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Molecular and Morphological Analyses Support Different Taxonomic Units for Asian and Australo-Pacific Forms of *Ischnura aurora* (Odonata, Coenagrionidae)

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Abstract: Despite the great technological progress that has aided taxonomical identification, taxonomical issues remain for certain species found in remote and/or understudied geographical areas. The damselfly species *Ischnura aurora* has been the subject of a long-standing taxonomical debate, focused mainly on the existence of morphological and behavioural differences between Asian and Australo-Pacific forms of this species that could justify their placement into two different species. Here, we carried out a comparative morphological analysis of specimens currently identified as *I. rubilio* from India and *I. aurora* from Asia and Oceania, combined with the analysis of mitochondrial and nuclear sequence data, both developed by us and available in public repositories. Our results split the Asian and Australo-Pacific forms of *I. aurora* into two well-differentiated taxonomic units and, hence, different (albeit closely related) species, and support the specific status of *I. rubilio*. The results of our genetic analyses suggest the existence of a third (and even fourth) taxonomic unit, stressing the need to revise all available material belonging to the different *I. aurora* subspecies that have been described. Finally, we have identified several questionable DNA sequences currently available in public repositories, upon which previous conclusions about the phylogenetic position of *I. rubilio* are based. Our study stresses the importance of being able to link available DNA sequence data with voucher specimens as well as to carry out a careful examination of DNA sequence data prior to their inclusion in taxonomical studies.

Keywords: Zygoptera; damselfly; integrative taxonomy; species delineation; barcoding; morphological analysis; DNA sequencing

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1. Introduction

The science of taxonomy is central to many disciplines within biology, and hence being able to correctly identify species and to associate a scientific name with a particular organism is a prerequisite for ecological and conservation studies [1,2]. Until the development of alternative techniques to define species boundaries, the identification of species by means of morphological analysis was the only option available to taxonomists, even though the existence of "cryptic" species constituted a clear limitation. Taxonomy has shown great advances in recent years with the incorporation of technological advances (the most relevant being DNA sequencing) and the possibility of virtually accessing museum collections for specimen examination [3], which has led to integrative taxonomy, i.e., the study of variation in different types of datasets to delineate species boundaries more accurately [4]. Since 2003, when the DNA-based approach to taxonomy was first proposed [5–7], the so-called DNA barcode—the mitochondrial *COI* gene—has been widely used for species descriptions. However, the well-known limitations of mitochondrial DNA (e.g., incomplete lineage sorting, introgression, or the presence of nuclear copies of mitochondrial genes [8–11]) may lead to erroneous conclusions in species delimitation studies (see for example Papakostas et al. [12] or Ožana et al. [13]), and therefore the use of information

from both mitochondrial and nuclear DNA markers is preferred, as it integrates information from two different genetic sources, hence allowing for a better support of taxa. Despite the acknowledged advantages of DNA sequences as a tool for species identification, species delineation is the crucial first step in taxonomy. To accurately delineate species boundaries, we need to be able to link a newly discovered species with its correct name, and describe its natural history, morphology, and behaviour [14].

Regardless of all the technological progress that has helped with taxonomic identification, the heterogeneous fieldwork effort carried out across the world may hamper the identification of specimens found in certain areas that, in some cases, host the highest diversity. Therefore, the combination of extensive fieldwork and modern technologies is necessary to clarify biodiversity, an essential matter nowadays due to the diversity loss produced because of climate change. Damselflies (Zygoptera: Odonata) constitute a good example to address taxonomic issues due to their high diversity in environments with difficult access and the high interspecific morphological similarities that exist in this insect group, sometimes only possible to unravel by genetic analysis [15]. Recent works have pointed to the genus *Ischnura* Charpentier, 1840, also known as forktails (Coenagrionidae), as a group with a non-resolved taxonomy, partly due to an incomplete knowledge of this genus in several areas of Asia [16,17]. *Ischnura* is a speciose genus with worldwide distribution, which has colonised many oceanic islands and shows great diversity in morphology, colouration, and behaviour. There are currently ca. 77 known species of *Ischnura*, which can be found in a range of diverse environments and spread across vast distribution areas [17–19]. One example of a species within this genus showing a wide geographical distribution and taxonomical issues is *Ischnura aurora* (Brauer, 1865). This species is a well-known migrant, passively dispersed throughout long distances as part of the aerial plankton [20,21] (pp. 396 and 409–411). Its ability to disperse over vast geographical areas explains its widespread distribution range that spans from the Pacific islands and Australia to the South-East Asiatic continent, India, Pakistan, and Iran [22,23] (Figure 1).

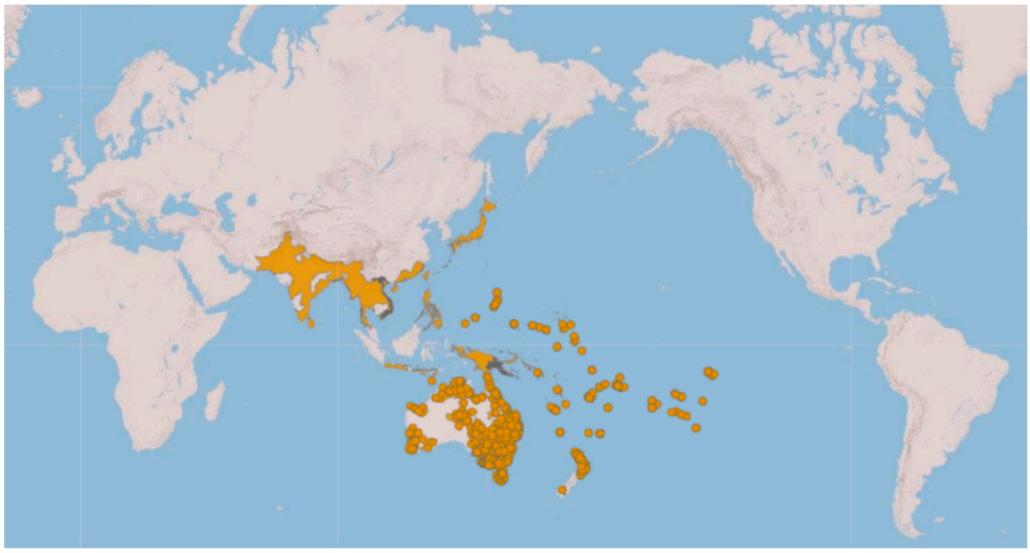

Figure 1. Map showing the geographic distribution of *Ischnura aurora* (orange dots). Modified from Dow et al. [22].

There has been a long-standing debate about which name should be used when referring to individuals of this species. In 1858, the name *Agrion delicatum* was given by Hagen to a damselfly found in Sri Lanka, Bengal (currently India and Bangladesh) and Australia [24]. In his work, Hagen included only data on body and wing sizes, but failed to provide an actual description of the species, which has remained as a *nomen nudum* since then. Later, *A. delicatum* was transferred to the genus *Ischnura* and the species *Ischnura delicata* was described using material from Asia and Australia [25]. *I. delicata*

was considered a senior synonym of *Agrion* (*Ischnura*) *aurora*, a very similar damselfly earlier described by Brauer [26] using material from Tahiti. In his description of *I. delicata*, Selys mentions a male of a "*variety or juvenile*" of the species from India, which he named *Ischnura rubilio*. Selys provided information on some key morphological characteristics of *I. rubilio*: "the basal articulation of the 4–6 segments not circled in black; the 8 segment entirely blue like the 9" [25] (p. 283). Even though this taxon is still awaiting formal description, it was considered a subspecies of *I. aurora* [27] until Kalkman et al. [28] raised it to specific status. Therefore, *I. rubilio* is currently recognised as a distinct species restricted to the Indian subcontinent, whereas *I. delicata* is considered a junior synonym of *I. aurora*. At the molecular level, there has been no consensus yet about the placement of *I. rubilio* within the genus *Ischnura*. A phylogenetic analysis of the genus by Dumont [23], based on nuclear (Internal Transcribed Spacer, *ITS*) and mitochondrial (Cytochrome Oxidase I, *COI*) DNA sequence data which included specimens of *I. rubilio* collected in Bhutan and India, concluded that this species was in fact distinct from *I. aurora*, being either basal to the genus *Ischnura* or a member of the *pumilio* group. However, a more recent phylogenetic analysis by Sánchez-Guillen et al. [18], based also on nuclear (*ITS*) and mitochondrial (Cytochrome B, *CYTB* and Cytochrome Oxidase II, *COII*) sequence data, has placed *I. rubilio* as a sister species of *I. aurora*.

