Molecular phylogeny of *Orthetrum* dragonflies reveals cryptic species of *Orthetrum pruinosum*

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Dragonflies of the genus *Orthetrum* are members of the suborder Anisoptera, family Libellulidae. There are species pairs whose members are not easily separated from each other by morphological characters. In the present study, the DNA nucleotide sequences of mitochondrial and nuclear genes were employed to elucidate the phylogeny and systematics of *Orthetrum* dragonflies. Phylogenetic analyses could not resolve the various subfamilies of the family Libellulidae unequivocally. The nuclear 28S rRNA gene is highly conserved and could not resolve congeneric species of *Orthetrum*. Individual mitochondrial genes (COI, COII, and 16S rRNA) and combination of these genes as well as the nuclear ITS1&2 genes clearly differentiate morphologically similar species, such as the reddish species pairs *O. chrysis* and *O. testaceum*, and the bluish-coloured species *O. glaucum* and *O. luzonicum*. This study also reveals distinct genetic lineages between *O. pruinosum schneideri* (occurring in Malaysia) and *O. pruinosum neglectum* (occurring north of Peninsular Malaysia from India to Japan), indicating these taxa are cryptic species.

Results

Aligned sequences and genetic divergence. The total length for each aligned sequences for various molecular markers and their parsimony information are summarised in Supplementary Table 1. The uncorrected 'p'-distance between *Orthetrum* species based on 16S rDNA, COI, combined COI + 16S rDNA, combined COI + COII + 16S rDNA, ITS1&2, and combined COI + COII + 16S rDNA + 28S rDNA + ITS1&2 nucleotide sequences are summarized in supplementary Tables 2–6 respectively. The interspecific 'p' distance was many folds larger than intraspecific 'p' distance. For COI, the intraspecific p-distance ranged from 0.00–3.99% (highest in *O. melania*), while interspecific p-distance ranged from 3.33% (*O. melania* and *O. triangulare*) to 17.29% (*O. chrysis* and *O. sabina*) (Supplementary Table 2). For 16S rDNA, the intraspecific p-distance ranged from 0.00–2.10% (highest in...
O. glaucum); the interspecific p-distance ranged from 0.60% (O. melania and O. triangularis) to 9.92% (O. abbotti and O. poecilops) (Supplementary Table 2).

The interspecific p-distance for ITS1&2 sequences ranged from 0.00–5.05% (highest in O. luzonicum); the interspecific p-distance ranged from 1.14% (O. pruinosum neglectum and O. testaceum) to 21.12% (O. sabina and O. chrysostigma) (Supplementary Table 3).

The interspecific p-distance for the combined COI + 16S rDNA sequences ranged from 0.00–1.78% (highest in O. sabina); the interspecific p-distance ranged from 1.15% (O. pruinosum neglectum and O. testaceum) to 12.23% (O. chrysis and O. Sabina; O. japonicum and O. Sabina) (Supplementary Table 4). For the combined mitochondrial markers (COI + COII + 16S rDNA) the intraspecific p-distance ranged from 0.00–1.94% (highest in O. pruinosum schneideri); the interspecific p-distance ranged from 7.32% (O. chrysis and O. pruinosum schneideri) to 12.58% (O. chrysis and O. sabina) (Supplementary Table 5).

For the combined five markers (COI + COII + 16S rDNA + ITS1&2) the intraspecific p-distance ranged from 0.00–1.55% (highest in O. pruinosum schneideri); the interspecific p-distance ranged from 4.20% (O. chrysis and O. sabina) to 9.51% (O. chrysis and O. sabina) (Supplementary Table 6).

Phylogenetic relationships based on 28S rDNA nucleotide sequences. There were no distinct nucleotide sequence divergence among the congeners of Orthetrum (supplementary Fig. 1). The various subfamilies of the family Libellulidae were not resolved unequivocally.

Phylogenetic relationships based on 16S rDNA nucleotide sequences. Orthetrum pruinosum schneideri clustered with O. chrysis and both were distinctly separated from O. testaceum and O. pruinosum neglectum (Fig. 1). O. sabina from Peninsular Malaysia was not grouped together with O. sabina of India, Japan and Fiji. Additionally, O. luzonicum from Peninsular Malaysia was distinct from O. luzonicum of China and Japan.

