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# 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.**

The genus *Copera* Kirby, 1890 is a member of the subfamily Platycnemidinae in the family Platycnemididae[1–3]. It is represented by nine species worldwide[4]. Another genus of the subfamily is *Platycnemis* Burmeister, 1839, with 30 species[4]. In Malaysia, only the genus *Copera* is represented from the subfamily Platycnemidinae[5,6]. Three species occur in Peninsular Malaysia – *C. ciliata* (Selys, 1863), *C. marginipes* (Rambur, 1842), and *C. vittata* (Selys, 1863); two of these (*C. marginipes* and *C. vittata*) also occur in Sabah-Sarawak (Borneo).

Taxonomic uncertainty has been noted at the species and generic level[1–3,7], particularly the generic status of the component genera (*Copera* and *Platycnemis*) from the subfamily Platycnemidinae[3]. In addition to the generic status, the taxonomic status of some species of *Copera* has been unclear. Before 1984, *C. annulata* and *C. ciliata* were considered the same species[8]. Recently, *C. ciliata* remains a synonym (as *Psilocnemis ciliata* Selys, 1863) of *A. annulata* in Korean Zygoptera[9], indicating the occurrence of *C. annulata* in Malaysia. Taxonomic uncertainty of *C. tokyoensis* is reflected by the non-monophyly of its mitochondrial gene (COI-COII) genealogy with *C. annulata*[10].

The most widespread species, *C. vittata*, is represented by no fewer than seven subspecies occurring in different parts of South-East Asia[11]. *C. vittata* is believed to contain several distinct species[12]. Red and black-legged forms occur in Borneo[5]. Such variants have been regarded as possibly representing separate species[6].

The present study examined the DNA sequences of mitochondrial COI, COII, 16S rRNA and nuclear 28S rRNA genes in the three component species of the genus *Copera* in Malaysia. Additionally, members of the genus *Coeliccia* (Platycnemididae, Calicnemiinae) and members of the genus *Prodasineura* of the Protoneuridae were included for comparison.

## Results

**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, 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.

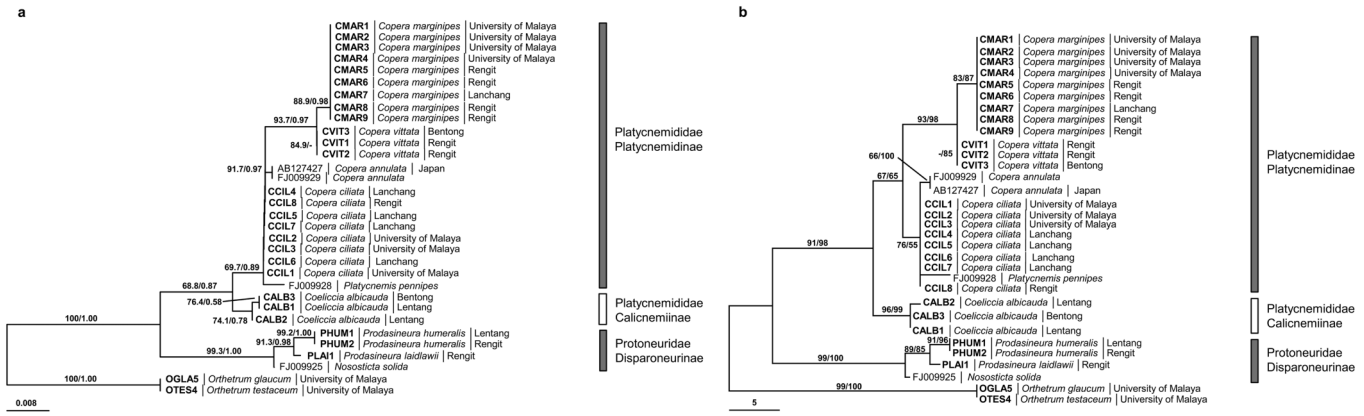


**Figure 1** | **Phylogeny of the genus *Copera* and platycnemine subfamilies based on 28S rDNA nucleotide sequences.** (a) Numeric values at nodes are arranged in order of ML bootstrap support/Bayesian posterior probabilities. (b) Numeric values at nodes are arranged in order of MP bootstrap support/NJ bootstrap support.