Beyond this debate on taxonomic hierarchy, other authors have focussed on the existence of morphological and behavioural differences between the Asian and Australo-Pacific forms of *I. aurora* that would justify their placement into two different species [27,29]. According to these authors, males of the Asian forms of the species possess large postocular spots, a completely blue eighth abdominal segment, a more pronounced dorsal tubercle in the 10th abdominal segment, acute cerci in its superior region, and narrower and pointer paraprocts, while the females mate in the adult stage. Australo-Pacific forms of *I. aurora*, on the other hand, possess small and circular postocular spots, one-third of the eighth abdominal segment with a blue colour, less pronounced dorsal tubercle in the 10th abdominal segment, rounded cerci in its superior region, and females that mate only in the teneral stage. While Papazian et al. [27] and Rowe [29] agree on the existence of morphological differences, there is no consensus on which name should be given to each of these forms. Papazian et al. [27] considered the Asian forms from the Indian subcontinent as a subspecies of *I. aurora*, named *I. aurora rubilio* (according to Selys description above), and in support of their argument, Dumont stated that "*I. aurora* is rare or totally absent West of the Wallace Line" [23] (p. 307). On the other hand, Rowe [29] suggested the existence of Asian forms of *I. aurora* in South and East Asia, as well as in Sri Lanka and India. He stated that "this 'species' should therefore be cited as *Ischnura delicata* (Hagen in Selys 1876)" [29] (p. 189), and, also, he highlighted the complex taxonomy of the Asian forms of *I. aurora*, for which three further names exist (*I. rubilio, I. amelia* and *I. bhimtalensis*), which have been assumed at some point to be either subspecies or junior synonyms of *I. aurora*.

Therefore, both Papazian et al. [27] and Rowe [29] coincide in restricting the "true" *Ischnura aurora* to the Australo-Pacific geographic area, but they disagree in the taxonomic classification and distribution of the Asian forms of this species. Here, we carried out a comparative morphological analysis of specimens currently identified as *I. rubilio* from India and *I. aurora* from Asia and Oceania, focusing on those characteristics traditionally used to distinguish among the Asian and Australo-Pacific forms of *I. aurora*. We combine the morphological analysis of male and female specimens with the analysis of mitochondrial and nuclear sequence data, both developed by us and available at public repositories, with the aim of clarifying the taxonomic status of these taxa.

2. Materials and Methods

2.1. Specimen Collection, DNA Extraction and Sequencing

A total of nine specimens of *I. aurora* from China (6 males, 1 female), Australia (1 male), and Fiji (1 male) belonging to ACR's collection and stored in 80% ethanol were selected

for DNA extraction. Additionally, four dried-preserved specimens of *I. rubilio* (2 males, 2 females) collected in India were also used for DNA extraction (see Table 1).

Table 1. Information on *Ischnura aurora* and *I. rubilio* specimens used for DNA extraction and sequencing as described in the main text. For each specimen, we list the voucher ID, sex, and collection locality. n.a. indicates that no sequence could be obtained for a particular marker and/or specimen; Accession numbers labelled with an asterisk are those that correspond to sequences annotated as *COI*-like sequences (see Appendix A and Table 2).

Species	Specimen ID	Sex	Collection Locality	GenBank Acc. Nos	
				COI	*ITS*
Ischnura aurora	ACR819	M	Pond at Bandiana, Wodonga, Victoria, Australia.	OM964934	OM964914
I. aurora	ACR2379	M	Stream at Xi Meng, Yunnan, China.	OM964933	OM964916
I. aurora	ACR2880	M	Pond at Meng Ding, Yunnan, China.	OM964932	OM964917
I. aurora	ACR2956	M	Pond at Na Bang, Yunnan, China.	OM964931	n.a.
I. aurora	ACR3503	M	Rice fields, Huaping, Yunnan, China.	OM964930	OM964918
I. aurora	ACR3888	M	Pond in agricultural area, Mengding, Yunnan, China.	OM964929	OM964919
I. aurora	ACR3998	F	River at Meng Lun, Yunnan, China.	OM964928	OM964920
I. aurora	ACR4067	M	Stream at Meng Lun, Yunnan, China.	OM964927	OM964921
I. aurora	ACR5010	M	Somosomo Damm, Chakaudrove, Taveuni, Fiji	n.a.	OM964915
Ischnura rubilio	MB-IrbKeM	M	Trivandrum, Kerala, South India.	OM964925 *	OM964922
I. rubilio	MB-IrbKeF	F		OM964924 *	n.a.
I. rubilio	MB-IrbTam	F	Tamil Nadu, South India.	OM964926 *	n.a.
I. rubilio	MB-IrbGir	M	Unknown locality, India.	OM964923 *	n.a.

Total genomic DNA was extracted from individual legs using the GeneJet DNA extraction kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Fragments of the mitochondrial *COI* gene and the nuclear *ITS* were amplified using previously published primer pairs (COI-S0: TACCAATTATAATTGGAGGATTYGG/COI-AS0: CTTCTGGATGTCCAAARAATCA and ITS-F0: GGAAAGATGGCCAAACTTGA/ITS-28S-AS0: CCTCCGCTTATTAATATGCTTAAATTC [30]). PCR reactions were carried out at specific annealing temperatures (48 °C for *COI* and 52 °C for *ITS*) using the DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA). Before sequencing, PCR products were purified with shrimp alkaline phosphatase and exonuclease I (New England Biolabs, Ipswich, MA, USA) to remove unincorporated primers and dNTPs. Cleaned PCR products were sequenced bidirectionally using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA) at either the Macrogen Laboratories in Spain or at the CACTI genomics facility from the University of Vigo.

2.2. Genetic Analyses

DNA chromatograms were visually inspected, trimmed and automatically assembled using Geneious v. 9.1.8 (https://www.geneious.com/). Previously published *COI* and *ITS* sequences from specimens identified as *I. aurora*, *I. rubilio* or *I. delicata*; together with representative species of the Coenagrionidae genera, *Ischnura*, *Aciagrion*, *Ceriagrion*, *Coenagrion*, *Enallagma*, *Erythromma*, *Mortonagrion* and *Pseudagrion*, were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and added to our datasets (see Appendix B Table A1). Prior to the genetic analyses, a quality control step was carried out to ensure that the sequences included in the final datasets were not derived from contaminations or, in the case of the mitochondrial DNA, also to rule out the amplification of paralogous copies of *COI* (nuclear mitochondrial DNA copies or *numts* [11,31] which were also recently described in odonates [13]). The quality control and data mining steps are described in detail in Appendix A.

After quality control, all sequences selected for inclusion in the final datasets were aligned using MAFFT [32], as implemented in Geneious v 9.1.8. Phylogenetic relationships

were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) approaches. ML analyses were carried out using IQTree v. 1.6.12 [33], with the best substitution model for each marker selected by ModelFinder [34]. Support of branches in the resulting tree was assessed by 10,000 ultrafast bootstrap replicates [35,36]. BI analyses were carried out using MrBayes 3.2.6 [37,38], as implemented in Geneious v 9.1.8. MCMC searches were run for 1.1 million generations, with default priors and with the GTR + I+G substitution model. Resulting phylogenetic trees were edited with FigTree v. 1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v. 1.0 [39].