Phylogenetic relationships based on COI nucleotide sequences. Orthetrum pruinosum schneideri clustered with O. chrysis and both were distinctly separated from O. testaceum and O. pruinosum neglectum (Fig. 2). The peninsular Malaysian taxon of O. luzonicum clustered with those of China and Japan. Likewise, O. sabina from Peninsular Malaysia clustered with O. sabina of India, Japan and Fiji.

Phylogenetic relationships based on COII nucleotide sequences. There were two major clusters of Orthetrum species (supplementary Fig. 2): (I) [O. pruinosum schneideri, O. chrysis], O. testaceum, O. melanina, O. luzonicum, O. glaucum, O. albistylum with weak support posterior probability (PP = 0.51) values and no support from maximum likelihood (ML); and (II) O. sabina.

Phylogenetic relationships based on ITS1 and ITS2 nucleotide sequences. The ITS nuDNA nucleotide sequences clearly separated O. pruinosum schneideri and O. pruinosum neglectum (Fig. 3) indicating distinct genetic lineages. O. pruinosum schneideri nested with O. chrysis while O. pruinosum neglectum nested with O. testaceum. The component taxa of Orthetrum were grouped in two distinct clades separated by a clade of other Libellulid genera. O. sabina was not nested with other Orthetrum taxa. The genus Orthetrum and the Libellulid subfamilies were not monophyletic.

Phylogenetic relationships based on combined nucleotide sequences. The combined COI and COII sequences yielded three major clusters (Fig. 4): (I) [O. pruinosum schneideri, O. chrysis], O. testaceum, O. triangularis, O. luzonicum with PP supprot of 0.92 and no support from ML; (II) O. glaucum; and (III) O. sabina.

Similar topology resulted from the combined COI + COII + 16S rDNA nucleotide sequences (supplementary Fig. 3). The combined 5 markers (supplementary Fig. 4) showed three clades: (I) O. chrysis, O. pruinosum schneideri, O. testaceum; (II) O. glaucum, O. sabina; and (III) O. luzonicum.

The combined COI + 16S rDNA sequences of Orthetrum taxa formed five major clusters (Fig 5): (I) [O. pruinosum schneideri, O. chrysis], O. testaceum, O. pruinosum neglectum, O. melanina; (II) [O. internum, O. japonicum], O. poecilops, O. albistylum; (III) O. luzonicum; (IV) O. glaucum; and (V) O. sabina. The first four clusters (I–IV) had full PP and high ML support except cluster V with moderate support of PP = 0.79 and ML = 79%.

Discussion

The phylogeny of the dragonflies (suborder Anisoptera) has been extensively studied4–10. Nine genera of Libellulidae have been reported to be monophyletic11. In the present study with more extensive taxon sampling, the various subfamilies of the family Libellulidae as well as the component taxa of the genus Orthetrum were not resolved unequivocally as monophyletic by the 28S rDNA (supplementary Fig. 1), 16S rDNA (Fig. 1), COI (Fig. 2), and ITS1&2 (Fig. 3) nucleotide sequences.

Species complexes in the genus Orthetrum have been uncovered by DNA sequence analyses. Based on molecular phylogeny and morphological characteristics, Orthetrum internum McLachlan, 1894 (previously regarded as O. japonicum internum McLachlan, 1894) is resolved as a genuine/distinct species from O. japonicum japonicum (Uhler, 1858)12,13. Likewise, O. triangulare and the allied taxon O. melanina are well separated by the nuclear (ITS1 and ITS2) and mitochondrial (COI and 16S rRNA) genes5. Additionally, O. melanina is separated into four subgroups: O. m. melanina (mainland Japan), O. m. continentale (China, Korea and Taiwan), O. m. yaeyamense (Yaeyama Island, Japan), and O. m. rukyuense (Amami, Kerama, Okinawa and Tokara, Japan).

In the present study, the nuclear 28S rDNA nucleotide sequences were highly conserved and could not resolve congenic species of Orthetrum (supplementary Fig. 1). The 28S rDNA gene has been found to be better for resolving deep branching in the Odonata15. However, the mitochondrial genes (COI, COII and 16S) and the nuclear ITS1&2 genes unequivocally separated morphologically similar species, such as the reddish-coloured O. chrysis and O. testaceum and the bluish-coloured species O. glaucum and O. luzonicum (Figs. 1–4, Supplementary Fig. 2). Additionally, the 16S rDNA sequences revealed distinct genetic lineages of (1) O. luzonicum from Peninsular Malaysia and China-Japan, and (2) O. sabina of Peninsular Malaysia and India-Japan-Fiji (Fig. 1).