**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

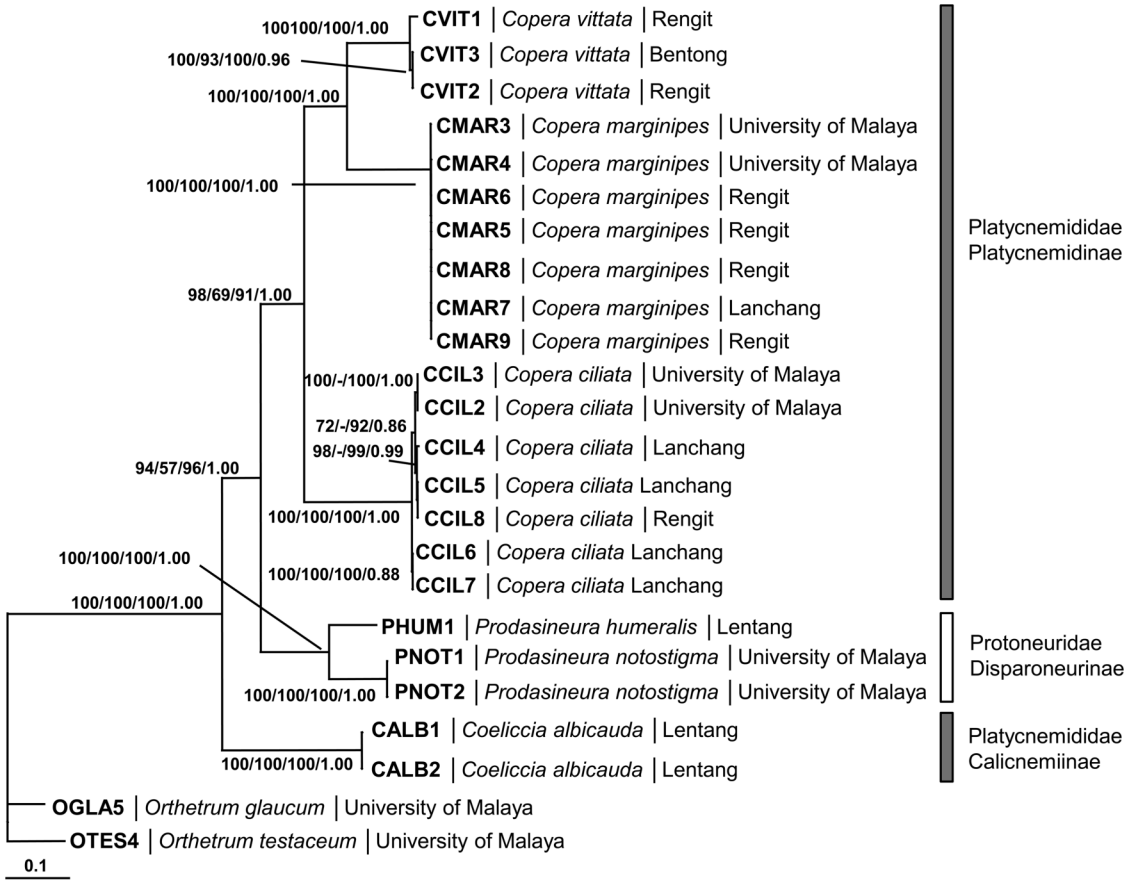


**Figure 2** | **Phylogeny of the genus *Copera* and platycnemine subfamilies based on combined COI + COII + 16S rDNA nucleotide sequences.** Numeric values at nodes are arranged in order of ML bootstrap support/MP bootstrap support/NJ bootstrap support/Bayesian posterior probabilities.