Genetic differentiation between *Ischnura* species (uncorrected p-distances) was estimated for each dataset in MEGA X [40] using the pairwise deletion option, which removes all ambiguous positions for each sequence pair. To further confirm the species delimitation within our datasets, we used the single-locus distance-based delimitation method implemented by the software *Assemble Species by Automatic Partitioning* (ASAP) [41]. Analyses were run at the ASAP web server (https://bioinfo.mnhn.fr/abi/public/asap/), using fasta files for each locus (*COI* and *ITS*) as the input files. The species delimitation analyses were carried out using only the sequences from *I. aurora*, *I. delicata* and *I. rubilio*, with the default options and with genetic distances computed under the Kimura (K80) model.

2.3. Morphological Analyses

For the morphological analyses, we examined a total of 31 ethanol-preserved individuals of *Ischnura aurora* (10 males and 9 females from China; 3 males and 2 females from Fiji; 3 males and 4 females from Australia; see Table A2), plus the four dried-preserved *Ischnura rubilio* specimens collected in India listed in Table 1. The aim of the morphological analysis was to determine whether the morphology of these specimens would corroborate the results of the genetic analyses and justify their placement as two different taxonomic units. Specimens were examined under an Olympus SZ60 stereoscopic microscope. Photographs of the individuals were taken using a Leica Flexacam C1 digital camera attached to the microscope at varying magnifications; and afterwards stacked and edited using the software GIMP v. 2.10 [42]. The male genital ligula of one individual of *I. rubilio* (MB-IrbkeM) and three *I. aurora* individuals (ACR2880 from China, ACR0818 from Australia and ACR5009 from Fiji) was dissected and observed under a scanning electron microscope (Philips XL30) at the CACTI microscopy service from the University of Vigo. The terminology used in the morphological descriptions follows that in Garrison et al. [43]. Abdominal segments are referred to as capital "S" plus the segment number. All measurements are given in millimetres.

3. Results

3.1. Genetic Analyses

All sequences generated in this study were deposited in GenBank with accession numbers OM964914-OM964934 (see Table 1). The *COI* sequences obtained from the *I. rubilio* samples collected in India were not included in the mtDNA dataset, as they were likely "*COI*-like" sequences or *numts*, rather than orthologous copies of the mitochondrial *COI* gene (Table 2; see also Appendix A). Regarding the sequences retrieved from GenBank, a total of thirteen sequences belonging to *I. aurora*, *I. delicata* and *I. rubilio* were also excluded from the datasets after the quality control steps carried out as described in Appendix A. Three *I. aurora COI* sequences were excluded because they corresponded to the 3′-end of the *COI* gene (Table 2 and Appendix A Figure A4). Five *COI* sequences belonging to *I. aurora* (N = 2) and *I. delicata* (N = 3) were discarded because they showed ambiguity-coded bases, which are not expected in a mitochondrial coding gene (Table 2). Three sequences belonging to *I. aurora* (N = 1) and *I. rubilio* (N = 2) were excluded from the datasets because they were likely the product of specimen misidentification or misplacement of individuals in the laboratory at the time of DNA extraction (Table 2; see also Appendix A). Finally, two *COI* sequences of *I. rubilio* were excluded from the mtDNA dataset because they showed several features consistent with these being "*COI*-like" sequences or *numts*. It is important

to note that three of the *I. rubilio* sequences that were excluded from our datasets were those included in the phylogeny of the genus *Ischnura* by Dumont [23].

Table 2. Sequences of *Ischnura aurora*, *I. delicata* and *I. rubilio* that were excluded from the final genetic analyses after the quality control steps that are described in detail in the Appendix A, including the *I. rubilio* "*COI*-like" sequences generated in this study.

Taxa	Data Source	GenBank No.	Marker	Reason for Exclusion
Ischnura aurora	Nolan et al. [44]	EU219876	*COI*	Sequence corresponds to the 3′ end of the *COI* gene
I. aurora	Nolan et al [44]	EU219877	*COI*	Sequence corresponds to the 3′ end of the *COI* gene
I. aurora	Mehmood et al. (unpublished)	LC198680	*COI*	Sequence corresponds to the 3′ end of the *COI* gene
I. aurora	Ramage et al. [45]	KX053527	*COI*	Ambiguity-coded bases in sequence
I. aurora	Ramage et al. [45]	KX053531	*COI*	Ambiguity-coded bases in sequence
I. aurora	Dumont et al. [46]	FN356100	*ITS*	Specimen misidentification and/or misplacement?
Ischnura delicata	Ashfaq et al. (unpublished)	KY832433	*COI*	Ambiguity-coded bases in sequence
I. delicata	Ashfaq et al. (unpublished)	KY838304	*COI*	Ambiguity-coded bases (insertion of 3 "Ns") in sequence
I. delicata	Ashfaq et al. (unpublished)	KY844428	*COI*	Ambiguity-coded bases in sequence
Ischnura rubilio	Pavithran et al. (unpublished)	MW143324	*COI*	Both sequences are identical. No stop codons, but sequences are odd compared to references. Similar to several marine sponge genera. "*COI*-like/*COI*-numt" sequence?
I. rubilio	Dumont [23]	MH449981	*COI*	
I. rubilio	Dumont [23]	MH449992	*COI*	Specimen misidentification and/or misplacement?
I. rubilio	Dumont [23]	MH447434	*ITS*	Specimen misidentification and/or misplacement?
I. rubilio	This study	OM964923	*COI*	Ambiguity codes in sequence. Similar to *Nesobasis* spp. Annotated as "*COI*-like/*COI*-numt" sequences
I. rubilio	This study	OM964924	*COI*	
I. rubilio	This study	OM964925	*COI*	
I. rubilio	This study	OM964926	*COI*	

After the quality control steps, the final number of sequences included in each dataset was 102 for *COI* (451 bp-long alignment) and 66 for *ITS* (639 bp-long alignment). The difference in size between both datasets stems mostly from the fact that the *COI* gene (and more specifically, the Folmer region) is the marker of choice in barcoding studies, hence the higher number of sequences from this marker that are available at public repositories.

The obtained phylogenetic trees were congruent between BI and ML methods and between nuclear and mitochondrial DNA markers, with strong support for the split of *Ischnura aurora* in two clades: the Australo-Pacific and the Asian clade (see Figures 2 and 3). The Asian clade included the individuals of *I. aurora* from China that were sequenced by us, together with *I. aurora* specimens from Thailand and individuals identified as *I. delicata* and *I. rubilio* collected in Pakistan and India, respectively, whose sequences were obtained from GenBank. For the *ITS* marker, the Asian clade also included the *I. rubilio* specimen from Kerala (India) sequenced by us. The Australo-Pacific clade included all *I. aurora* individuals from Australia and Fiji sequenced by us, plus all individuals identified as *I. aurora* whose sequences were downloaded from GenBank, and which were collected in the Australo-Pacific distribution area of the species (i.e., Australia, Japan, Samoa, Tonga, French Polynesia, Fiji, Wallis and Futuna, Guam, and New Guinea; see Figures 2 and 3, Table A1). For the *COI* dataset, there were three exceptions to this pattern: the first one was an individual identified as *I. aurora* from Kerala in India (GenBank No KR149808) that falls within the Australo-Pacific clade (see Figure 2 and Table A1). The other exceptions corresponded with two other individuals identified as *I. aurora* from India (GenBank No MT511656) and New Guinea (GenBank No MH449994) which also fall outside their expected clades in the phylogenetic analyses: both individuals appear as basal to the rest of the Australo-Pacific *I. aurora* (see Figure 2 and Table A1).