In the phylogeny based on nine Japanese Orthetrum species, O. pruinosum neglectum clusters with O. melanina15. The present study based on the ITS1&2 (Fig. 3), COI (Fig. 2), 16S rDNA (Fig. 1) and combined COI + 16S rDNA (Fig. 5) nucleotide sequences and with more extensive taxon sampling indicates that O. pruinosum neglectum clusters nearer to O. testaceum than O. melanina. The allied/sibling taxon O. pruinosum schneideri is grouped with O. chrysis (Figs. 1–5, Supplementary Figs. 2–4). It is distinctly separated from O. pruinosum neglectum. The two taxa are, without reasonable doubt, cryptic species of a species complex. In the African dragonfly genus Trithemis, COI and ND1 genes reveal three distinct genetic clusters of T. stricta but these taxa could not be identified by using classical taxonomic characters19.

In summary, phylogenetic analyses of a more extensive taxon sampling based on nucleotide sequences of mitochondrial and nuclear genes indicate that the various subfamilies of the family Libellulidae and the genus Orthetrum are not resolved unequivocally as monophyletic. The nuclear 28S rRNA gene is highly conserved and could not resolve congenic species of Orthetrum. Individual mitochondrial genes (COI, COII, and 16S rRNA) and combination
Figure 1 | BI tree based on 16S rDNA nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.
Figure 2 | BI tree based on COI nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.
of these genes as well as the nuclear ITS1&2 genes clearly differentiate morphologically similar species, such as the reddish species pairs *O. chrysis* and *O. testaceum*, and the bluish-coloured species *O. glaucum* and *O. luzonicum*. This study also reveals distinct genetic lineages between *O. pruinosum schneideri* (occurring in Malaysia) and *O. pruinosum neglectum* (occurring north of Peninsular Malaysia from India to Japan), indicating these taxa are cryptic species. The finding of *O. pruinosum* occurring as a species complex paves the way for an

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Figure 3 | BI tree based on ITS1&2 nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.
in-depth phylogeographical study to determine the systematic status of the component taxa. Likewise, phylogeographical studies are needed for *O. luzonicum* and *O. sabina*.

**Methods**

**Ethics statement.** No specific permits were required for the described field studies. The dragonflies were collected in disturbed habitats such as open ditches and ponds. No specific permissions were required and the dragonflies are not endangered or protected species.

**Specimens.** Specimens of the *Orthetrum* dragonflies for the present study were collected using sweep net or plastic bag. They were identified with established literature15,16. In addition, *Ictinogomphus decoratus* (Anisoptera, Gomphidae) was included for comparison. Two species of *Ceriagrion* (Zygoptera, Coenagrionidae) were used as outgroup. Details of the species studied are listed in Table 1.

**DNA extraction, PCR amplifications and DNA sequencing.** The genomic DNA was extracted and PCR amplification was performed as described in Lim et al.17 except with variations in annealing temperature for different primers. The primers and annealing temperature for PCR were: COI –F: 5'-ATAATTGGRGGRTTYGGRAAYTGG-3', R: 5'- CCAAARAATCAAAATAARTGT TG-3'; COII: C2-J-3102: 5'-AAATGGCAACATGAGCACAAYT-3', TK-N-3773: 5'-GAGACCCAGTACTTGCTTTCAGTCATC-3' at 50°C; 16S rDNA: 5'-TTGACTGTACAAGGTAGC-3' and 5'-GATATTACGCTGTTATCCC-3' at 50°C; 28S rDNA: 28sf, 5'-AAGGTAGCCAAATGCCTCATC-3' and 28sr, 5'-AGTAGGGTAAA-3' at 52°C; ITS1: CAS18sF,5'-TACACACCGCCCGTCGCTACTA-3' and CAS5p8sB1d, 5'-ATGTGCGTTCRAAATGTCGATGTTCA-3' at 67°C; and ITS2: CAS5p8sFc, 5'-TGAACATCGACATTTYGAACGCACAT-3' and CAS28sB1d, 5'-TTCTTTTCCTCCSCTTAYTRATATGCTTAA-3' at 55°C.