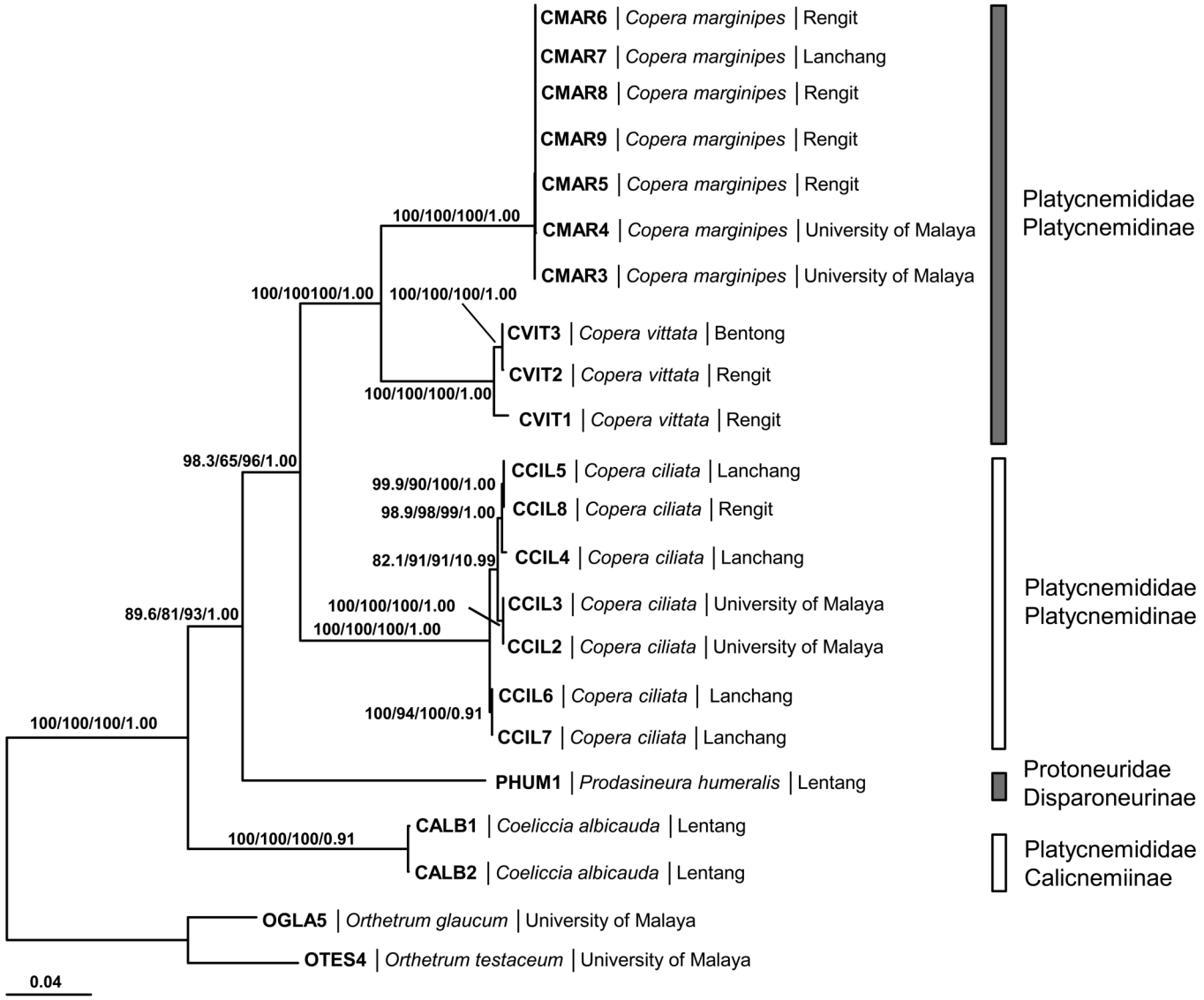


**Figure 3 | Phylogeny of the genus *Copera* and platycnemine subfamilies based on combined COI + COII + 16S rDNA + 28S rDNA nucleotide sequences.** Numeric values at nodes are arranged in order of ML bootstrap support/MP bootstrap support/NJ bootstrap support/Bayesian posterior probabilities.

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[10,13]. Additionally, the slower-evolving nuclear 28S rRNA gene has been used for determining odonate phylogeny[13].

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[14]. By contrast, male-limited polymorphisms are not as common[15]. As in the larvae of *Ceriagrion chaoi*[16], 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[17] and libellulid dragonflies[18]. 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,