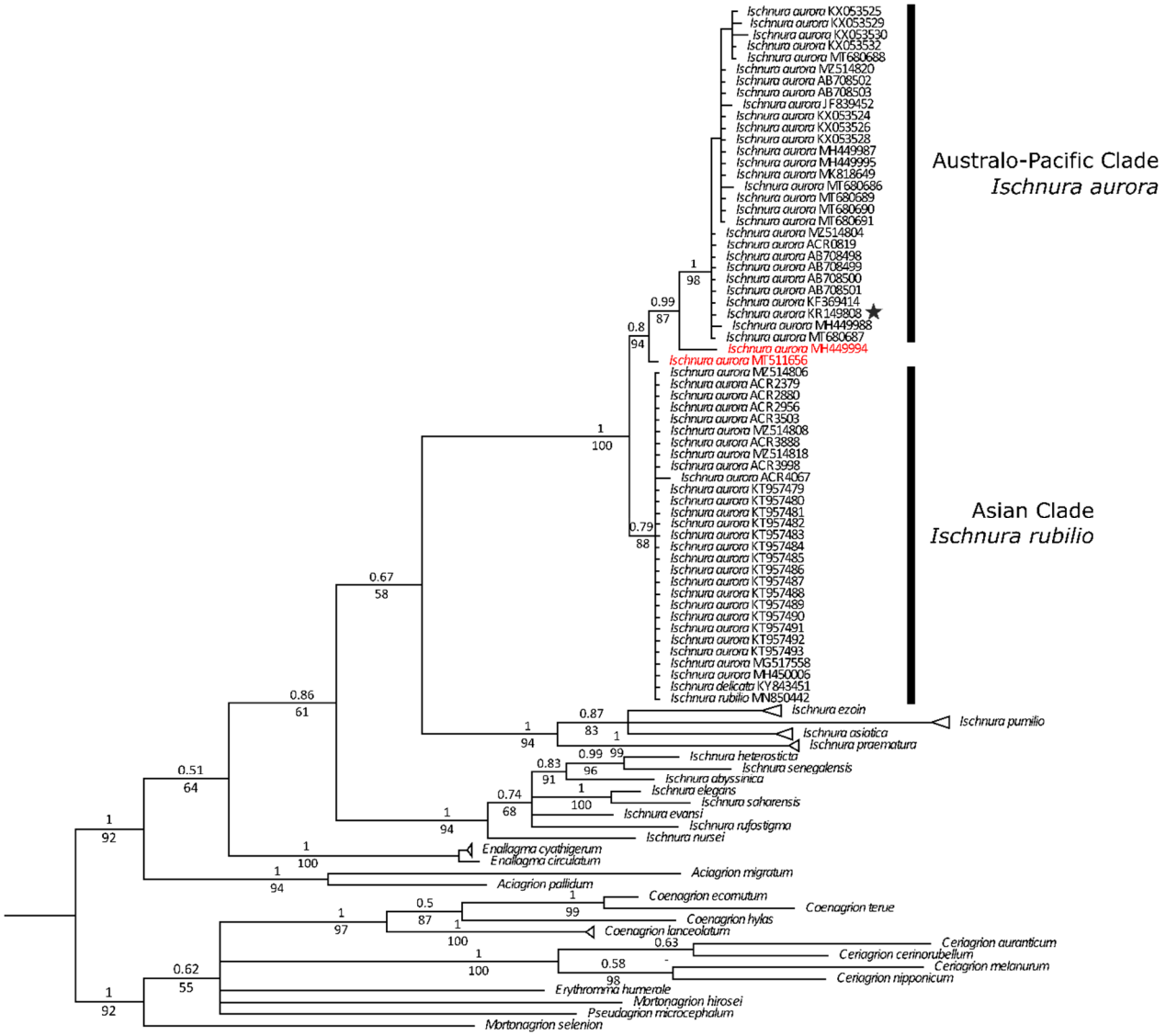

Figure 2. Tree representing the phylogenetic relationships among the *Ischnura* species analysed in this study, using mitochondrial DNA (*COI*) sequence data. Numbers above and below branches represent Bayesian posterior probability and maximum likelihood bootstrap values, respectively. Clades are labelled according to the ASAP proposed species delimitation. The star within the Australo-Pacific clade indicates the *I. aurora* individual from Kerala (India) retrieved from GenBank, while the specimens in red are the two *I. aurora* from New Guinea and India that are identified as different taxonomic units by ASAP (see also Table A1 and Figure A7a).

The ASAP species delimitation analyses were congruent with the results of the phylogenetic analyses: for both the nuclear and mitochondrial DNA datasets, partitions with the best ASAP score split the Australo-Pacific and continental Asian forms of *I. aurora* in two different taxonomic units (see Figure A7), the latter also including the specimens currently under the name *I. rubilio* and *I. delicata*. For the *COI* marker, two sequences were identified as belonging to different taxonomic units in the analysis. The first sequence was that of a specimen identified as *I. aurora* collected at Baliem River in New Guinea with accession no MH449994, which was identified as a different species in the partition with the best ASAP score. The second one was that of an *I. aurora* specimen from India with accession no MT511656, which according to the partition with the second best ASAP score would correspond to a fourth species (see Table A1 and Figure A7a).