The PCR products 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 puri-
The purified PCR products were sent to a commercial company for sequencing. The same set of PCR primers were used for DNA sequencing. Samples were sequenced using BigDye Terminator v3.1 Sequencing Kit and analysed on an ABI PRISM 377 Genetic Analyser.

To assess the parsimony information of the sequences of the data sets and species level variation of Orthetrum species, selected specimens were used to measure the uncorrected (p) pairwise genetic distances using PAUP* 4.0b10 software. All individual markers and combined mitochondrial markers (COI + 16S rDNA; COI + COII + 16S rDNA; and COI + COII + 16S rDNA + 28S rDNA) were used to estimate uncorrected (p) pairwise genetic distances.

To elucidate the phylogenetic relationship among the different species of Orthetrum species, sequences generated from this study were used to elucidate the phylogenetic relationship among the different species of Orthetrum species, sequences generated from this study were

Figure 5 | BI tree based on COI + 16S rDNA nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.
In the preliminary alignment for ITS1 and ITS2, the flanking sequences of Australia) software. The datasets for all genetic markers were aligned using ClustalX2. In the generated forward and reverse sequences were combined with GenBank sequences (Table 1 and Supplementary Table 7) to construct phylogenetic trees. The generated forward and reverse sequences were manually edited and assembled using ChromasPro v1.5 (Technelysium Pty Ltd, Australia) software. The datasets for all genetic markers were aligned using ClustalX2. In the preliminary alignment for ITS1 and ITS2, the flanking sequences of 18S rDNA and 5.8S rDNA were included as the guide and were only being trimmed into account the secondary structure of the RNA. The generated aligned sequences were subjected for the search of the best model to be used for maximum likelihood and morphological comparison with allied species. Part I. Tombo, Fukui

| No. | Sample Name | Sampling Location | Collection Code | GenBank/DDBJ Accession Number |
|-----|-------------|--------------------|-----------------|------------------------------|
| 1   | Orthetrum chrysis | University Malaya | OCHR1           | AB860015 AB860016 AB860042 AB860043 AB860070 AB860071 AB860099 KJ802958 KJ802986 |
| 2   | Orthetrum chrysis | University Malaya | OCHR3           | AB860017 AB860044 AB860071 AB860099 KJ802959 KJ802987 |
| 3   | Orthetrum chrysis | University Malaya | OCHR5           | AB860018 AB860045 AB860072 AB860100 KJ802960 KJ802988 |
| 4   | Orthetrum chrysis | Lanchang, Pahang | OCHR6           | AB860019 AB860046 AB860073 KJ802961 KJ802989 |
| 5   | Orthetrum glaucum | University Malaya | OGLA1           | AB860020 AB860047 AB860074 AB860102 KJ802963 KJ802991 |
| 6   | Orthetrum glaucum | University Malaya | OGLA2           | AB860021 AB860048 AB860075 AB860103 KJ802964 KJ802992 |
| 7   | Orthetrum glaucum | University Malaya | OGLA3           | AB860022 AB860049 AB860076 AB860104 KJ802965 KJ802993 |
| 8   | Orthetrum glaucum | University Malaya | OGLA4           | AB860030 AB860050 AB860077 KJ802966 KJ802994 |
| 9   | Orthetrum glaucum | University Malaya | OGLA5           | AB860031 AB860051 AB860078 KJ802967 KJ802995 |
| 10  | Orthetrum glaucum | University Malaya | OGLA6           | AB860032 AB860052 AB860079 KJ802968 KJ802996 |
| 11  | Orthetrum glaucum | Lanchang, Pahang | OGLA7           | AB860034 AB860053 AB860080 KJ802969 KJ802997 |
| 12  | Orthetrum testaceum | University Malaya | OTES1           | AB860024 AB860051 AB860078 KJ802970 KJ802998 |
| 13  | Orthetrum testaceum | University Malaya | OTES2           | AB860025 AB860052 AB860079 KJ802971 KJ802999 |
| 14  | Orthetrum testaceum | University Malaya | OTES3           | AB860026 AB860053 AB860080 KJ802972 KJ803000 |
| 15  | Orthetrum testaceum | University Malaya | OTES4           | AB860027 AB860054 AB860081 KJ802973 KJ803001 |
| 16  | Orthetrum testaceum | University Malaya | OTES5           | AB860028 AB860055 AB860082 KJ802974 KJ803002 |
| 17  | Orthetrum testaceum | University Malaya | OTES6           | AB860029 AB860056 AB860083 KJ802975 KJ803003 |
| 18  | Orthetrum luzonicum | Pahang | OLUZ1           | AB860037 AB860064 AB860091 KJ802976 KJ803004 |
| 19  | Orthetrum luzonicum | Pahang | OLUZ2           | AB860038 AB860065 AB860092 KJ802977 KJ803005 |
| 20  | Orthetrum pruinosum | Pahang | OPRU1           | AB860039 AB860063 AB860090 KJ802978 KJ803006 |
| 21  | Orthetrum pruinosum | Pahang | OPRU2           | AB860040 AB860064 AB860091 KJ802979 KJ803007 |
| 22  | Orthetrum pruinosum | Pahang | OPRU3           | AB860041 AB860064 AB860091 KJ802980 KJ803008 |
| 23  | Orthetrum pruinosum | Pahang | OPRU4           | AB860042 AB860062 AB860092 KJ802981 KJ803009 |
| 24  | Orthetrum pruinosum | Pahang | OPRU5           | AB860043 AB860062 AB860092 KJ802982 KJ803010 |
| 25  | Orthetrum sabina | Kamar, Perak | OSAB1           | AB860038 AB860067 AB860094 KJ802983 KJ803011 |
| 26  | Orthetrum sabina | Lanchang, Pahang | OSAB2           | AB860039 AB860066 AB860093 KJ802984 KJ803012 |
| 27  | Gomphidae | - | - | - |
| 28  | Gomphidae | - | - | - |
| 29  | Ceriagrionoides | University Malaya | CCHA20          | AB860041 AB860068 AB860095 KJ802985 KJ803013 |
| 30  | Ceriagrionoides | University Malaya | CCER1           | AB860031 AB860067 AB860094 KJ802986 KJ803014 |