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 |
| **Samples derived from this study** | | | | | | | |
| **Platycnemididae** | | | | | | | |
| **Platycnemidinae** | | | | | | | |
| 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 |
| **Platycnemididae** | | | | | | | |
| **Calicnemiinae** | | | | | | | |
| 21 | *Coeliccia albicauda* | Lentang, Pahang | CALB1 | KF248083 | KF248110 | KF248136 | KF581182 |
| 22 | *Coeliccia albicauda* | Lentang, Pahang | CALB2 | KF248084 | KF248111 | KF248137 | KF581183 |
| 23 | *Coeliccia albicauda* | Bentong, Pahang | CALB3 | - | - | KF248138 | KF581184 |
| **Protoneuridae** | | | | | | | |
| **Disparoneurinae** | | | | | | | |
| 24 | *Prodasineura humeralis* | Lentang, Pahang | PHUM1 | KF248080 | KF248107 | KF248135 | KF581179 |
| 25 | *Prodasineura humeralis* | Rengit, Pahang | PHUM2 | KF248081 | KF248108 | - | KF581180 |
| 26 | *Prodasineura laidlawii* | Rengit, Pahang | PLAI1 | KF248082 | KF248109 | - | KF581181 |
| 27 | *Prodasineura notostigma* | University Malaya | PNOIT1 | KF248078 | KF248105 | KF248133 | - |
| 28 | *Prodasineura notostigma* | University Malaya | PNOT2 | KF248079 | KF248106 | KF248134 | - |
| **Libellulidae** | | | | | | | |
| 29 | *Orthetrum testaceum* | University Malaya | OTES4 | KF248085 | KF248112 | KF248139 | KF581185 |
| 30 | *Orthetrum glaucum* | University Malaya | OGLA5 | KF248086 | KF248113 | KF248140 | KF581186 |
| **Samples taken from GenBank** | | | | | | | |
| **Platycnemididae** | | | | | | | |
| **Platycnemidinae** | | | | | | | |
| 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 tokyonensis* | 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* | NA | - | - | - | - | FJ009929 |
| **Platycnemididae** | | | | | | | |
| **Platycnemididae** | | | | | | | |
| **Calicnemiinae** | | | | | | | |
| 42 | *Coeliccia flavicauda* | Japan | - | AB446426 | - | - | - |
| 43 | *Coeliccia flavicauda* | Japan | - | AB446427 | - | - | - |
| 44 | *Coeliccia cyanomelas* | NA | - | - | EU055373 | - | |
| **Platycnemididae** | | | | | | | |
| **Platycnemidinae** | | | | | | | |
| 45 | *Platycnemis foliacea* | Japan | - | JF288876 | JF288876 | - | - |
| 46 | *Platycnemis foliacea* | Japan | - | JF288877 | JF288877 | - | - |
| 47 | *Platycnemis foliacea* | Japan | - | JF288875 | JF288875 | - | - |
| 48 | *Platycnemis pennipes* | NA | - | - | EU055397 | - | - |

Table 1 | Cont.

| No. | Sample Name | Sampling Location | Collection Code | GenBank Accession Number | | | |
|---|---|---|---|---|---|---|---|
| | | | | COI | COII | 16S | 28S |
| 49 | *Platycnemis latipes* | France | - | - | - | EU477625 | - |
| 50 | *Platycnemis pennipes* | Greece | - | - | - | EU477627 | - |
| 51 | *Platycnemis pennipes* | NA | - | - | - | - | FJ009928 |
| 52 | *Platycnemis acutipennis* | France | - | - | - | EU477626 | - |
| **Protoneuridae** | | | | | | | |
| **Disparoneurinae** | | | | | | | |
| 53 | *Nosostica solida* | NA | - | - | EU055351 | - | - |
| 54 | *Nososticta solida* | Australia | - | - | - | - | FJ009925 |
| 55 | *Phylloneura westermanni* | NA | - | - | EU055389 | - | - |

MP, NJ or BI methods are concordant (Figs. 2, 3). Among the four markers used, COI appears to be the most variable (based on the genetic p-distance and support values of the phylogenetic trees) and is thus suitable as the single marker of choice for species differentiation and phylogenetic analysis of Odonata.

The present molecular data (COI, COII, 16S rDNA and 28S rDNA nucleotide sequences) clearly separate the marginipes group (*C. marginipes* and *C. vittata*), with coloured legs and the tibiae only moderately distended, from the annulata group (including *C. ciliata*), with white legs and greatly dilated tibiae (Figs. 1–3). A genetic difference between *C. marginipes* and *C. annulata* has also been reported based on the DNA sequences of the large and small subunit nuclear and mitochondrial ribosomal RNAs and part of the nuclear EF-1α[19]. 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*[6]. 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[19]. Based on molecular evidence, the annulata group of the genus *Copera* should perhaps be placed in the genus *Platycnemis* as originally indicated[1].

