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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 *Coelliccia*, 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 worldwide⁴. Another genus of the subfamily is *Platycnemis* Burmeister, 1839, with 30 species⁴. 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 Platycnemidinae³. 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 species⁸. Recently, *C. ciliata* remains a synonym (as *Psilocnemis ciliata* Selys, 1863) of *A. annulata* in Korean Zygotera⁹, 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 Asia¹¹. *C. vittata* is believed to contain several distinct species¹². Red and black-legged forms occur in Borneo⁵. Such variants have been regarded as possibly representing separate species⁶.

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 *Coelliccia* (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.

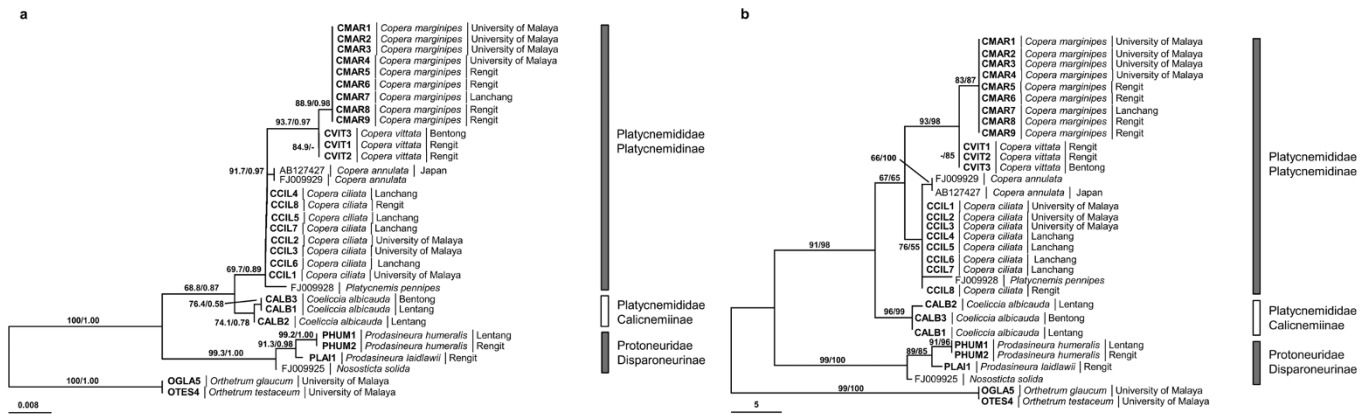


Figure 1 | Phylogeny of the genus *Copera* and platycnemine subfamilies based on 28S rDNA nucleotide sequences. (a) Numeric values at nodes are arranged in order of ML bootstrap support/Bayesian posterior probabilities. (b) Numeric values at nodes are arranged in order of MP bootstrap support/NJ bootstrap 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 *Coelliccia*; 13.66% to 14.79% between the genera *Copera* and *Prodasineura*; and 14.43% to 14.46% between the genera *Coelliccia* 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 CVIT2) 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

