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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.

Dragonflies of the genus *Orthetrum* Newman, 1833 are members of the suborder Anisoptera, family Libellulidae. The genus contains some 61 species spread across the Old World[1]. Among these *Orthetrum* dragonflies, there are species pairs whose members are not easily separated from each other by morphological characters, e.g. the reddish-coloured species *O. chrysis* and *O. testaceum*, and the bluish-coloured species *O. glaucum* and *O. luzonicum*.

The Crimson-tailed Marsh Hawk *Orthetrum pruinosum* (Burmeister, 1839) is a widespread species occurring from west India to Japan and south to Malaysia and the Sunda Islands. The subspecies in Malaysia is *O. p. schneideri* Förster, 1903 and that north of Peninsular Malaysia (India to Japan) is *O. p. neglectum* (Rambur, 1842).

The DNA nucleotide sequences of mitochondrial and nuclear genes have been employed to elucidate the phylogeny and systematics of *Orthetrum* dragonflies[2,3]. To-date the most comprehensive phylogenetic study of *Orthetrum* dragonflies involves all the nine Japanese species[2]. In the present study, the DNA nucleotide sequences of mitochondrial and nuclear genes were employed to elucidate the phylogeny and systematics of *Orthetrum* dragonflies. This study, covering a more extensive taxon sampling, provides a new insight to the evolutionary relationships of *Orthetrum* dragonflies. The molecular phylogeny based on ITS1&2, COI, COII and 16S nucleotide sequences, reveals the occurrence of cryptic species in *O. pruinosum*.

Results

Aligned sequences and genetic divergence. The total length for each aligned sequences for various molecular markers and their parsimony information are sumarised 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. triangulare*) to 9.92% (*O. abbotti* and *O. poecilops*) (Supplementary Table 2).

The intraspecific 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 intraspecific 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 + 28S 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. melania*, *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. triangulare*, *O. luzonicum* with PP supoprt 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. melania*; (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 studied[4–10]. Nine genera of Libellulidae have been reported to be monophyletic[11]. 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)[2,12]. Likewise, *O. triangulare* and the allied taxon *O. melania* are well separated by the nuclear (ITS1 and ITS2) and mitochondrial (COI and 16S rRNA) genes[3]. Additionally, *O. melania* is separated into four subgroups: *O. m. melania* (mainland Japan), *O. m. continentale* (China, Korea and Taiwan), *O. m. yaeyamense* (Yaeyama Island, Japan), and *O. m. ryukyuense* (Amami, Kerama, Okinawa and Tokara, Japan).

In the present study, the nuclear 28S rDNA nucleotide sequences were highly conserved and could not resolve congeneric species of *Orthetrum* (supplementary Fig. 1). The 28S rRNA gene has been found to be better for resolving deep branching in the Odonata[13]. 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-coloued 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. melania*[2]. 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. melania*. 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 characters[14].

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 congeneric species of *Orthetrum*. Individual mitochondrial genes (COI, COII, and 16S rRNA) and combination

