Distinct genetic clades of Malaysian Copera damselflies and the phylogeny of platycnemine subfamilies

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The phylogenetic relationships of some taxa in the Platycnemidinae at the species and generic levels have been investigated. Phylogenetic trees were generated from both individual mitochondrial encoded COI, COII, 16S rDNA and nuclear encoded 28S rDNA and also combined sequences; these data indicate that the component taxa of the genus Copera belong to two distinct genetic clades – the marginipes group and the annulata group. There was no distinct genetic difference between the red-legged and yellow-legged morphs of C. vittata. Molecular data showed that the annulata group is considered a member of the genus Platycnemis, as originally proposed. The genus Coeliccia, a member of the subfamily Calicnemiinae (Platycnemididae), is not grouped with the Platycnemidinae. The Disparoneurinae of the 'Protoneuridae' showed a closer relationship to the Platycnemidinae than the Calicnemiinae. The dataset supports the placement of the Disparoneurinae as a subfamily of the Platycnemididae. This resolves the monophyly of Platycnemididae.

Sequence alignment and statistics. The COI and 16S rDNA nucleotide sequences appeared to be more variable and parsimony informative among all the data sets as shown by the statistics of the MP analyses. The consistency indices (CI) for COI, COII, 16S rDNA and nuclear 28S rDNA and COI + COII + 16S rDNA + 28S rDNA nucleotide sequences were 0.5685, 0.5042, 0.6880, 0.9157 and 0.7537, respectively; whereas the respective retention indices (RI) were 0.8736, 0.8290, 0.8696, 0.9721 and 0.8976.
Genetic divergence. The uncorrected p-distances of *Copera* and its related taxa based on COI, COII, 16S rDNA, 28S rDNA, and COI + COII + 16S rDNA + 28S rDNA are summarised in Supplementary Table 1. Based on combined COI, COII, 16S rDNA and 28S rDNA sequences, the intraspecific p-distance varied from 0% to 1.08% (Supplementary Table 1e). The interspecific p-distance was many times larger: 9.41% to 12.82% for congeneric species of *Copera*; 13.90% to 15.39% between the genera *Copera* and *Coeliccia*; 13.66% to 14.79% between the genera *Copera* and *Prodasineura*; and 14.43% to 14.46% between the genera *Coeliccia* and *Prodasineura* (Supplementary Table 1e).

Phylogenetic relationships based on 28S rDNA nucleotide sequences. The annulata group of the genus *Copera* (*C. ciliata*) clustered with *Platycnemis pennipes* and was distinctly separated from the marginipes group (*C. marginipes* and *C. vittata*) (Fig. 1). The yellow-legged (CVIT3) and red-legged (CVIT1 and CIT2) morphs of *C. vittata* shared identical sequences.

Phylogenetic relationships based on combined COI, COII and 16S rDNA nucleotide sequences. The annulata group (*C. ciliata*) of the genus *Copera* was distinctly separated from the marginipes group (*C. marginipes* and *C. vittata*) (Fig. 2). The yellow-legged (CVIT3) and...
red-legged (CVIT1 and CVIT2) morphs of *C. vittata* grouped in a highly supported clade in all analyses, indicating their genetic similarity. The Calicnemiinae (*Coeliccia albicauda*) appeared to be non-monophyletic with respect to the Platycnemidinae, and the Disparoneurinae (Protoneuridae) showed a closer relationship with the Platycnemidinae.

**Phylogenetic relationships based on combined COI, COII, 16S and 28S nucleotide sequences.** The annulata group (*C. ciliata*) of the genus *Copera* was distinctly separated from the marginipes group (*C. marginipes* and *C. vittata*) (Fig. 3). The yellow-legged morph (CVIT3) of *C. vittata* was genetically similar to the red-legged morph (CVIT1 and CVIT2), and they were highly supported as monophyletic in all analyses. Calicnemiinae (*Coeliccia albicauda*) was not monophyletic with respect to the Platycnemidinae, and the Disparoneurinae (Protoneuridae) showed a closer relationship with the Platycnemidinae.