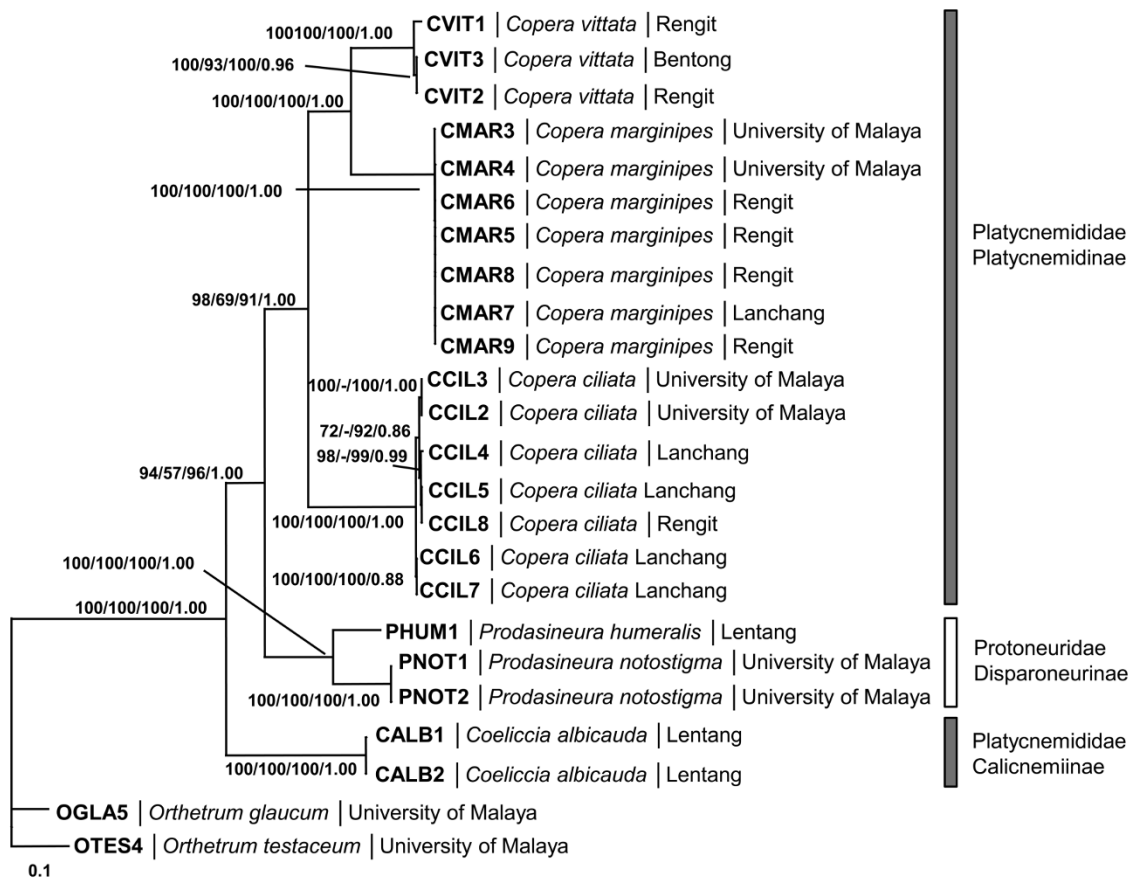


Figure 2 | Phylogeny of the genus *Copera* and platycnemine subfamilies based on combined COI + COII + 16S rDNA nucleotide sequences. Numeric values at nodes are arranged in order of ML bootstrap support/MP bootstrap support/NJ bootstrap support/Bayesian posterior probabilities.