combined with GenBank sequences (Table 1 and Supplementary Table 7) to construct phylogenetic trees. The generated forward and reverse sequences were manually edited and assembled using ChromasPro v1.5 (Technelysium Pty Ltd, Australia) software. The datasets for all genetic markers were aligned using ClustalX2. In the preliminary alignment for ITS1 and ITS2, the flanking sequences of 18S rDNA and 5.8S rDNA were included as the guide and were only being trimmed off after final alignment before subjected for phylogenetic analysis. For 28S and 16S, the sequences were trimmed using MAFFT 6’s, with Q-INS-i strategy in order to take into account the secondary structure of the RNA. The generated aligned sequences were subjected for the search of the best model to be used for maximum likelihood (ML) and Bayesian Inference (BI) analyses using Kassan v. 3.2. Best fit models were evaluated using the corrected Akaike Information Criterion for ML and the Bayesian Information Criterion (BIC) for BI with nonpartitioned on the whole sequence. The selected models for ML and BI of each data set are summarised in Supplementary Table 1. ML analysis was performed via Treefinder version October27 and BI analysis was performed using MrBayes 3.1.27. Bayesian analyses were initiated with a random starting tree and two parallel runs, each of which consisted of running four chains of Markov chain Monte Carlo (MCMC) iterations for 6x106 generations. The trees in each chain were sampled every 200th generation. Likelihood values for all post-analysis trees and parameters were evaluated for convergence and burn-in using the “sumt” command in MrBayes and the computer program Tracer ver. 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The first 30,000 trees were discarded as burn-in (where the likelihood values were stabilized prior before the burn in), and the remaining trees after burn-in were used to calculate posterior probabilities using the “sum” command.