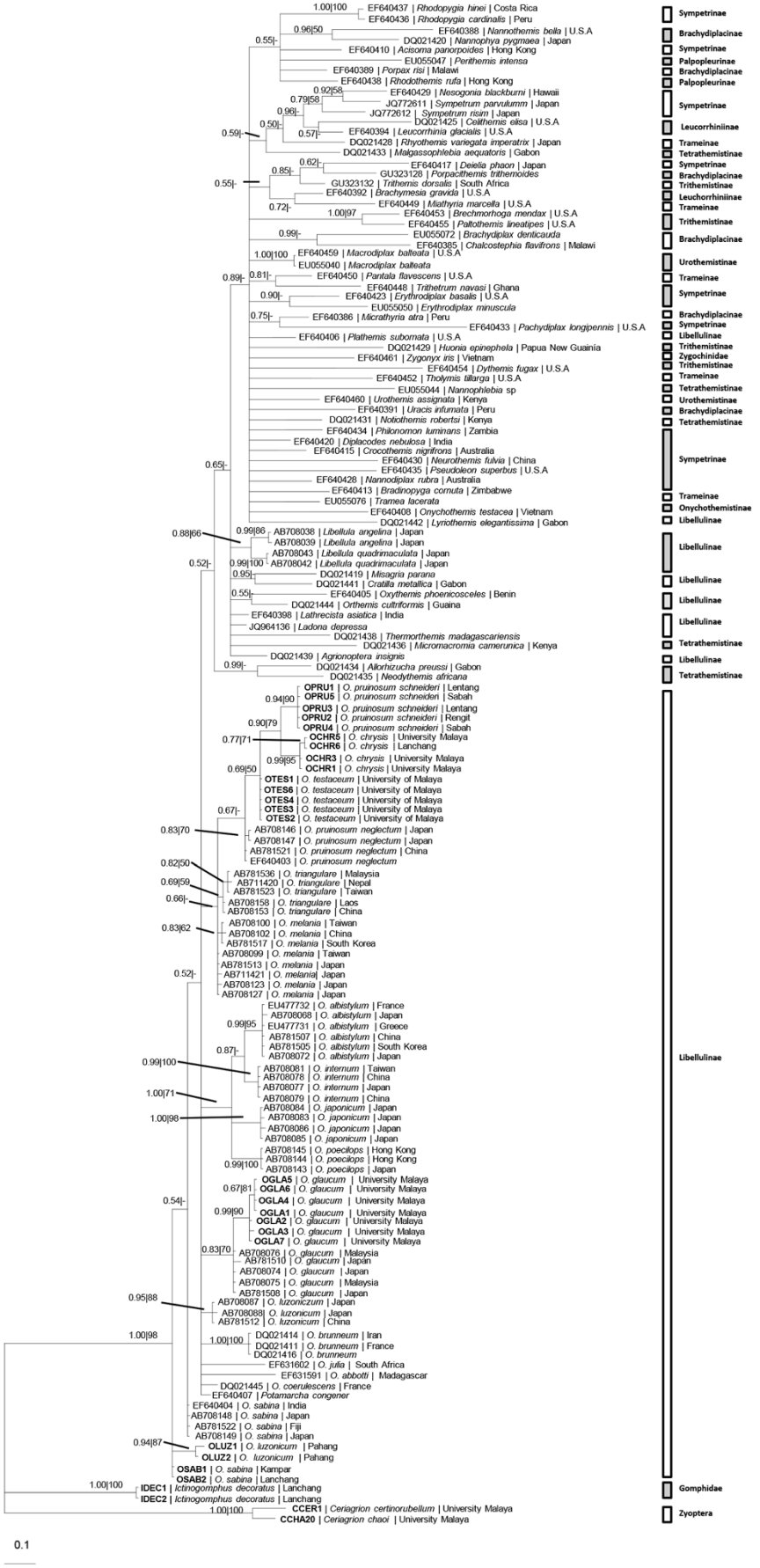

Figure 1 | BI tree based on 16S rDNA nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.