The family Platycnemididae, as currently delimited, is not monophyletic. Based on mitochondrial genes (COI, COII and 16S rRNA), the genus *Coeliccia*, 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). This discrepancy reflects the need to use multiple genes and extensive taxon sampling to arrive at a more accurate phylogenetic relationship.

Nucleotide sequences of the nuclear ribosomal genes 5.8S, 18S, and ITS1 and 2 indicate that the Platycnemididae is non-monophyletic because of several representatives of uncertain placement, but the true Platycnemididae and the non-American protoneurids are closely related[20]. An earlier study from 122 phylogenetically informative characters (skeletal morphology and wing venation of adults, and a few larval characters) also indicates that the family Platycnemididae is not demonstrably monophyletic[21].

The Paleotropical component of the Protoneuridae appears to be more closely related to the Platycnemididae and the Isostictidae[22]. Based on the finding of the Old World disparoneurine protoneurids nesting within the Platycnemididae and well-separated from the New World protoneurine *Neoneura aaroni*, it has been suggested that the Disparoneurinae be regarded as a subfamily of the Platycnemididae[19]. The present analysis, involving *Prodasineura* spp., *Nososticta solida* and *Phylloneura westermanni* of the Disparoneurinae ('Protoneuridae'), concurs with previous findings of their close relationship with the Platycnemididae.

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[5,12]. Additionally, *Coeliccia albicauda* (Förster, 1907), a member of the Calicnemiinae (Platycnemididae) and three species of *Prodasineura* (Protoneuridae, 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[23]. except with variations in annealing temperature for different primers. The primers and annealing temperature for PCR were: COI – COS2265 (forward): GCACAAGAAAG AGGGAAAAAAGA, COA3625 (reverse): GCCCCACAAATTTCGGAACATTG, at 50°C[10,24]; COII – C2-J-3102: AAATGGCAACATGAGCACAAYT, TK-N-3773: GAGACCAGTACTTGCTTTCAGTCATC, at 50°C[25] 16S – LR-J-13756 (16S-F): TAGTTTTTTTAGAAATAAATTTAATTTA, LR-N-13308 (16S-R): GCCTTCAATTAAAAGACTAA, at 42°C[26], and Hym_16S_F: TTGACTGTA-CAAAGGTAGC, Hym_16S_R: GATATTACGCTGTTATCCC, at 50°C[27] and 28S rDNA – 28sf, 5′-AAGGTAGCCAAATGCCTCATC-3′; 28sr, 5′-AGTAGGG-TAAAACTAACCT-3′ at 50°C[28].

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 LaboPassTM PCR purification kit (Cosmo Genetech, South Korea). The purified PCR products were sent to a commercial company for sequencing. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit and analysed on an ABI PRISMH 377 Genetic Analyser.

**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[29]. All individual markers, combined mitochondrial markers COI + COII + 16S rDNA and combined COI + COII + 16S rDNA + 28S rDNA were used to estimate uncorrected (p) pairwise genetic distances.

**Sequence alignment and phylogenetic analysis.** The COI, COII, 16S rDNA and 28S rDNA nucleotide sequences were initially aligned using the CLUSTAL X program[30] and subsequently manually aligned. The combined COI + COII + 16S rDNA and COI + COII + 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)[31,32]. The PHT was performed in PAUP* 4.0b10[29] using 100 replicates and the heuristic standard search options.

Maximum likelihood (ML) analysis was performed via Treefinder version October 2008[33]. Bayesian (BI) analysis was performed using MrBayes 3.1.2[34]. The best fit nucleotide substitution model was determined using KAKUSAN v.3[35], which also generated input files for ML and BI. Best fit models were evaluated using the corrected Akaike Information Criterion[36,37] for ML and the Bayesian Information Criterion (BIC) with significance determined by Chi-square analysis.

The best model for COI, combined COI + COII + 16S rDNA and combined COI + COII + 16S rDNA + 28S rDNA was the general time-reversible (GTR) model of DNA evolution with a gamma shape parameter (G); the best model for COII was J2 with a gamma shape parameter (G); the best model for mitochondrial 16S rDNA and nuclear 28S rDNA was the HKY model with a gamma-shaped parameter (G).