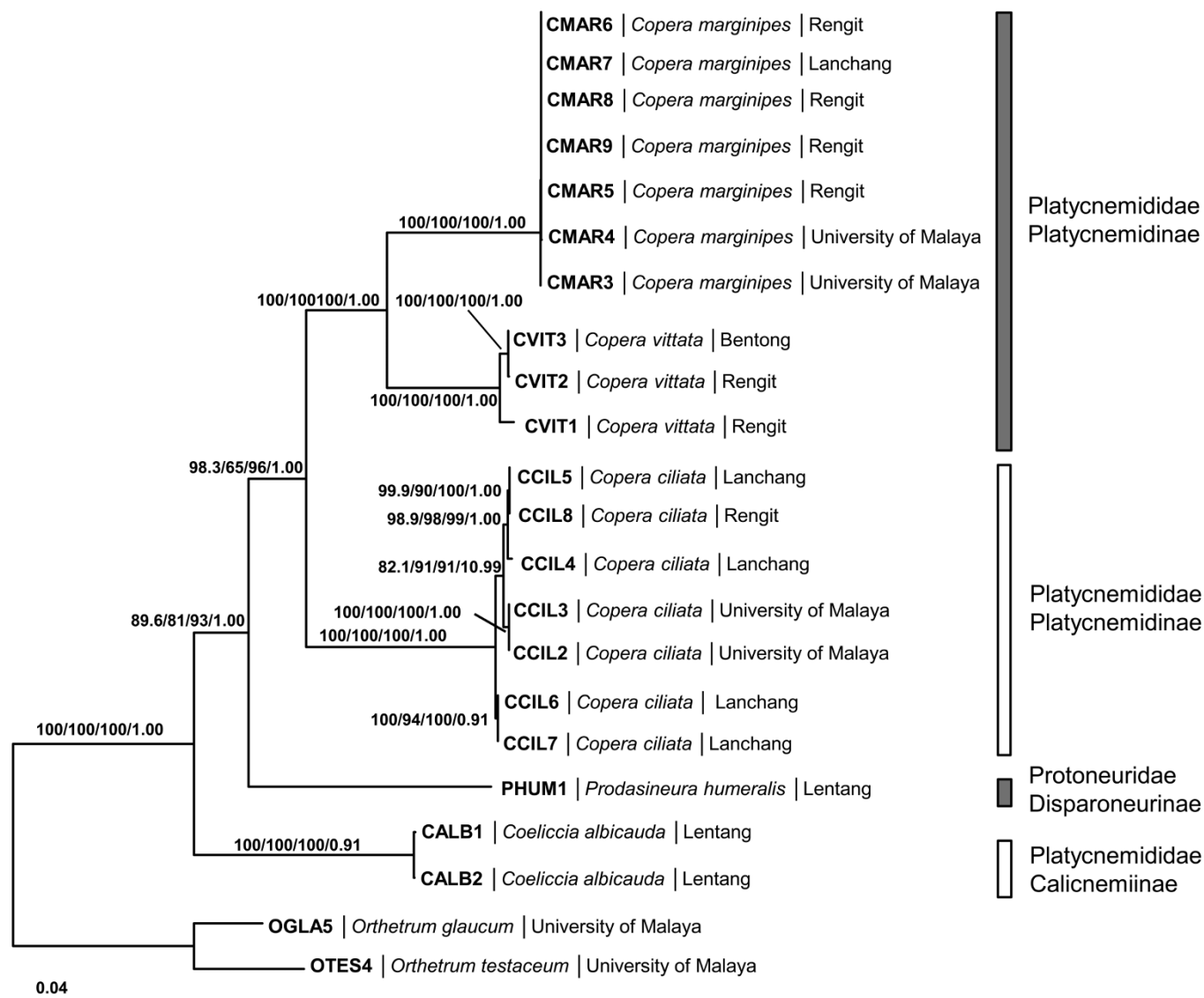


Figure 3 | Phylogeny of the genus *Copera* and platycnemine subfamilies based on combined COI + COII + 16S rDNA + 28S rDNA nucleotide sequences. Numeric values at nodes are arranged in order of ML bootstrap support/MP bootstrap support/NJ bootstrap support/Bayesian posterior probabilities.

red-legged (CVIT1 and CVIT2) morphs of *C. vittata* grouped in a highly supported clade in all analyses, indicating their genetic similarity. The Calicnemiinae (*Coelliccia 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 (*Coelliccia 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 phylogeny¹³.