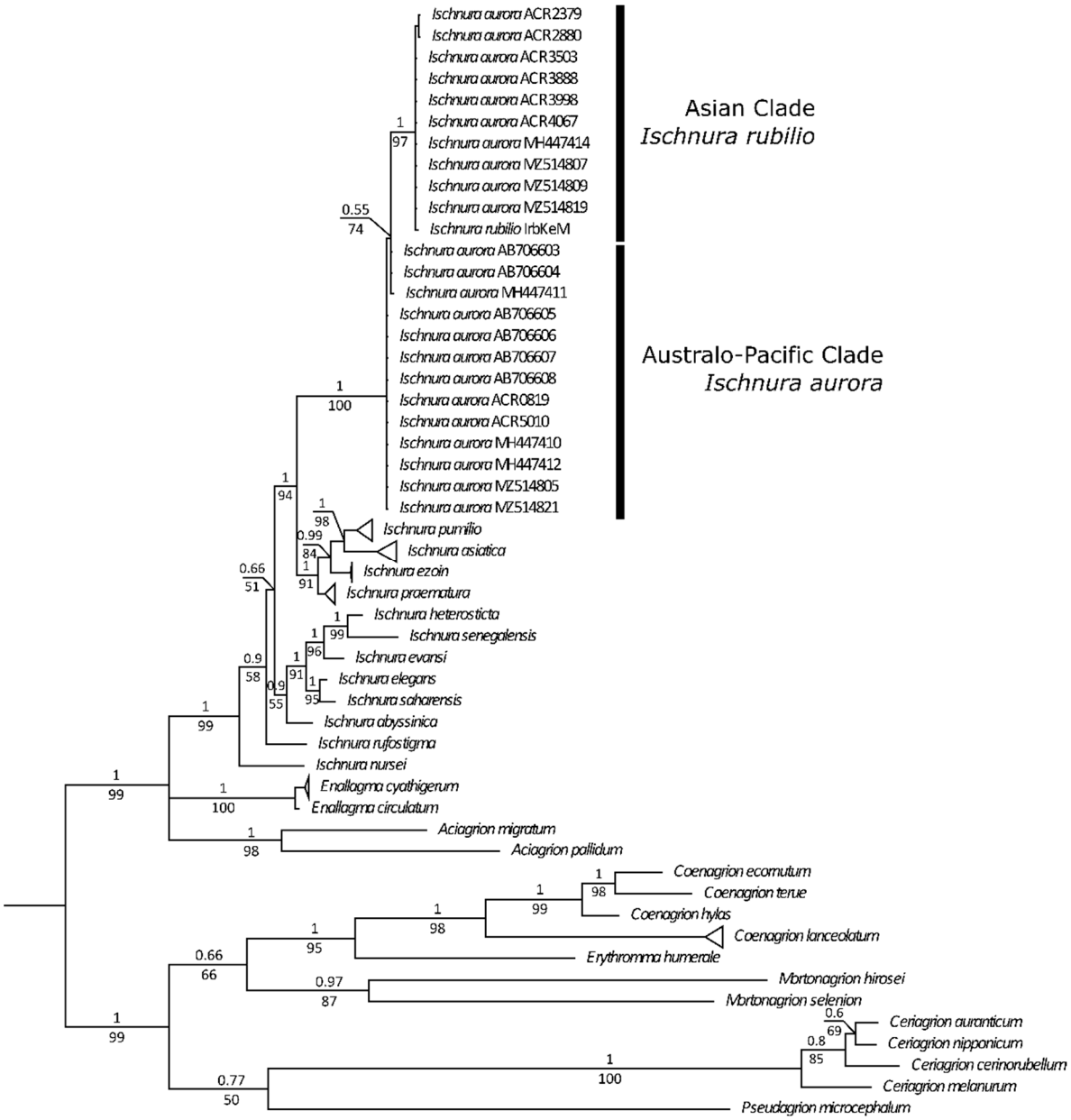

Figure 3. Tree representing the phylogenetic relationships among the *Ischnura* species analysed in this study, using nuclear DNA (*ITS*) sequence data. Numbers above and below branches represent Bayesian posterior probability and maximum likelihood bootstrap values, respectively. Clades are labelled according to the ASAP proposed species delimitation (see also Table A1 and Figure A7b).

The results presented above were further supported by the genetic distances: the average p-distance between Asian and Australo-Pacific *I. aurora* was 2% for the *ITS* marker and 3.1% for the *COI* marker. Similar values were found between the specimens labelled as *I. delicata* and *I. rubilio* and the Australo-Pacific *I. aurora*, whereas genetic differentiation between these two species and the Asian *I. aurora* was nearly zero in all cases (see Table A2). For the *COI* marker, genetic distances between the two identified clades and the *I. aurora* sequences with GenBank numbers MH449994 and MT511656 from New Guinea and India, respectively, were comparable to the distances found between the two clades (~2% in all cases; see Table A2).

3.2. Morphological Analyses

Ischnura aurora specimens from China, Australia, and Fiji all showed similar body length (males from China: 25.0 ± 0.9, N = 10; Australia: 25.3 ± 0.4, N = 2; Fiji: 24.8 ± 1.0, N = 3; females from China: 25.4 ± 1.0, N = 9; Australia: 25.7 ± 0.5, N = 5; Fiji: 23.0 ± 0.0, N = 2) and hindwing length (males from China: 11.5 ± 0.4, N = 10; Australia: 12.6 ± 0.9, N = 2; Fiji: 12.3 ± 0.4, N = 3; females from China: 13.4 ± 0.9, N = 9; Australia: 15.0 ± 0.5, N = 5; Fiji: 12.6 ± 1.3, N = 2; Figure 4). Due to their poor state of preservation, it was not possible to measure the *I. rubilio* specimens.

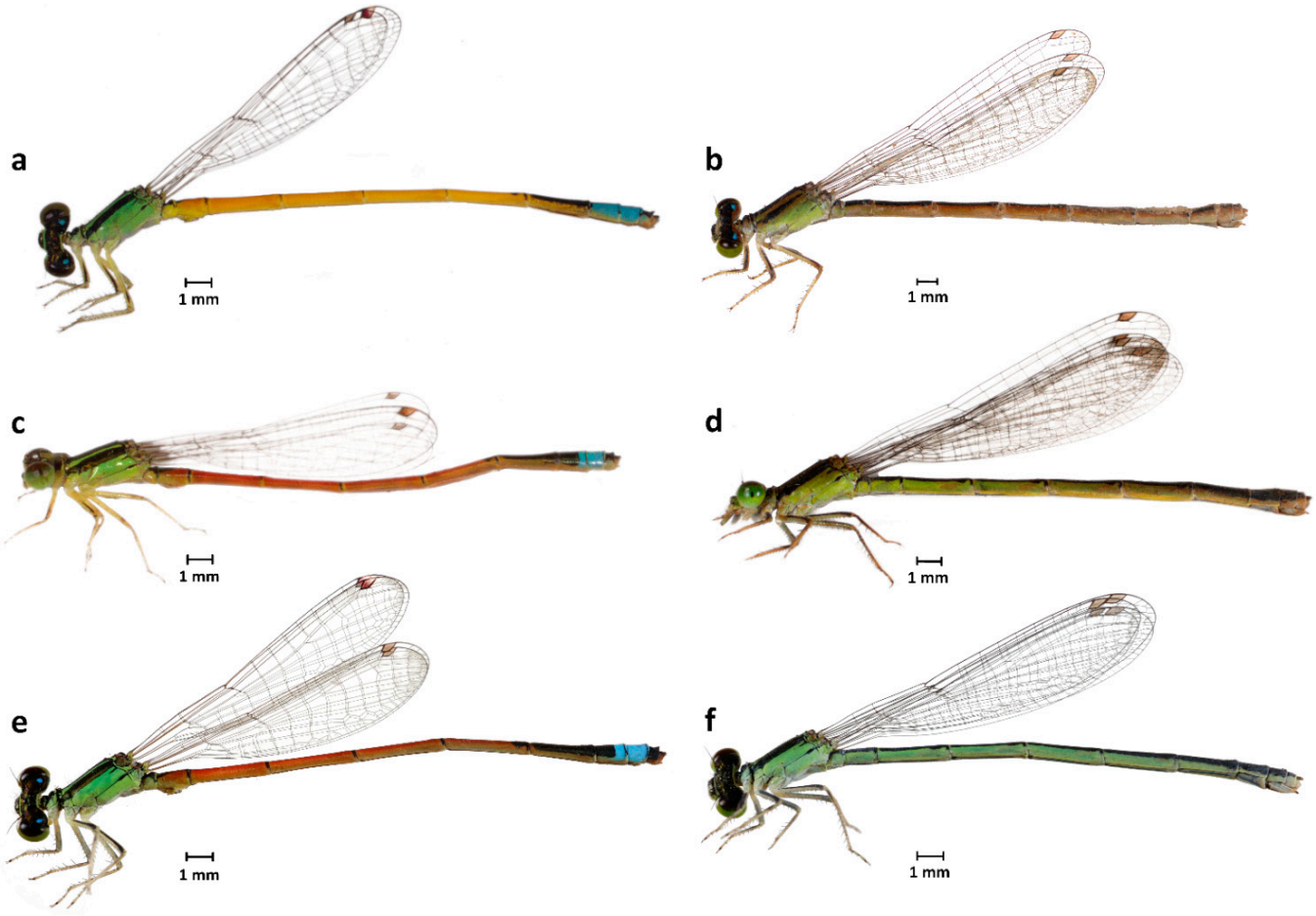

Figure 4. Lateral view of specimens of *Ischnura aurora* from China ((**a**) male; (**b**) female), Australia ((**c**) male, (**d**) female), and Fiji ((**e**) male, (**f**) female). Due to the poor state of preservation of the *I. rubilio* specimens, it was not possible to add an image of this species, but its morphology was similar to that observed in the *I. aurora* from China.

Head: No differences in colour patterns were observed between *I. rubilio* and the examined *I. aurora* specimens, independently of their origin. All specimens observed possessed blue small and rounded postocular spots. Some *I. aurora* females from China show a green-brownish colouration of the occipital region of the head which, in some individuals, reached the postocular spots.