ML analyses were performed with 1000 bootstrap replicates. Two parallel runs were performed in MrBayes using four Markov chain Monte Carlo (MCMC) chains. One million MCMC generations were run, with convergence diagnostics calculated every 1000th generation for monitoring the stabilisation of log likelihood scores. Trees in each chain were sampled every 100th generation. A 50% majority rule consensus tree was generated from the sampled trees after discarding the first 20%.

Maximum Parsimony (MP) analyses were performed using PAUP* 4.0b10[29] using a heuristic search with 100 random sequence addition replicates and a tree bisection reconnection (TBR) branch-swapping algorithm. Gaps in the alignment were treated as missing data. All characters were treated as unordered and equally weighted, the Multrees option active and branches with a maximum length of zero collapsed to yield polytomies. To assess support for the resulting nodes, bootstrap percentage (BP) was computed with 1000 replications using one random taxon addition under the heuristic search method with TBR swapping. For the datasets that yielded more than one tree, the trees sharing the same topology with ML and BI analyses were chosen.


1. Fraser, F. C. *A reclassification of the order Odonata* (Royal Zoological Society of New South Wales, Sydney, 1957).
2. Gassman, D. The Phylogeny of Southeast Asian and Indo-Pacific Calicnemiinae (Odonata, Platycnemididae). *Bonner zool. Beiträge* **53**, 37–80 (2004).
3. Hämäläinen, M. *PIatycnemis phasmovolans* sp. nov. – an extraordinary damselfly from Laos with notes on its East Asian congeners (Odonata: Platycnemididae). *Tombo* **46**, 1–7 (2003).
4. Martens, A. *Die Federlibellen Europas* (Westarp Wissenschaften, Magdeburg, 1996).
5. Orr, A. G. *A guide to the dragonflies of Borneo: their identification and biology* (Natural History Publications (Borneo), Kota Kinabalu, 2003).
6. Orr, A. G. *Dragonflies of Peninsular Malaysia and Singapore* (Natural History Publications (Borneo), Kota Kinabalu, 2005).
7. Dijkstra, K. D. B., Clausnitzer, V. & Martens, A. Tropical African *Platycnemis* damselflies (Odonata: Platycnemididae) and the biogeographical significance of a new species from Pemba Island, Tanzania. *Syst. Biodiv.* **5**, 187–198 (2007).
8. Asahina, S. *Copera annulata* (Selys) composed of two different species! *Chô Chô* **7**, 2–10 (1984).
9. Yum, J. W., Lee, H. Y. & Bae, Y. J. Taxonomic review of the Korean Zygoptera (Odonata). *Ent. Res. Bull.* **26**, 41–55 (2010).
10. Kiyoshi, T. *et al*. Taxonomic uncertainty of a highly endangered brook damselfly, *Copera tokyoensis* Asahina, 1948 (Odonata: Platycnemididae), revealed by mitochindrial gene genealogy. *Conserv. Genet.* **12**, 845–849 (2011).
11. Silsby, J. *Dragonflies of the world* (CSIRO Publishing, Collingwood, 2001).
12. Tang, H. B., Wang, L. K. & Hämäläinen, M. *A photographic guide to the dragonflies of Singapore* (The Raffles Museum of Biodiversity Research, Singapore, 2010).
13. Bybee, S. M., Ogden, T. H., Branham, M. A. & Whiting, M. F. Molecules, morphology and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. *Cladistics* **24**, 477–514 (2008).
14. Fincke, O. M., Jodicke, R., Paulson, D. & Schultz, T. D. The evolution and frequency of female colour morphs in Holartic Odonata: Why are male-like females typically the minority? *Int. J. Odonatol.* **8**, 183–212 (2005).
15. van Gossum, H., Sherratt, T. N. & Cordero-Rivera, A. The evolution of sex-limited colour polymorphism. *Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research*. Córdoba-Aguilar, A. (ed.) 219–229 (Oxford University Press, Oxford, 2008).
16. Yong, H. S., Lim, P. E., Tan, J. & Eamsobhana, P. Genetically determined colour polymorphism in larvae of *Ceriagrion chaoi* (Insecta: Odonata: Coenagrionidae). *Raffles Bull. Zool.* **61**, 47–51 (2013).