**Discussion**

Species identification based on morphological characters has proven to be problematic in sibling and polymorphic odonate taxa and in other organisms. Morphological characters have also posed problems at higher taxonomic levels. Currently, molecular sequence data are used for determining the systematic status and phylogenetic relationship at various taxonomic levels. The mitochondrial COI, COII and 16S rRNA genes have been commonly used to study the phylogenetics of odonate species. Additionally, the slower-evolving nuclear 28S rRNA gene has been used for determining odonate phylogeny.

Here, the genetic similarity of the yellow-legged and red-legged forms of *C. vittata* indicates that these two morphs are conspecific rather than members of a species complex. Female-limited colour polymorphism is common in adult odonates. By contrast, male-limited polymorphisms are not as common. As in the larvae of *Ceriagrion chaoi*, the colour morphs of *C. vittata* are not sex-limited.

It can be expected that the topology of the phylogenetic trees that were generated by different methods may vary (Fig. 1 and Supplementary Fig. 1). Such variation has been reported for other organisms, e.g., in filarial parasites and libellulid dragonflies. However, the phylogenetic trees produced from the combined analyses of the mitochondrial encoded COI + COII + 16S rDNA and the COI + COII + 16S rDNA + nuclear encoded 28S rDNA generated by ML,

Figure 3 | Phylogeny of the genus *Copera* and platycnemine subfamilies based on combined COI + COII + 16S rDNA + 28S rDNA nucleotide sequences. Numeric values at nodes are arranged in order of ML bootstrap support/MP bootstrap support/NJ bootstrap support/Bayesian posterior probabilities.
Table 1 | Nucleotide sequences of COI, COII, 16S rRNA and/or 28S rRNA genes for 55 taxa of odonates used in the present study. Orthetrum testaceum and Orthetrum glaucum were used as outgroups. NA, not available