1. Silby, J. Dragonflies of the world (CSIRO Publishing, Collingwood, 2001).
2. Karube, H., Futahashi, R., Sasamoto, A. & Kawashima, T. Taxonomic revision of Japanese odonate species, based on nuclear and mitochondrial gene genealogies and morphological comparison with allied species. Part I. Tombo, Fukui 54 , 75–106 (2012).
3. Sasamoto, A. & Futahashi, R. Taxonomic revision of the status of Orthetrum triangulare and melania group (Anisoptera: Libellulidae) based on molecular phylogenetic analyses and morphological comparisons, with a description of three new subspecies of melania. Tombo, Fukui 55 , 57–82 (2013).
4. Arnt, T., Schultz, T. R., Polhemus, D. A. & Simon, C. Molecular phylogenetic analysis of the dragonfly genera Libellula, Ladona, and Plathemis (Odonata: Libellulidae) based on mitochondrial cytochrome oxidase I and 16S RNA sequence data. Mol. Phylogenet. Evol. 18, 348–361 (2001).
5. Ware, J., May, M. & Kjer, K. Phylogeny of the higher Libellulioidea (Anisoptera: Odonata); an exploration of the most speciose superfamily of dragonflies. Mol. Phylogenet. Evol. 45, 289–310 (2007).
6. Fleck, G., Breck, M. & Misof, B. Larval and molecular characters help to solve phylogenetic puzzles in the highly diverse dragonfly family Libellulidae (Insecta: Odonata: Anisoptera): The Tetrathemistinae are a polyphyletic group. Org. Divers. Evol. 8, 1–16 (2008).

7. Fleck, G. et al. A phylogeny of anisopterous dragonflies (Insecta, Odonata) using mtRNA genes and mixed nucleotide/doublet models. J. Zool. Syst. Evol. Res. (2008) 46, 310–322 (2008).

8. Dijkstra, K.-D. B. & Vick, G. S. Inflation by venation and the bankruptcy of traditional genera: the case of Neodythemis and Micromacromia, with keys to the continental African species and the description of two new Neodythemis species from the Albertine Rift (Odonata: Libellulidae). Int. J. Odonatol. 9, 51–70 (2006).

9. Pilgrim, E. M. & von Dohlen, T. H., Branhama, M. A. & Whiting, M. F. Molecules, morphologies and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. Cladistics 24, 477–514 (2008).

10. Blanke, A., Greve, C., Molko, R., Beckman, F. & Misof, B. An updated phylogeny of Anisoptera including formal convergence analysis of morphological characters. Syst. Ent. 38, 474–490 (2013).

11. 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).

12. Futahashi, R. A. A revisional study of Japanese dragonflies based on DNA analyses. Tombo. Fukuı 53, 67–74 (2011). (in Japanese).

13. 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).

14. Damm, S., Schierwater, B. & Hadrys, H. An integrative approach to species discovery in odonates from character-based DNA barcoding to ecology. Mol. Ecol. 19, 3881–3893 (2010).

15. Orr, A. G. Dragonflies of Peninsular Malaysia and Singapore (Natural History Publications (Borneo), Kota Kinabalu, 2005).

16. 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).

17. Jobb, G., von Haeseler, A. & Strimmer, K. Treefinder: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 18 (2004).

18. Hayashi, F., Dobata, S. & Futahashi, R. Disturbed population genetics: suspected introgressive hybridization between two Mnais damselfly species (Odonata). Zool. Sci. 22, 869–881 (2005).

19. Jordan, S., Simon, C. & Polhemus, D. Molecular systematics and adaptive radiation of Hawaii’s endemic damselfly genus Megalagron (Odonata: Coenagrionidae). Syst. Biol. 52, 89–109 (2003).

20. Jobb, G., von Haeseler, A. & Strimmer, K. Treefinder: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 18 (2004).

21. Ji, Y.-J., Zhang, D.-X. & He, L.-J. Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. Mol. Ecol. Notes 3, 581–585 (2003).

22. Swoford, D. L. PAUP: Phylogenetic analysis using parsimony (and other methods) (Sinauer Associates, Sunderland, Massachusetts, 2002).

23. 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).

24. Katoh, K., Asimenos, G. & Toh, H. Multiple alignment of DNA sequences with MAFFT. Bioinformatics for DNA Sequence Analysis. Posada, D. (ed.) 39–54 (Humana Press, New York, 2009).

25. 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).

26. Jobb, G., von Haeseler, A. & Strimmer, K. Treefinder: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 18 (2004).

27. Hueslenbeck, J. P. & Ronquist, F. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755 (2001).

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Author contributions
H.S.Y. and P.E.L. conceived the research in collaboration with J.T., Y.F.N., P.E. and I.W.S. H.S.Y., Y.F.N. and I.W.S. collected the specimens. H.S.Y. 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
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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