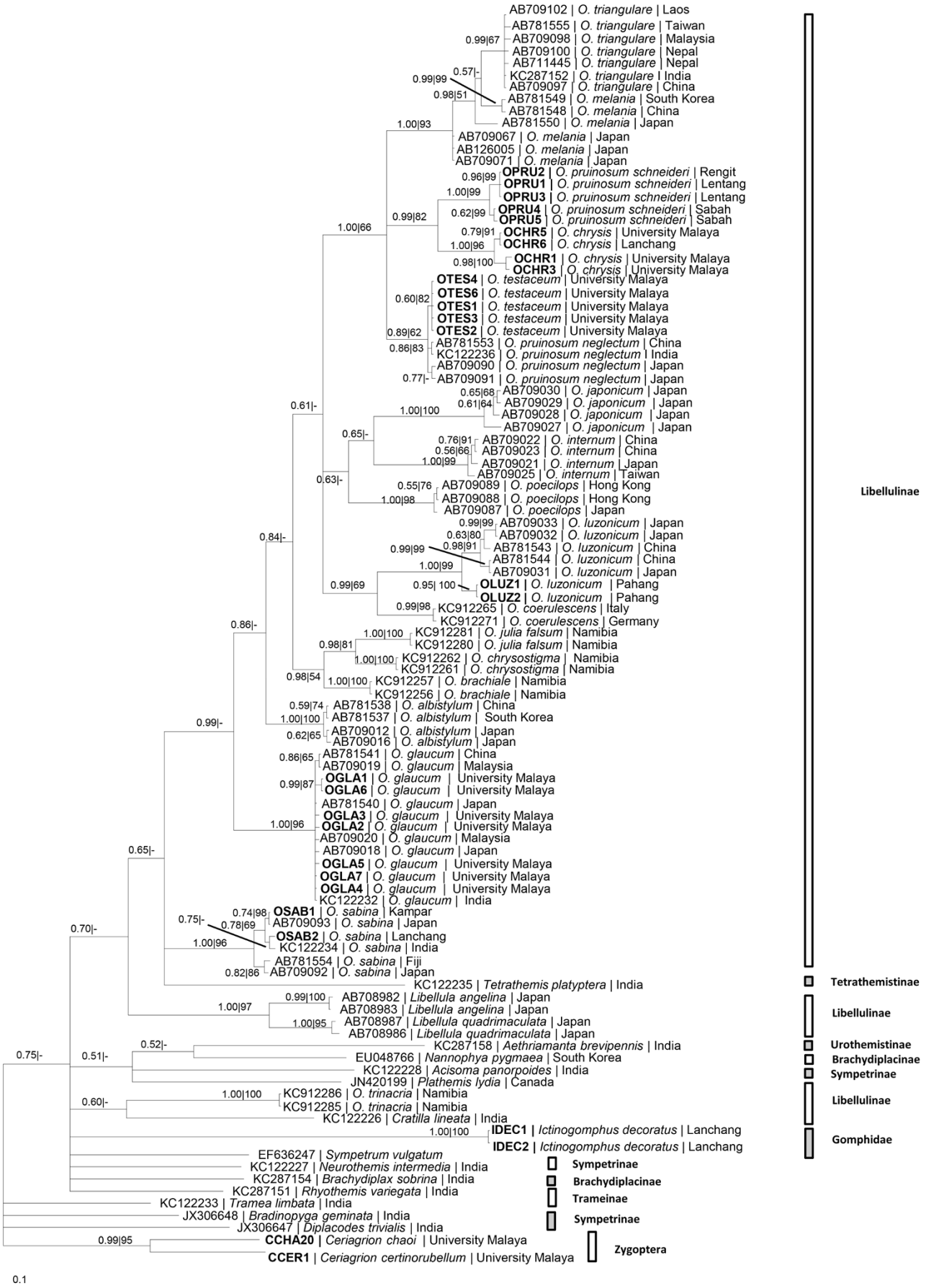

Figure 2 | **BI tree based on COI nucleotide sequences.** Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.