| No. | Sample Name          | Sampling Location | Collection Code | GenBank Accession Number |
|-----|----------------------|-------------------|-----------------|-------------------------|
|     |                      |                   |                 | COI         | COII    | 16S      | 28S      |
| 1   | Copera ciliata       | University Malaya | CCIL1           | KF248070   | -       | KF248125 | KF581171 |
| 2   | Copera ciliata       | University Malaya | CCIL2           | KF248071   | KF248098 | KF248126 | KF581172 |
| 3   | Copera ciliata       | University Malaya | CCIL3           | KF248072   | KF248099 | KF248127 | KF581173 |
| 4   | Copera ciliata       | Lanchang, Pahang  | CCIL4           | KF248073   | KF248100 | KF248128 | KF581174 |
| 5   | Copera ciliata       | Lanchang, Pahang  | CCIL5           | KF248074   | KF248101 | KF248129 | KF581175 |
| 6   | Copera ciliata       | Lanchang, Pahang  | CCIL6           | KF248075   | KF248102 | KF248130 | KF581176 |
| 7   | Copera ciliata       | Lanchang, Pahang  | CCIL7           | KF248076   | KF248103 | KF248131 | KF581177 |
| 8   | Copera ciliata       | Rengit, Pahang    | CCIL8           | KF248077   | KF248104 | KF248132 | KF581178 |
| 9   | Copera marginipes    | University Malaya | CMAR1           | KF248058   | KF248087 | -       | KF581159 |
| 10  | Copera marginipes    | University Malaya | CMAR2           | KF248059   | KF248088 | -       | KF581160 |
| 11  | Copera marginipes    | University Malaya | CMAR3           | KF248060   | KF248089 | KF248115 | KF581161 |
| 12  | Copera marginipes    | University Malaya | CMAR4           | KF248061   | KF248090 | KF248116 | KF581162 |
| 13  | Copera marginipes    | Rengit, Pahang    | CMAR5           | KF248062   | KF248091 | KF248117 | KF581163 |
| 14  | Copera marginipes    | Rengit, Pahang    | CMAR6           | KF248063   | KF248092 | KF248118 | KF581164 |
| 15  | Copera marginipes    | Lanchang, Pahang  | CMAR7           | KF248064   | KF248093 | KF248119 | KF581165 |
| 16  | Copera marginipes    | Rengit, Pahang    | CMAR8           | KF248065   | KF248094 | KF248120 | KF581166 |
| 17  | Copera marginipes    | Rengit, Pahang    | CMAR9           | KF248066   | KF248095 | KF248121 | KF581167 |
| 18  | Copera vittata       | Rengit, Pahang    | CVIT1           | KF248067   | KF248096 | KF248122 | KF581168 |
| 19  | Copera vittata       | Rengit, Pahang    | CVIT2           | KF248068   | KF248097 | KF248123 | KF581169 |
| 20  | Copera vittata       | Bentong, Pahang   | CVIT3           | KF248069   | KF248114 | KF248124 | KF581170 |
|     |                      |                   |                 |            |         |         |         |
| 21  | Coeliccia albicauda  | Lantang, Pahang   | CALB1           | KF248083   | KF248110 | KF248136 | KF581182 |
| 22  | Coeliccia albicauda  | Lantang, Pahang   | CALB2           | KF248084   | KF248111 | KF248137 | KF581183 |
| 23  | Coeliccia albicauda  | Bentong, Pahang   | CALB3           | -          | -       | KF248138 | KF581184 |
|     |                      |                   |                 |            |         |         |         |
| 24  | Prodasinoneura humeralis | Lantang, Pahang | PHUM1           | KF248080   | KF248107 | KF248135 | KF581179 |
| 25  | Prodasinoneura humeralis | Rengit, Pahang  | PHUM2           | KF248081   | KF248108 | -       | KF581180 |
| 26  | Prodasinoneura laidlawii | Rengit, Pahang | PLA1           | KF248082   | KF248109 | -       | KF581181 |
| 27  | Prodasinoneura notostigma | University Malaya | PNOIT1          | KF248078   | KF248105 | KF248133 | -         |
| 28  | Prodasinoneura notostigma | University Malaya | PNOIT2          | KF248079   | KF248106 | KF248134 | -         |
|     |                      |                   |                 |            |         |         |         |
| 29  | Orthetrum testaceum  | University Malaya | OTES4           | KF248085   | KF248112 | KF248139 | KF581185 |
| 30  | Orthetrum glaucum    | University Malaya | OGLA5           | KF248086   | KF248113 | KF248140 | KF581186 |
|     |                      |                   |                 |            |         |         |         |
| 31  | Copera tokyonensis   | Japan             | -               | JF288853   | JF288853 | -       | -         |
| 32  | Copera tokyonensis   | Japan             | -               | JF288856   | JF288856 | -       | -         |
| 33  | Copera tokyonensis   | Japan             | -               | JF288859   | JF288859 | -       | -         |
| 34  | Copera tokyonensis   | Japan             | -               | JF288861   | JF288861 | -       | -         |
| 35  | Copera annulata      | Japan             | -               | JF288868   | JF288868 | -       | -         |
| 36  | Copera annulata      | Japan             | -               | JF288870   | JF288870 | -       | -         |
| 37  | Copera annulata      | Japan             | -               | JF288872   | JF288872 | -       | -         |
| 38  | Copera annulata      | Japan             | -               | JF288873   | JF288873 | -       | -         |
| 39  | Copera annulata      | Japan             | -               | JF288862   | JF288862 | -       | -         |
| 40  | Copera annulata      | Japan             | -               | -          | -       | -       | AB127427 |
| 41  | Copera annulata      | Japan             | NA              | -          | -       | -       | FJ009929 |
|     |                      |                   |                 |            |         |         |         |
| 42  | Coeliccia flavicauda | Japan             | -               | AB446426   | -       | -       | -         |
| 43  | Coeliccia flavicauda | Japan             | -               | AB446427   | -       | -       | -         |
| 44  | Coeliccia cyanomelas | NA               | -               | -          | EU055373 | -       | -         |
|     |                      |                   |                 |            |         |         |         |
| 45  | Platycnemis foliacea | Japan             | -               | JF288876   | JF288876 | -       | -         |
| 46  | Platycnemis foliacea | Japan             | -               | JF288877   | JF288877 | -       | -         |
| 47  | Platycnemis foliacea | Japan             | -               | JF288875   | JF288875 | -       | -         |
| 48  | Platycnemis pennipes | NA               | -               | -          | EU055397 | -       | -         |
The present molecular data (COI, COII, 16S rDNA and 28S rDNA) shows a closer relationship of the annulata group of the genus Coelia to two distinct genetic lineages that most likely warrant separate generic status. Additionally, the larvae of C. marginipes and C. vittata possess fringes of long filaments at the margins of the caudal lamellae; this structure is not present in C. ciliata. Additionally, ghost forms occur in the immatures of C. marginipes and C. vittata. All lines of evidence support the conclusion that the present component species of the genus Copera belong to two distinct genetic lineages that most likely warrant separate generic status.