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 odonates¹⁴. By contrast, male-limited polymorphisms are not as common¹⁵. 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 parasites¹⁷ and libellulid dragonflies¹⁸. 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	<i>Copera ciliata</i>	University Malaya	CCIL1	KF248070	-	KF248125	KF581171
2	<i>Copera ciliata</i>	University Malaya	CCIL2	KF248071	KF248098	KF248126	KF581172
3	<i>Copera ciliata</i>	University Malaya	CCIL3	KF248072	KF248099	KF248127	KF581173
4	<i>Copera ciliata</i>	Lanchang, Pahang	CCIL4	KF248073	KF248100	KF248128	KF581174
5	<i>Copera ciliata</i>	Lanchang, Pahang	CCIL5	KF248074	KF248101	KF248129	KF581175
6	<i>Copera ciliata</i>	Lanchang, Pahang	CCIL6	KF248075	KF248102	KF248130	KF581176
7	<i>Copera ciliata</i>	Lanchang, Pahang	CCIL7	KF248076	KF248103	KF248131	KF581177
8	<i>Copera ciliata</i>	Rengit, Pahang	CCIL8	KF248077	KF248104	KF248132	KF581178
9	<i>Copera marginipes</i>	University Malaya	CMAR1	KF248058	KF248087	-	KF581159
10	<i>Copera marginipes</i>	University Malaya	CMAR2	KF248059	KF248088	-	KF581160
11	<i>Copera marginipes</i>	University Malaya	CMAR3	KF248060	KF248089	KF248115	KF581161
12	<i>Copera marginipes</i>	University Malaya	CMAR4	KF248061	KF248090	KF248116	KF581162
13	<i>Copera marginipes</i>	Rengit, Pahang	CMAR5	KF248062	KF248091	KF248117	KF581163
14	<i>Copera marginipes</i>	Rengit, Pahang	CMAR6	KF248063	KF248092	KF248118	KF581164
15	<i>Copera marginipes</i>	Lanchang, Pahang	CMAR7	KF248064	KF248093	KF248119	KF581165
16	<i>Copera marginipes</i>	Rengit, Pahang	CMAR8	KF248065	KF248094	KF248120	KF581166
17	<i>Copera marginipes</i>	Rengit, Pahang	CMAR9	KF248066	KF248095	KF248121	KF581167
18	<i>Copera vittata</i>	Rengit, Pahang	CVIT1	KF248067	KF248096	KF248122	KF581168
19	<i>Copera vittata</i>	Rengit, Pahang	CVIT2	KF248068	KF248097	KF248123	KF581169
20	<i>Copera vittata</i>	Bentong, Pahang	CVIT3	KF248069	KF248114	KF248124	KF581170
Platycnemididae							
Calicnemiinae							
21	<i>Coelliccia albicauda</i>	Lentang, Pahang	CALB1	KF248083	KF248110	KF248136	KF581182
22	<i>Coelliccia albicauda</i>	Lentang, Pahang	CALB2	KF248084	KF248111	KF248137	KF581183
23	<i>Coelliccia albicauda</i>	Bentong, Pahang	CALB3	-	-	KF248138	KF581184
Protoneuridae							
Disparoneurinae							
24	<i>Prodasineura humeralis</i>	Lentang, Pahang	PHUM1	KF248080	KF248107	KF248135	KF581179
25	<i>Prodasineura humeralis</i>	Rengit, Pahang	PHUM2	KF248081	KF248108	-	KF581180
26	<i>Prodasineura laidlawii</i>	Rengit, Pahang	PLAI1	KF248082	KF248109	-	KF581181
27	<i>Prodasineura notostigma</i>	University Malaya	PNOIT1	KF248078	KF248105	KF248133	-
28	<i>Prodasineura notostigma</i>	University Malaya	PNOT2	KF248079	KF248106	KF248134	-
Libellulidae							
29	<i>Orthetrum testaceum</i>	University Malaya	OTES4	KF248085	KF248112	KF248139	KF581185
30	<i>Orthetrum glaucum</i>	University Malaya	OGLA5	KF248086	KF248113	KF248140	KF581186
Samples taken from GenBank							
Platycnemididae							
Platycnemidinae							
31	<i>Copera tokyonensis</i>	Japan	-	JF288853	JF288853	-	-
32	<i>Copera tokyonensis</i>	Japan	-	JF288856	JF288856	-	-
33	<i>Copera tokyonensis</i>	Japan	-	JF288859	JF288859	-	-
34	<i>Copera tokyonensis</i>	Japan	-	JF288861	JF288861	-	-
35	<i>Copera annulata</i>	Japan	-	JF288868	JF288868	-	-
36	<i>Copera tokyonensis</i>	Japan	-	JF288870	JF288870	-	-
37	<i>Copera annulata</i>	Japan	-	JF288872	JF288872	-	-
38	<i>Copera annulata</i>	Japan	-	JF288873	JF288873	-	-
39	<i>Copera annulata</i>	Japan	-	JF288862	JF288862	-	-
40	<i>Copera annulata</i>	Japan	-	-	-	-	AB127427
41	<i>Copera annulata</i>	NA	-	-	-	-	FJ009929
Platycnemididae							
Calicnemiinae							
42	<i>Coelliccia flavicauda</i>	Japan	-	AB446426	-	-	-
43	<i>Coelliccia flavicauda</i>	Japan	-	AB446427	-	-	-
44	<i>Coelliccia cyanomelas</i>	NA	-	-	EU055373	-	-
Platycnemididae							
Platycnemidinae							
45	<i>Platycnemis foliacea</i>	Japan	-	JF288876	JF288876	-	-
46	<i>Platycnemis foliacea</i>	Japan	-	JF288877	JF288877	-	-
47	<i>Platycnemis foliacea</i>	Japan	-	JF288875	JF288875	-	-
48	<i>Platycnemis pennipes</i>	NA	-	-	EU055397	-	-