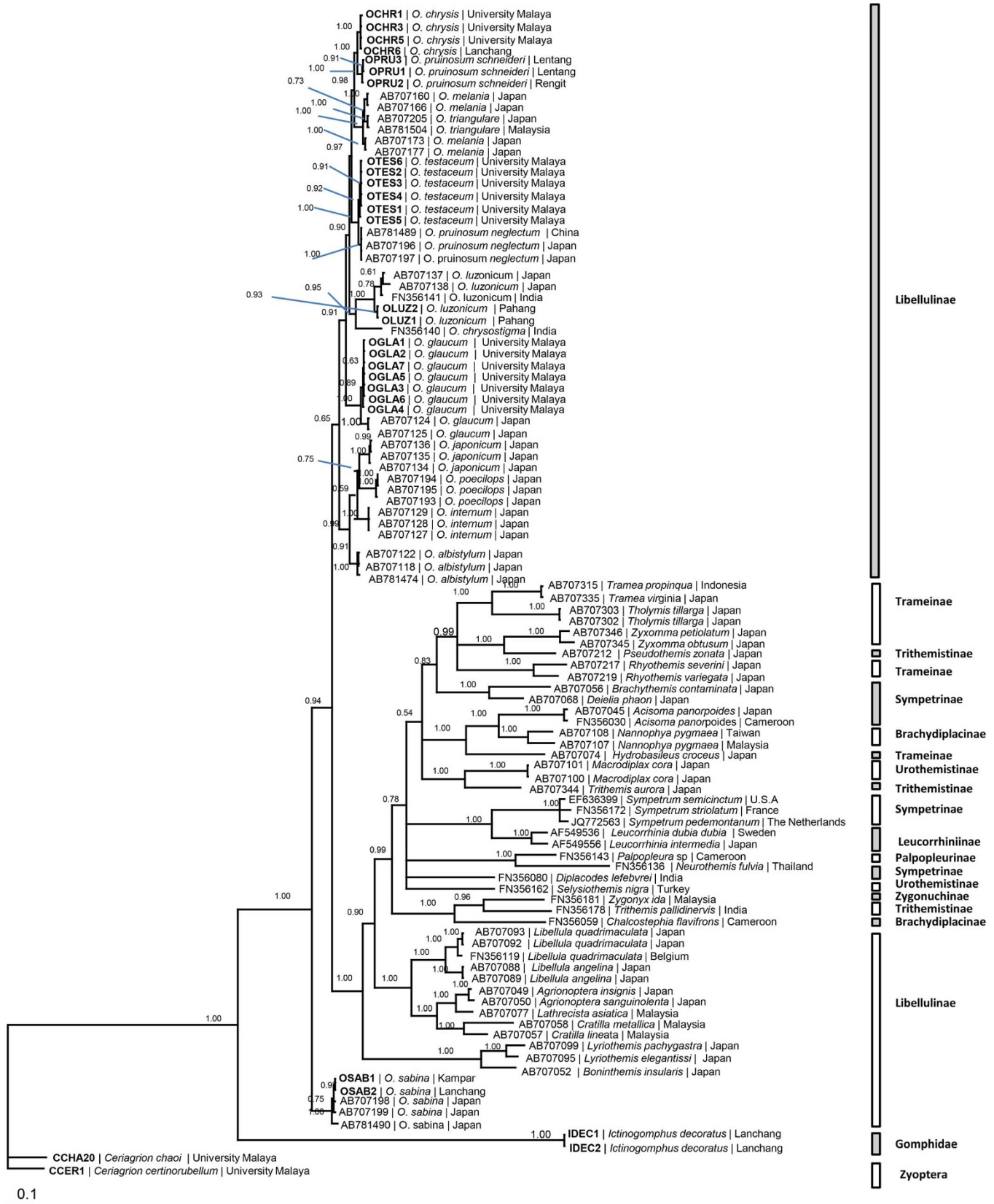

Figure 3 | BI tree based on ITS1&2 nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.