Phylogenetic analyses based on COI, COII and 16S rDNA nucleotide sequences invariably indicate that the annulata group containing C. ciliata is more closely related to the genus Platycnemis than the marginipes group (Fig. 2, Supplementary Figs. 1–3). This relationship is reflected by the separation of C. marginipes from the grouping of C. annulata with Platycnemis pennipes based on the large and small subunit nuclear and mitochondrial ribosomal RNAs and part of the nuclear EF-1α. Based on molecular evidence, the annulata group of the genus Copera should perhaps be placed in the genus Platycnemis as originally indicated.

The family Platycnemididae, as currently delimited, is not monophyletic. Based on mitochondrial genes (COI, COII and 16S rRNA), the genus Coelia, a member of the subfamily Calicnemiinae (Platycnemididae), is not recovered with the Platycnemidinae. The Disparoneurinae of the ‘Protoneuridae’ (represented by Prodasineura spp.) shows a closer relationship to the Platycnemidinae than to the Calicnemiinae. However, the phylogenetic relationships of Platycnemidinae and Calicnemiinae are not concordant based on mitochondrial genes (COI, COII and 16S rRNA) (Fig. 2, Supplementary Figs. 1–3) or the nuclear 28S rRNA gene (Fig. 1).

In summary, the yellow-legged and red-legged forms of C. vittata are most likely conspecific. The present dataset supports the inclusion of the annulata group of the genus Copera (C. ciliata and C. annulata/C. tokyoensis) in the genus Platycnemis and Disparoneurinae of the Old World ‘protoneurids’ as a subfamily of Platycnemididae. The Disparoneurinae appear to be more closely related to the Platycnemidinae than to the Calicnemiinae. The inclusion of the Disparoneurinae as a subfamily of the Platycnemididae renders Platycnemididae monophyletic.

**Methods**

**Ethics statement.** No specific permits were required for the described field studies. The damselflies were collected in open ditches and ponds and not from any national parks or protected areas. No specific permissions were required, and the damselflies are not endangered or protected species.

**Specimens.** Specimens were collected using sweep nets or plastic bags. All three Copera species inhabit sluggish channels and shallow pools in swampy areas. They were identified with established literature. Additionally, Coelia albicauda (Forster, 1907), a member of the Calicnemiinae (Platycnemididae) and three species of Prodasineura (Prodisnereinae, Disparoneurinae) were included for comparison. Two species of Orthetrum (Anisoptera) were used as an outgroup. Details of the species studied are listed in Table 1.

**DNA extraction, polymerase chain reaction, and sequencing.** Genomic DNA was extracted and PCR amplification was performed as described in Lim et al., except with variations in annealing temperature for different primers. The primers and annealing temperature for PCR were: COI – COS2265 (forward): GCACAAGAAAGAGGGAAAAAAGA, COA3625 (reverse): GCCCCACAAATTTCGGAACATTG, at 50°C; COII – C2-J-3102: AAATGGCAACATGAGCACAAYT, TK-N-3773: GAGACGAGTACTGGCTTCATGCATC, at 50°C; 16S – EU477625 (forward): GAGACGAGTACTGGCTTCATGCATC, at 50°C; 28S – EM_16S_F: TTGACTGTA-9, EM_16S_R: GATATTACGGCTGT7TACCC, at 50°C and 28S rRNA – 28sF, 5′-AGGGATCCATCGGATGTCGATATGC, at 50°C; 28S rRNA – 28sR, 5′-AGGGATCCATCGGATGTCGATATGC, at 50°C.