Thorax: Males of both *I. rubilio* and *I. aurora* from China showed the posterior dorso-lateral part of the pronotum (greenish) to be less elevated but the dorsal part (black) to be more expanded towards the mesothorax compared to that of *I. aurora* males from Australia and Fiji (Figure 5). The posterior dorso-lateral elevations of the pronotum of females of both *I. rubilio* and *I. aurora* from China (greenish-brownish) are connected or nearly connected by an arch of the same colour (although this character shows high interindividual variability), while both elevations are restricted to the lateral border in the

I. aurora females from Australia and Fiji (Figure 6). Additionally, in females of *I. rubilio* and *I. aurora* from China, the dorsal expansion towards the mesothorax is wider in than in females from Australia and Fiji (Figure 6). Mesostigmal male protuberances and female plates show similarities between *I. rubilio* and *I. aurora* from all studied populations. Some Australian specimens show completely black mesostigmal protuberances, while tips are greenish in the other specimens. Females of *Ischnura rubilio* and *I. aurora* from China showed a narrower black humeral stripe and a less expanded black colouration in the junction between the metepisternum and metepimeron (see Figure 4d–f).

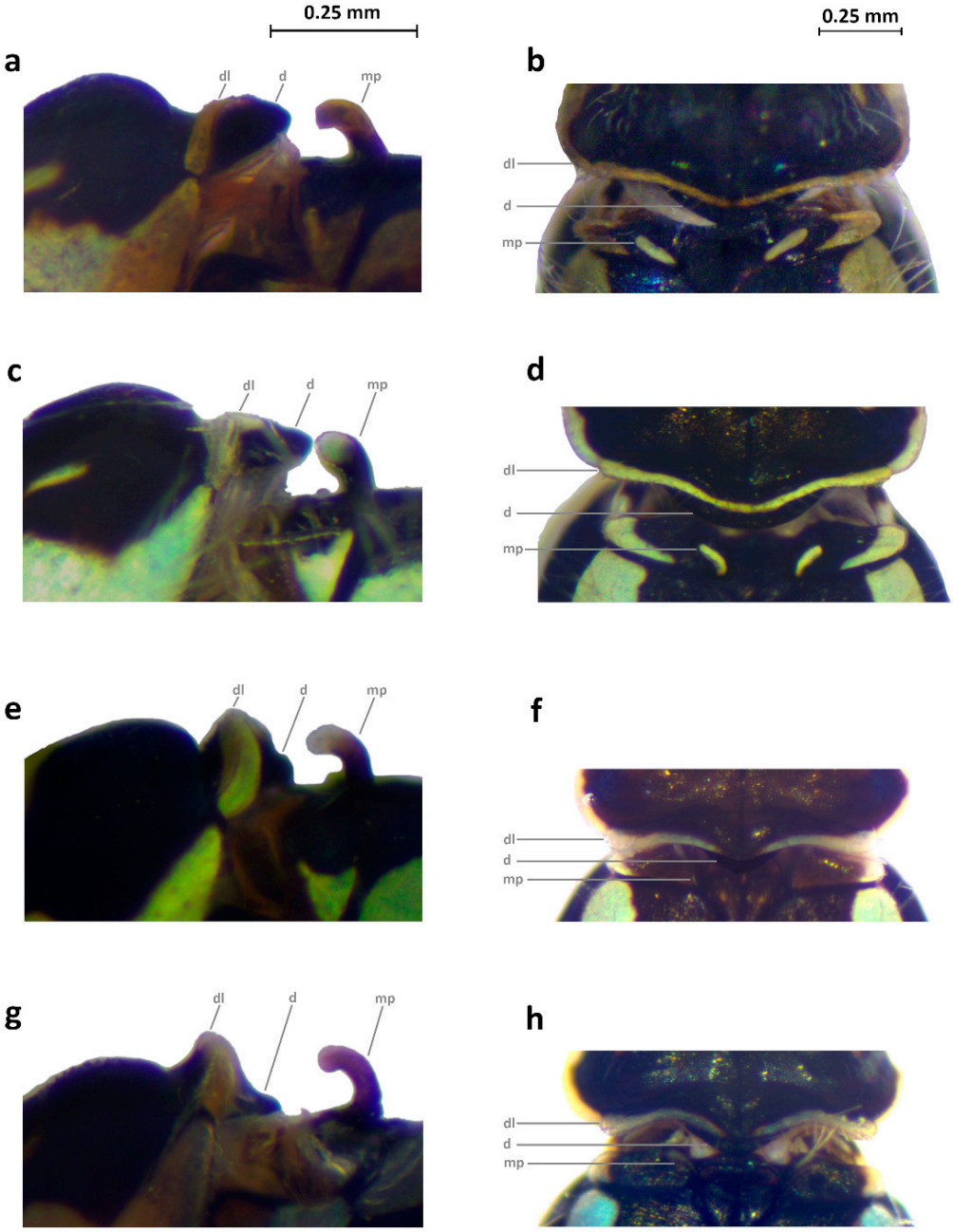

Figure 5. Posterior part of the pronotum and the anterior part of the mesothorax in males of *Ischnura rubilio* (lateral: (**a**), dorsal: (**b**)) and *Ischnura aurora* from China (lateral: (**c**), dorsal: (**d**)), Australia (lateral: (**e**), dorsal: (**f**)), and Fiji (lateral: (**g**), dorsal: (**h**)). *dl*: dorso-lateral elevation. *d*: dorsal expansion. *mp*: mesostigmal protuberance.