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

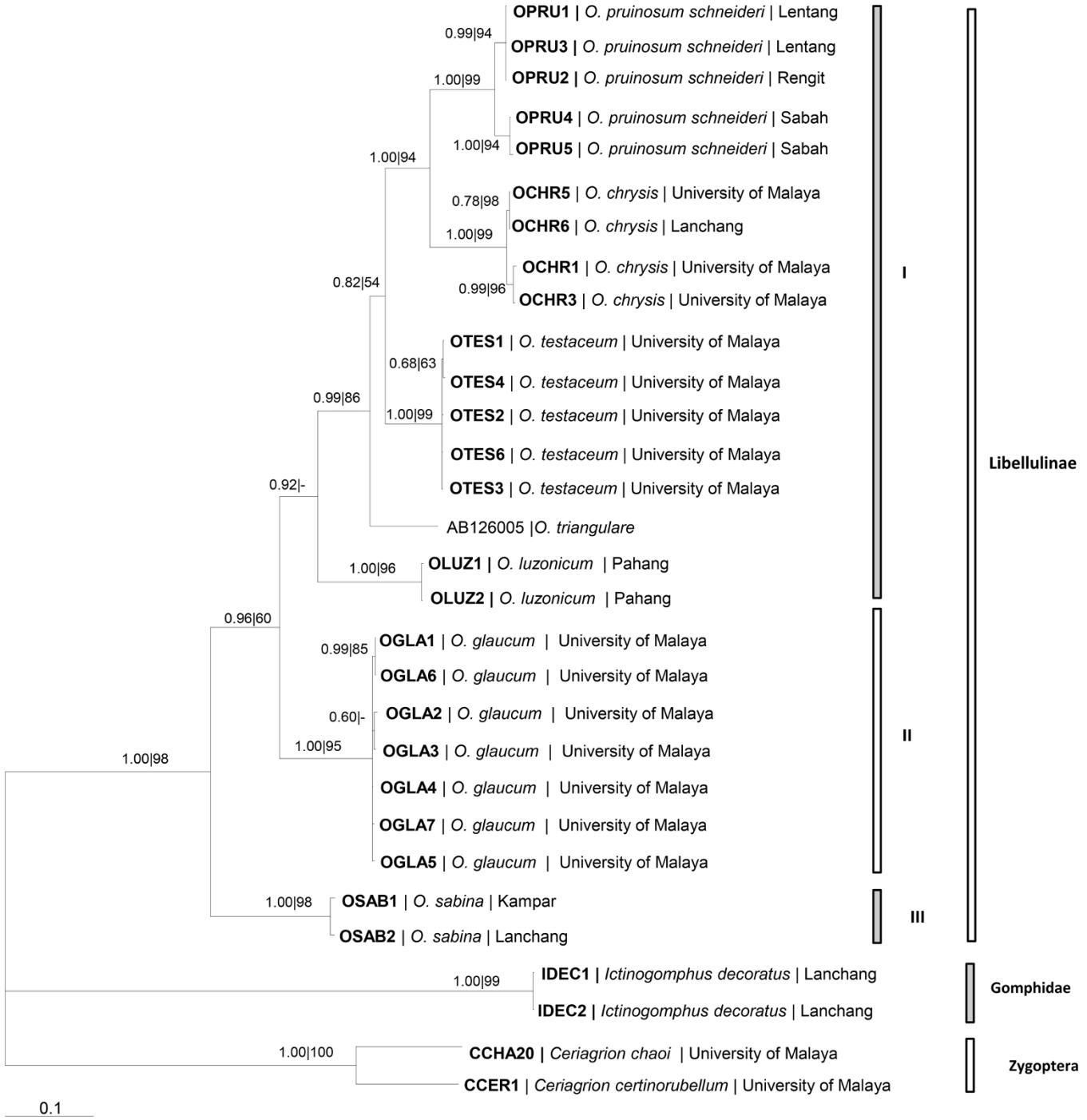

Figure 4 | BI tree based on COI + COII nucleotide sequences. Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.

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 literature[15,16]. 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′- ATAATTGGRGGRTTYGGRAAY TG-3′ and R: 5′- CCAAARAATCAAAATAARTGT TG-3′[18], at 50°C; COII: C2-J-3102: 5′-AAATGGCAACATGAGCACAAYT-3′ and TK-N-3773: 5′-GAGA-CCAGTACTTGCTTTCAGTCATC-3′[19] at 50°C; 16S rDNA: 5′-TTGACTGTACA-AAGGTAGC-3′ and 5′-GATATTACGCTGTTATCCC-3′[20] at 50°C; 28S rDNA: 28sf, 5′-AAGGTAGCCAAATGCCTCATC-3′ and 28sr, 5′-AGTAGGGTAAA-ACTAACCT-3′ at 52°C[13]; ITS1: CAS18sF,5′- TACACACCGCCCGTCGCTACTA-3′ and CAS5p8sB1d, 5′- ATGTGCGTTCRAAATGTCGATGTTCA-3′[21] at 67°C; and ITS2: CAS5p8sFc, 5′-TGAACATCGACATTTYGAACGCACAT-3′ and CAS28sB1d, 5′-TTCTTTTCCTCCSCTTAYTRATATGCTTAA-3′[21] 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-