17. Morales-Hojas, R. Molecular systematics of filarial parasites, with an emphasis on groups of medical and veterinary importance, and its relevance for epidemiology. *Inf. Genet. Evol.* **9**, 748–759 (2009).
18. Zhang, D.-Z., Dai, J.-X. & Zheng, Z.-M. Phylogeny of Libellulidae based on mitochondrial cytochrome b nucleotide sequences (Odonata: Anisoptera). *Sizhuan J. Zool.* **25**, 695–699 (2006).
19. Carle, F. L., Kjer, K. M. & May, M. L. Evolution of Odonata, with special reference to Coenagrionoidea (Zygoptera). *Arthropod Syst. Phy.* **66**, 37–44 (2008).
20. Dumont, H. J., Vierstraete, A. & Vanfleteren, J. R. A molecular phylogeny of the Odonata (Insecta). *Syst. Ent.* **35**, 6–18 (2010).
21. Rehn, A. C. Phylogenetic analysis of higher-level relationships of Odonata. *Syst. Ent.* **28**, 181–239 (2003).
22. Pessacq, P. Phylogeny of Neotropical Protoneuridae (Odonata: Zygoptera) and a preliminary study of their relationship with related families. *Syst. Ent.* **33**, 511–528 (2008).
23. Lim, P. E., Tan, J., Suana, I. W., Eamsobhana, P. & Yong, H. S. Distinct genetic lineages of *Bactrocera caudata* (Insecta: Tephritidae) revealed by COI and 16S DNA sequences. *PLoS ONE* **7**, e37276 (2012).
24. Kiyoshi, T. & Sota, T. Differentiation of the dragonfly genus *Davidius* (Odonata: Gomphidae) in Japan inferred from mitochondrial and nuclear gene genealogies. *Zool. Sci.* **23**,1–8 (2006).
25. Jordan, S., Simon, C. & Polhemus, D. Molecular systematics and adaptive radiation of Hawaii's endemic damselfly genus *Megalagrion* (Odonata: Coenagrionidae). *Syst. Biol.* **52**, 89–109 (2003).
26. Smith, P. T., Kambhampati, S. & Armstrong, K. A. Phylogenetic relationships among *Bactrocera* species (Diptera: Tephritidae) inferred from mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* **26**, 8–17 (2003).
27. Schmitz, J. & Moritz, R. F. A. Molecular phylogeny of Vespidae (Hymenoptera) and the evolution of sociality in wasps. *Mol. Phylogenet. Evol.* **9**, 183–191 (1998).
28. Hasegawa, E. & Kasuya, E. Phylogenetic analysis of the insect order Odonata using 28S and 16S rDNA sequences: a comparison between data sets with different evolutionary rates. *Entomol. Sci.* **9**, 55–66 (2006).
29. Swofford, D. L. *PAUP: Phylogenetic analysis using parsimony (and other methods)* (Sinauer Associates, Sunderland, Massachusetts, 2002).
30. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **24**, 4876–4882 (1997).
31. Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. Constructing a significance test for incongruence. *Syst. Biol.* **44**, 570–572 (1995).
32. Huelsenbeck, J. P., Bull, J. J. & Cunningham, C. W. Combining data in phylogenetic analysis. *TREE* **11**, 152–158 (1996).
33. Jobb, G., von Haeseler, A. & Strimmer, K. Treefinder: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol. Biol.* **4**, 18 (2004).
34. Huelsenbeck, J. P. & Ronquist, F. MrBayes: Bayesian Inference of phylogenetic trees. *Bioinformatics* **17**, 754–755 (2001).
35. Tanabe, A. S. Kakusan: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. *Mol. Ecol. Notes* **7**, 962–964 (2007).
36. Akaike, H. Information Theory and an Extension of the Maximum Likelihood Principle. *Proceedings of the 2nd International Symposium on Information Theory*. Petrov, B. N. & Csaki, F. (eds.) 267–281 (Akademia Kiado, Budapest, 1973).
37. Shono, H. Efficiency of the finite correction of Akaike's information criteria. *Fish. Sci.* **66**, 608–610 (2000).


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## Author contributions

H.S.Y. and P.E.L. conceived the research in collaboration with J.T. and P.E. H.S.Y. collected and identified the specimens. J.T. conducted the PCR and P.E.L., J.T. and P.E. performed the phylogenetic analyses. H.S.Y. and P.E.L. wrote the paper in collaboration with the co-authors. H.S.Y. and P.E.L. were responsible for the final manuscript version.

## Additional information

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