The PCR amplicons were assayed by electrophoresis on 1.0% agarose mini gels stained with SYBR® Safe DNA gel stain (Invitrogen, USA) and visualised under UV light. The amplicons were isolated and purified using the LaboPass™ PCR purification kit (Cosmo Genetech, South Korea). The purified PCR products were sent to a commercial company for sequencing. Samples were sequenced using BigDye terminator v3.1 Sequencing Kit and analysed on an ABI PRISM 377 Genetic Analyser.

**Table 1 | Cont.**

| No. | Sample Name | Sampling Location | Collection Code | GenBank Accession Number |
|-----|-------------|-------------------|-----------------|-------------------------|
| 49  | Platycnemis latipes | France | - | EU477625 |
| 50  | Platycnemis pennipes | Greece | - | EU477627 |
| 51  | Platycnemis pennipes | NA | - | FJ009928 |
| 52  | Platycnemis acutipennis | France | - | |

**Prodisnereinae**

| No. | Sample Name | Sampling Location | Collection Code | GenBank Accession Number |
|-----|-------------|-------------------|-----------------|-------------------------|
| 53  | Nosostica solida | NA | - | EU055351 |
| 54  | Nosostica solida | Australia | - | FJ009925 |
| 55  | Phylloneura westermannii | NA | - | EU055389 |
DNA sequences from GenBank. To elucidate the phylogenetic relationship among the different species of Copera and related taxa, sequences generated from this study were combined with GenBank sequences (Table 1) to construct phylogenetic trees.

Genetic divergence. To assess the species level variation of Copera and related taxa, selected specimens were used to measure the uncorrected (p) pairwise genetic distances using PAUP* 4.0b10 software. All individual markers, combined mitochondrial markers COI + COI + 16S rDNA and combined the corrected mitochondrial rDNA + 28S rDNA were used to estimate uncorrected (p) pairwise genetic distances.

Sequence alignment and phylogenetic analysis. The COI, COI, 16S rDNA and 28S rDNA nucleotide sequences were initially aligned using the CLUSTAL X program and subsequently manually aligned. The combined COI + COI + 16S rDNA and COI + COI + 16S rDNA + 28S rDNA nucleotide sequences were also analysed to better understand the systematic relationships among different Copera species and related taxa. To investigate the utility of combining sequences from different molecular markers, statistical congruence was tested using a partition homogeneity test (PHT). The PST was performed in PAUP* 4.0b10 using 100 replicates and the heuristic standard search options.

Maximum likelihood (ML) analysis was performed via Treefinder version October 2008. Bayesian (BI) analysis was performed using MrBayes 3.1.2. The best fit nucleotide substitution model was determined using KAKUSAN v.3, which also generated input files for ML and BI. Best fit models were evaluated using the corrected Akaike Information Criterion (AIC) for ML and the Bayesian Information Criterion (BIC) with significance determined by Chi-square analysis.

To elucidate the phylogenetic relationship among the different species of Copera and related taxa, selected specimens were used to measure the uncorrected (p) pairwise genetic distances using PAUP* 4.0b10 software. All individual markers, combined mitochondrial markers COI + COI + 16S rDNA and combined the corrected mitochondrial rDNA + 28S rDNA were used to estimate uncorrected (p) pairwise genetic distances.

Sequence alignment and phylogenetic analysis. The COI, COI, 16S rDNA and 28S rDNA nucleotide sequences were initially aligned using the CLUSTAL X program and subsequently manually aligned. The combined COI + COI + 16S rDNA and COI + COI + 16S rDNA + 28S rDNA nucleotide sequences were also analysed to better understand the systematic relationships among different Copera species and related taxa. To investigate the utility of combining sequences from different molecular markers, statistical congruence was tested using a partition homogeneity test (PHT). The PST was performed in PAUP* 4.0b10 using 100 replicates and the heuristic standard search options.

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To elucidate the phylogenetic relationship among the different species of Copera and related taxa, selected specimens were used to measure the uncorrected (p) pairwise genetic distances using PAUP* 4.0b10 software. All individual markers, combined mitochondrial markers COI + COI + 16S rDNA and combined the corrected mitochondrial rDNA + 28S rDNA were used to estimate uncorrected (p) pairwise genetic distances.