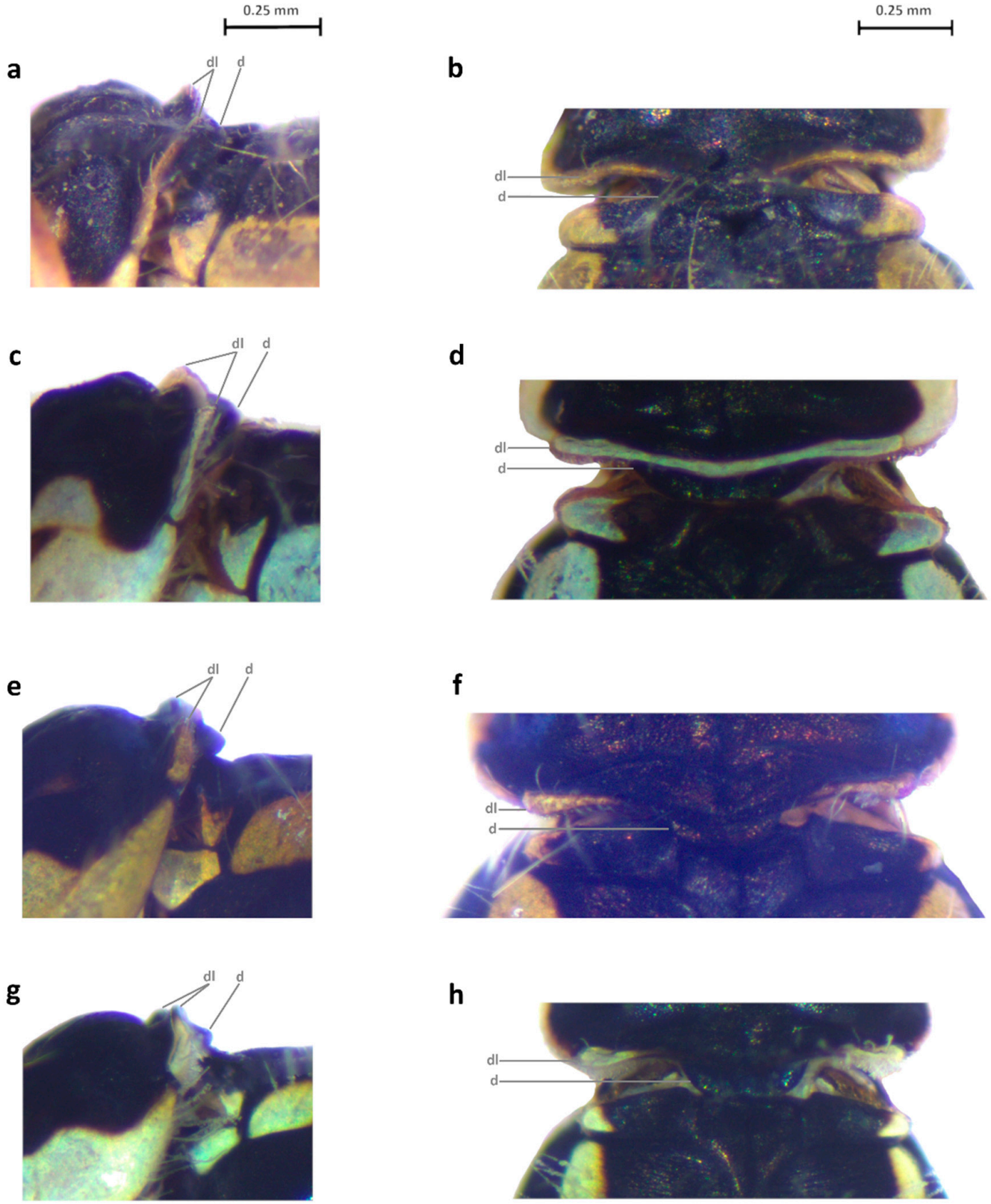

Figure 6. Posterior part of the pronotum and the anterior part of the mesothorax in females of *Ischnura rubilio* (lateral: (**a**), dorsal: (**b**)) and *Ischnura aurora* from China (lateral: (**c**), dorsal: (**d**)), Australia (lateral: (**e**), dorsal: (**f**)), and Fiji (lateral: (**g**), dorsal: (**h**)). *dl*: dorso-lateral elevation. *d*: dorsal expansion. Note that no mesostigmal protuberances exist in females.

Legs: No differences in leg colouration were found between the examined specimens.

Wings: Cell number was variable within and between all the studied populations.

Abdomen: *Ischnura rubilio* and *I. aurora* males from China show a less expanded black colouration on the dorsal part of S2 than that observed in *I. aurora* from China and Fiji. In the former, the apical stripe on S2 expands into a mid-dorsal stripe that reaches to approximately half of the segment, whereas in the *I. aurora* specimens from Australia and Fiji, this mid-dorsal stripe is much wider and may reach up to the end of S2 in some individuals (Figure 7). The basal articulation of S2 appears to be circled in black in the *I. aurora* individuals from Australia and Fiji, whereas it has a lighter colour in *I. rubilio* and *I. aurora* from China (Figure 7). S3 to S6 are citron yellow in males of *I. rubilio* and *I. aurora* collected in China and yellow-orange in males of *I. aurora* from Australia and Fiji, except for the black intersegment areas, which appear also of a lighter colour in *I. rubilio* and *I. aurora* from China (Figure 4). S6 is dorsally black in its posterior part and S7 is dorsally black in all examined individuals.

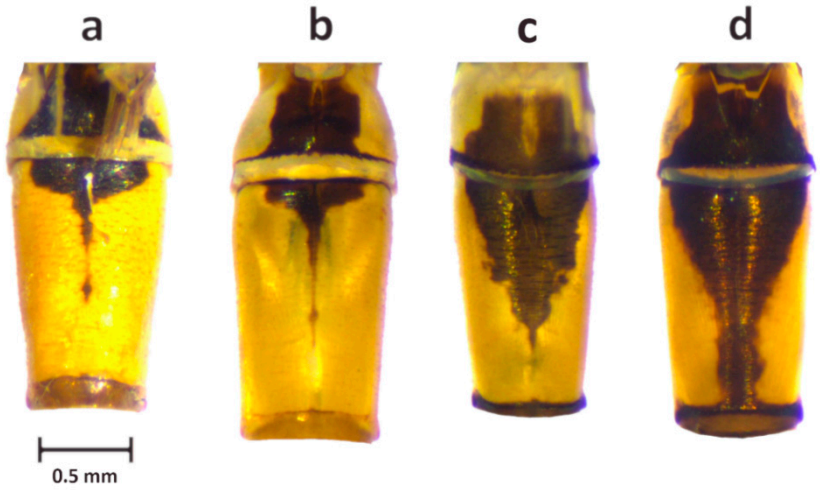

Figure 7. Dorsal view of the first and second abdominal segments of *Ischnura rubilio* (**a**) and *Ischnura aurora* from China (**b**), Australia (**c**), and Fiji (**d**).

Ischnura rubilio and *I. aurora* males from China show blue colouration in the dorsal and lateral part of S8 (except its anterior dorsal part, which shows a delta-shaped black colour) and S9, and laterally in the S10 (which vary between individuals) (Figure 7). Some individuals show blue spots in the lateral part of S7. *Ischnura aurora* males from Australia and Fiji show blue colour in the dorsal and lateral part of the S9 but only in the posterior dorso-lateral part of the S8 (1/3 or less of the segment length; see Figure 7). Individuals from Australia show certain variability in these colourations, showing black colour interrupting the blue bands of S8 and S9 but also showing blue colour in the lateral part of the S10. The dorsal tubercle of S10 is more pronounced in *I. rubilio* and *I. aurora* males from China than in *I. aurora* males from Australia and Fiji (Figure 7). All females examined showed a vulvar spine, except for one examined female from Australia. No differences were found in the length of the female genital valves.

Annal appendages: *Ischnura rubilio* and *I. aurora* males from China show more acute dorsal expression in their cerci and narrower paraprocts than males of *I. aurora* from Australia and Fiji (Figure 8). *I. aurora* from Fiji show a marked depression in the middle of the cerci under a lateral view (albeit this character shows high variability; Figure 8k), while the cerci in the rest of the specimens showed a straight border.

