the present study also show Tv values that exceed Ts ones when sequences are compared between distantly-related species (Fig. 2). By contrast, the COI and EF-1 α genes (more conser-

vative genes) show Ts values that exceed the Tv ones, which indicates that these genes were not yet saturated with transitions. As a result of this phenomenon, the COI and EF-1 α genes contribute to resolving the relationships at basal nodes whereas COII contributes to resolving the relationships among closely-related species (Figs 3, 4). These results are quite similar to those found in a study of the tribe Attacini (Saturnidae), in which EF-1 α serves well to resolve relationships among genera but is often poor in resolving phylogeny within a genus (Friedlander *et al.* 1998).

The most significant difference between the topologies based on molecular and morphological data concerns the relationships among the groups within the genus *Glyphodes* and also within the clade formed by *Glyphodes* Group 3, *Agrioglypta*, and *Talanga*. In the earlier morphological analysis, Sutrisno (2002) failed to find any single good character to support the monophyly of *Glyphodes*. As a result, the genus *Glyphodes* could not be shown to be monophyletic. In addition, the morphological characters that had been regarded by Sutrisno (2002) as synapomorphies to support the monophyly of *Glyphodes* Group 3+*Agrioglypta*+*Talanga*, such as a narrow bulbus ejaculatorius, a bulbus ejaculatorius much longer than the aedeagus, a ductus bursae five times longer than the longitudinal diameter of the corpus bursae, and a funnel-shaped thin membrane in the posterior part of the ductus bursae (from ostium to antrum), are homoplasious characters. These characters evolved independently in *Glyphodes* group 3, *Agrioglypta*, *Talanga*, and *Achyra nudalis* Hübner, 1825 (Pyraustinae) (Maes 1995; Sutrisno 2002). This suggests that the clade based on the above morphological characters is not a monophyletic group. Sutrisno's (2002) morphological analysis failed to resolve relationships among species within *Glyphodes* Group 3 because the characters of the male genitalia of this group were difficult to score informatively. Still, there were enough morphological synapomorphies to support the monophyly of this clade, such as the well developed, brush-like extension at the distal end of the male patagia, the large, globose head of the uncus with a series of thorn-like spines at the apex, and the rounded to ovate signum with pointed denticles (Sutrisno 2002).

The combination of morphological and molecular data presented here shows more clearly the relationships among *Glyphodes*, *Agrioglypta*, and *Talanga*. *Glyphodes* comprises three distinct clades. The best support for monophyly was found for Group 3, and the relationships among the species in this group are very clear: *G. bivitalis* Guenée, 1854 is the sister species of *G. actorionalis* Walker, 1859, and *G. doleschalli* Lederer, 1863 occupies its basal node. *Agrioglypta* consistently appears to be a paraphyletic group, forming a clade together with *Talanga* and occupying that clade's basal node. *Agrioglypta* is thus not a valid genus.

The combination of morphological and molecular data in the present study suggests that the genus *Glyphodes* is monophyletic. Each group of *Glyphodes*, especially Group 3, might conceivably be raised to the generic level. The position of the genus *Dysallacta* is still unclear, since there is no available material for molecular studies. Further studies including all species of the four genera are still needed to test the present hypothesis.

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