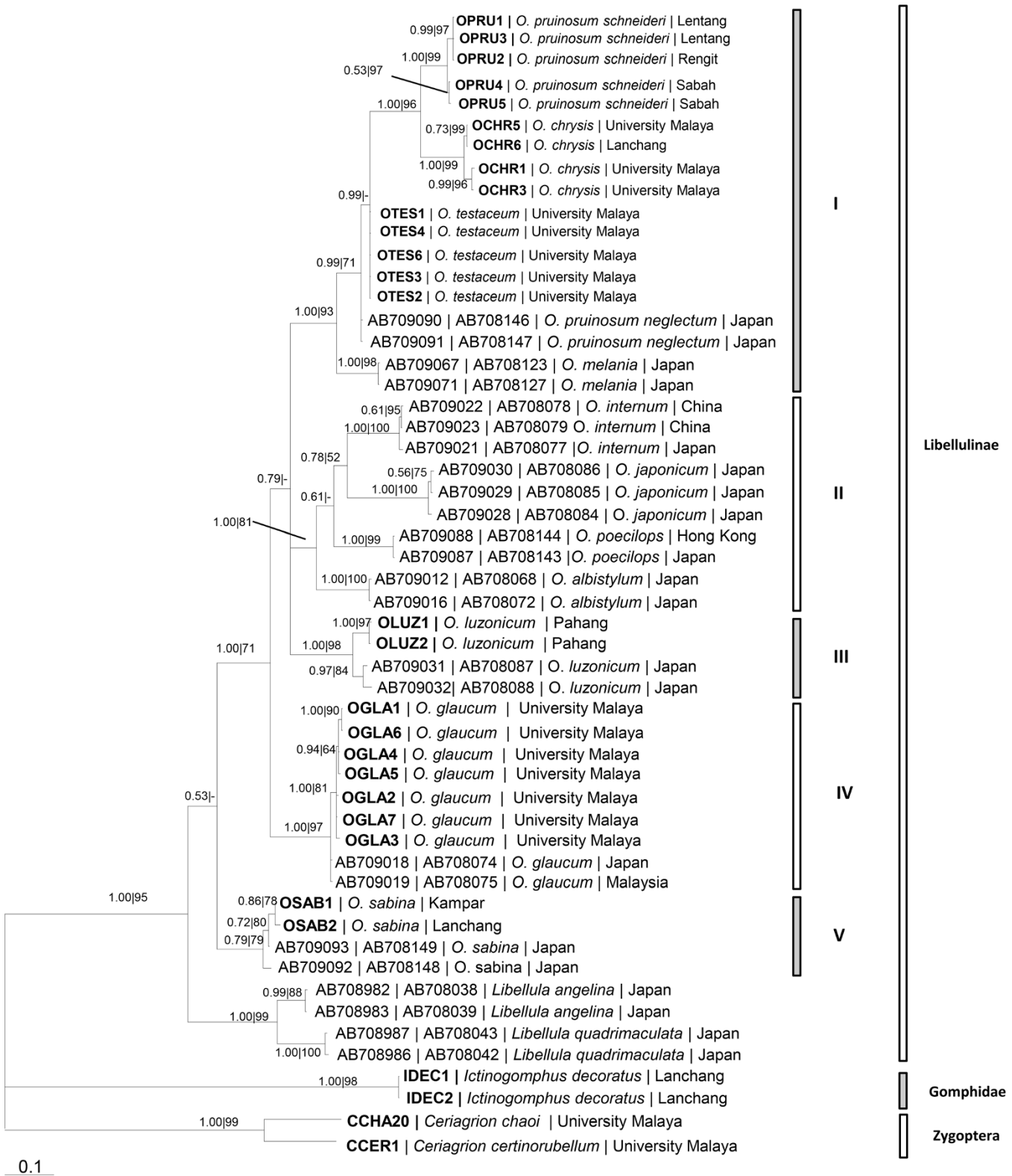

Figure 5 | **BI tree based on COI + 16S rDNA nucleotide sequences.** Numeric values at the nodes are Bayesian posterior probabilities/ML bootstrap.

fication kit (Cosmo Genetech, South Korea). 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 BigDyeH Terminator v3.1 Sequencing Kit and analysed on an ABI PRISMH 377 Genetic Analyser.

Genetic divergence. 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[22]. 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.

Phylogenetic analysis. To elucidate the phylogenetic relationship among the different species of *Orthetrum* species, sequences generated from this study were

Table 1 | Nucleotide sequences of COI, COII, 16S rRNA, 28S rRNA, ITS1 and/or ITS2 sequences for the taxa of *Orthetrum* of the family Libellulidae used in the present study. *Ictinogomphus decoratus* (family Gomphidae), *Ceriagrion chaoi* and *C. cerinorubellum* (suborder Zygoptera) were used as outgroups. NA, not available