Table 1 | Cont.

No.	Sample Name	Sampling Location	Collection Code	GenBank Accession Number			
				COI	COII	16S	28S
49	<i>Platycnemis latipes</i>	France	-	-	-	EU477625	-
50	<i>Platycnemis pennipes</i>	Greece	-	-	-	EU477627	-
51	<i>Platycnemis pennipes</i>	NA	-	-	-	-	FJ009928
52	<i>Platycnemis acutipennis</i>	France	-	-	-	EU477626	-
Protoneuridae							
Disparoneurinae							
53	<i>Nososticta solida</i>	NA	-	-	EU055351	-	-
54	<i>Nososticta solida</i>	Australia	-	-	-	-	FJ009925
55	<i>Phylloneura westermanni</i>	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-1 α 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-1 α ¹⁹. Based on molecular evidence, the annulata group of the genus *Copera* should perhaps be placed in the genus *Platycnemis* as originally indicated¹.

The family Platycnemididae, as currently delimited, is not monophyletic. Based on mitochondrial genes (COI, COII and 16S rRNA), the genus *Coelicia*, 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 related²⁰. 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 monophyletic²¹.

The Palearctic component of the Protoneuridae appears to be more closely related to the Platycnemididae and the Isostictidae²². 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 Platycnemididae¹⁹. 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, *Coelicia 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 al²³, except with variations in annealing temperature for different primers. The primers and annealing temperature for PCR were: COI – COS2265 (forward): GCACAAGAAAG AGGGAAAAAGA, COA3625 (reverse): GCCCCACAAATTTCCGGAACATTG, at 50°C^{19,24}; COII – C2-J-3102: AAATGGCAACATGAGCACAAYT, TK-N-3773: GAGACCAGTACTTGCTTTCAGTCATC, at 50°C²⁵ 16S – LR-J-13756 (16S-F): TAGTTTTTTTAGAAATAAATTTAATTTA, LR-N-13308 (16S-R): GCCTTCAATTTAAAGACTAA, at 42°C²⁶, and Hym_16S_F: TTGACTGTACAAAGGTAGC, Hym_16S_R: GATATTACGCTGTTATCCC, at 50°C²⁷ and 28S rDNA – 28sf, 5'-AAGGTAGCCAAATGCCTCATC-3'; 28sr, 5'-AGTAGGG-TAAACTAACCT-3' at 50°C²⁸.

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 BigDyeH Terminator v3.1 Sequencing Kit and analysed on an ABI PRISM 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 software²⁹. 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 program³⁰ 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* 4.0b10²⁹ using 100 replicates and the heuristic standard search options.

Maximum likelihood (ML) analysis was performed via Treefinder version October 2008³³. Bayesian (BI) analysis was performed using MrBayes 3.1.2³⁴. The best fit nucleotide substitution model was determined using KAKUSAN v.3³⁵, 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* 4.0b10²⁹ 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.

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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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