No.	Sample Name	Sampling Location	Collection Code	GenBank/DDBJ Accession Number					
				COI	COII	16S	28S	ITS1	ITS2
Samples derived from this study									
Odonata									
Libellulidae									
1	*Orthetrum chrysis*	University Malaya	OCHR1	AB860015	AB860042	AB860069	AB860097	KJ802958	KJ802986
2	*Orthetrum chrysis*	University Malaya	OCHR3	AB860016	AB860043	AB860070	AB860098	KJ802959	KJ802987
3	*Orthetrum chrysis*	University Malaya	OCHR5	AB860017	AB860044	AB860071	AB860099	KJ802960	KJ802988
4	*Orthetrum chrysis*	Lanchang, Pahang	OCHR6	AB860018	AB860045	AB860072	AB860100	KJ802961	KJ802989
5	*Orthetrum glaucum*	University Malaya	OGLA1	AB860019	AB860046	AB860073	AB860101	KJ802962	KJ802990
6	*Orthetrum glaucum*	University Malaya	OGLA2	AB860020	AB860047	AB860074	AB860102	KJ802963	KJ802991
7	*Orthetrum glaucum*	University Malaya	OGLA3	AB860021	AB860048	AB860075	AB860103	KJ802964	KJ802992
8	*Orthetrum glaucum*	University Malaya	OGLA4	AB860022	AB860049	AB860076	AB860104	KJ802965	KJ802993
9	*Orthetrum glaucum*	University Malaya	OGLA5	AB860308	KF248113	KF248140	KF581186	KJ802966	KJ802994
10	*Orthetrum glaucum*	University Malaya	OGLA6	AB860023	AB860050	AB860077	AB860106	KJ802967	KJ802995
11	*Orthetrum glaucum*	Lentang, Pahang	OLGA7	AB860024	AB860051	AB860078	AB860107	KJ802968	KJ802996
12	*Orthetrum testaceum*	University Malaya	OTES1	AB860025	AB860052	AB860079	AB860108	KJ802969	KJ802997
13	*Orthetrum testaceum*	University Malaya	OTES2	AB860026	AB860053	AB860080	AB860109	KJ802970	KJ802998
14	*Orthetrum testaceum*	University Malaya	OTES3	AB860027	AB860054	AB860081	AB860110	KJ802971	KJ802999
15	*Orthetrum testaceum*	University Malaya	OTES4	AB860028	KF248112	KF248139	KF581185	KJ802972	KJ803000
16	*Orthetrum testaceum*	University Malaya	OTES5	-	-	-	-	KJ802973	KJ803001
17	*Orthetrum testaceum*	University Malaya	OTES6	AB860029	AB860056	AB860083	AB860112	KJ802974	KJ803002
18	*Orthetrum luzonicum*	Pahang	OLUZ1	AB860037	AB860064	AB860091	AB860118	KJ802980	KJ803008
19	*Orthetrum luzonicum*	Pahang	OLUZ2	AB860038	AB860065	AB860092	AB860119	KJ802981	KJ803009
20	*Orthetrum pruinosum schneideri*	Lentang, Pahang	OPRU1	AB860032	AB860059	AB860086	AB860115	KJ802977	KJ803005
21	*Orthetrum pruinosum schneideri*	Rengit, Pahang	OPRU2	AB860033	AB860060	AB860087	AB860116	KJ802978	KJ803006
22	*Orthetrum pruinosum schneideri*	Lentang, Pahang	OPRU3	AB860034	AB860061	AB860088	AB860117	KJ802979	KJ803007
23	*Orthetrum pruinosum schneideri*	Maliau, Sabah	OPRU4	AB860035	AB860062	AB860089	-	-	-
24	*Orthetrum pruinosum schneideri*	Maliau, Sabah	OPRU5	AB860036	AB860063	AB860090	-	-	-
25	*Orthetrum sabina*	Kampar, Perak	OSAB1	AB860030	AB860057	AB860084	AB860113	KJ802975	KJ803003
26	*Orthetrum sabina*	Lanchang Pahang	OSAB2	AB860031	AB860058	AB860085	AB860114	KJ802976	KJ803004
Odonata									
Gomphidae									
27	*Ictinogomphus decoratus*	Lanchang, Pahang	IDEC1	AB860039	AB860066	AB860093	AB860120	KJ802982	KJ803010
28	*Ictinogomphus decoratus*	Lanchang, Pahang	IDEC2	AB860040	AB860067	AB860094	AB860121	KJ802983	KJ803011
Odonata									
Coenagrionidae									
29	*Ceriagrion chaoi*	University Malaya	CCHA20	AB860041	AB860068	AB860095	AB860122	KJ802984	KJ803012
30	*Ceriagrion cerinorubellum*	University Malaya	CCER1	AB860310	AB860307	AB860096	AB860123	KJ802985	KJ803013

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 ClustalX[23]. 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 aligned using MAFFT 6[24], 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 Kakusan v. 3[25]. 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 October[26] and BI analysis was performed using MrBayes 3.1.2[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 $6x10^6$ 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 "sump" 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 "sumt" command.

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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

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