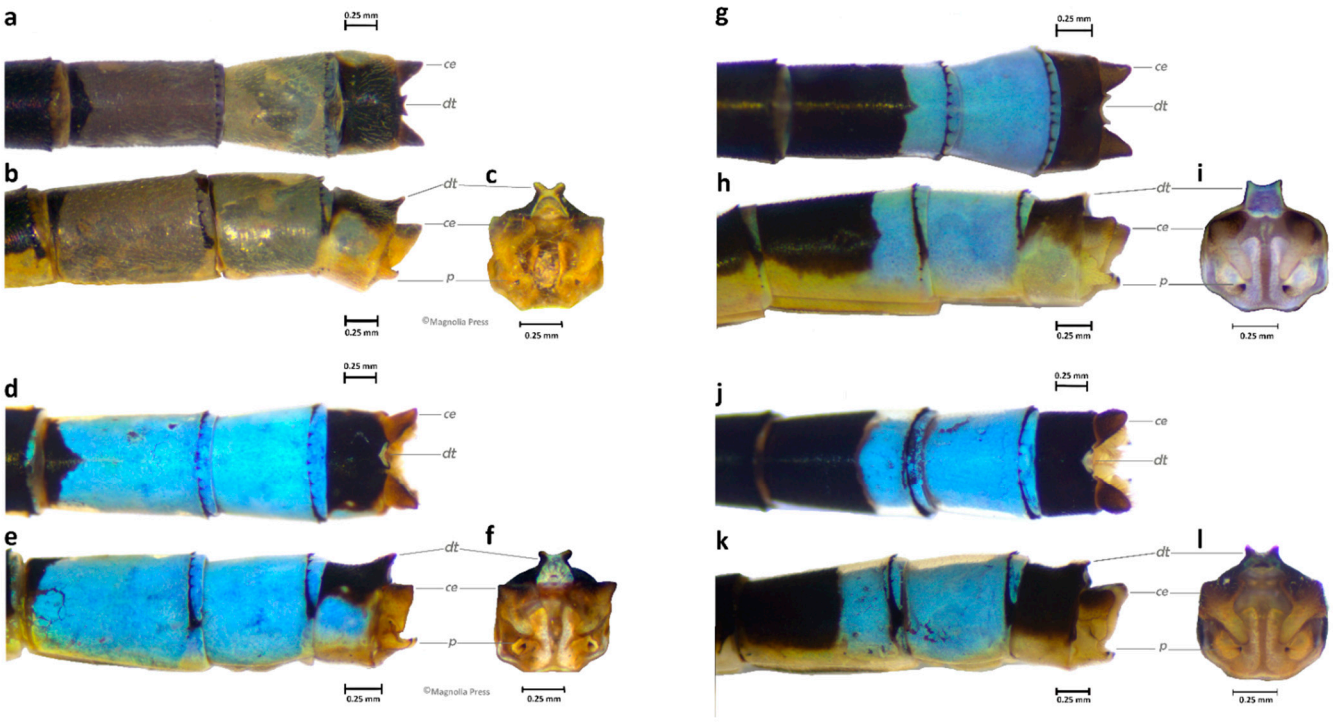

Figure 8. Dorsal, lateral, and posterior view of the abdominal appendages of *I. rubilio* (**a**–**c**) and *I. aurora* from China (**d**–**f**), Australia (**g**–**i**), and Fiji (**j**–**l**). *dt*: dorsal tubercle; *ce*: cerci; *p*: paraprocts. Images (**a**–**f**) are partly reproduced from Sanmartín-Villar et al. [14] with permission from the copyright holder.

Male genital ligula: No differences were observed between the male ligula of the analysed specimens (see Figure 9).

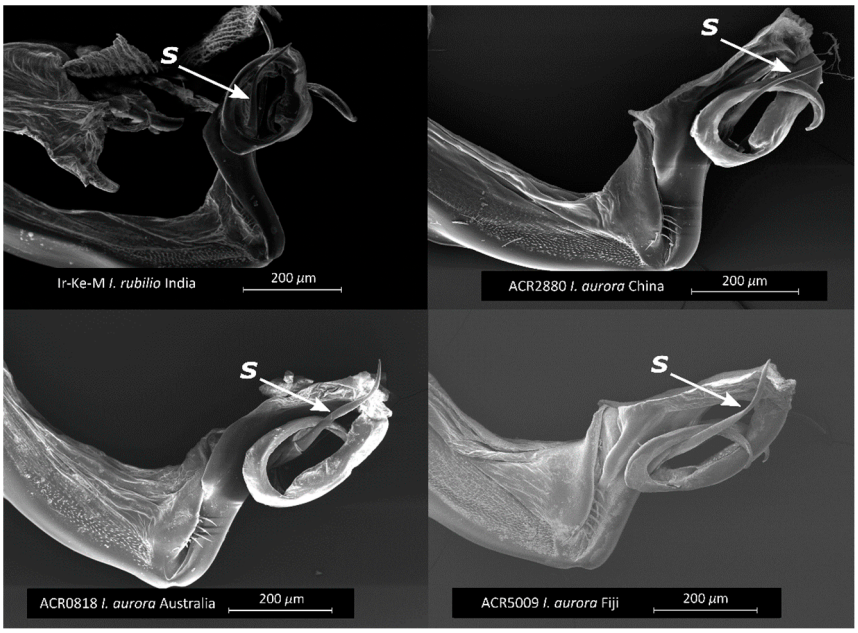

Figure 9. Scanning electron images showing the male genital ligula of *Ischnura rubilio* from India and *I. aurora* from China, Australia, and Fiji. The arrows in each image point to the spine (*s*) proximal to the genital ligula flexure, typical of most *Ischnura* species.

4. Discussion

The results of our genetic analyses indicate that the samples currently identified as *I. aurora* found within the Asian distribution area of this species all belong to a clade that also includes specimens currently under the names *I rubilio* and *I. delicata*. This clade appears to be closely related to that comprising the *I. aurora* individuals from the Australo-Pacific distribution area of the species. This split into two well-differentiated clades is supported by high bootstrap and posterior probability values for both nuclear and mitochondrial DNA (see Figures 2 and 3). Genetic distances between *I. rubilio*, *I. delicata* and *I. aurora* from China were nearly zero in all cases, whereas genetic distances between the Asian and the Australo-Pacific clade were ~3% for *COI* and ~2% for *ITS* (see Table A3). These distances found between both clades are similar to those found between other closely related species within the genus *Ischnura* (e.g., *I. elegans*/*I. saharensis*, Table A3; *I. elegans*/*I. graelsii* [47], or between closely related species in other Coenagrionidae genera (e.g., *Paracercion* [48]). The species delimitation analyses have provided further support for the placement of *I. aurora* from Asia within the same group as *I. rubilio* and *I delicata*, separated from the Australo-Pacific *I. aurora* (see Figure A7).

In agreement with the results of our molecular analyses, the morphological examination of material from Asia and Oceania points also to a closer relationship between the specimens currently under the name *I. aurora* collected in China and *I. rubilio* from India, than to *I. aurora* from Australia and Fiji. These morphological similarities also include the characteristics described above, which consistently differentiate *I. aurora* from China and *I. rubilio* from India from *I. aurora* from Australia and Fiji: in males, the less elevated but more expanded posterior part of the pronotum found in *I. rubilio* and *I. aurora* from China; and in females, the connectivity between the lateral elevations and the wider expansion, differing from the same structure in *I. aurora* females from Australia and Fiji (see Figures 5 and 6).

Beyond these morphological differences in the shape of the pronotum, all Asian and Australo-Pacific specimens examined by us differ mostly in their colouration pattern: *Ischnura rubilio* shows the same colour pattern as *I. aurora* from China, which differs from that observed in *I. aurora* from Australia and Fiji. Males of *I. rubilio* and *I. aurora* from China show a more expanded blue colouration on S8–S10. The abdominal blue colouration is highly variable in some Asian *Ischnura* [16] and similar between species (for instance, *I. praematura* Sanmartín-Villar & Zhang, 2022 shows an intermediate colouration between *I. aurora* from China and from the Australo-Pacific forms [17]), which might represent one of the main factors hindering the identification of these species. Nevertheless, we have found that all examined specimens from *I. rubilio* and *I. aurora* from China share the same colour pattern, which is different from that of *I. aurora* specimens collected in Australia and Fiji. These observations agree with those of Papazian et al. [27] and Rowe [29] regarding their examination of Asian and Australo-Pacific *I. aurora*; and with Selys in his description of *I. rubilio* [25]. Other differences that we have found between the Asian and Australo-Pacific material, and which have also been pointed out by some or all of these authors, include: (i) differences in the colour pattern of S1–S2 [27]; (ii) the paler basal articulations of the abdominal segments found in both *I. rubilio* and *I. aurora* from China when compared to Australo-Pacific *I. aurora* [25]; (iii) a more prominent dorsal tubercle in S10 in *I. rubilio* and *I. aurora* from China than in *I. aurora* from Australia and Fiji [29]; and (iv) the differences found in the anal appendages between the Asian and Australo-Pacific material that we have examined [29].

Contrary to what has been previously reported, we did not find differences in the postocular spots between *I. aurora* and *I. rubilio*. Papazian et al. [27] suggested that *I. rubilio* showed larger postocular spots and pointed out that they were "sometimes confluent across the occiput" [27] (p. 60). Even though we have observed that the occipital green-brownish colour in some females of *I. aurora* from China reaches the postocular spots, we highlight the different colouration of the postocular spots (blue) and the occipital region (green-brownish) and hence, no confluence between the postocular spots in the examined

specimens. Considering the high variability that this character shows in *Ischnura* [49], including the species we examined here; and given that we found no consistent differences across the examined specimens, we conclude that postocular spots cannot be used as a trait to distinguish between *I. rubilio* and *I. aurora*.

Our morphological analysis of Asian and Australo-Pacific material allowed us to find some morphological differences in the females, which have not been addressed previously. Thus, we found differences in the thoracic colouration pattern (the black colour is less expanded in females of *I. rubilio* and *I. aurora* from China; compared to females of *I. aurora* from Australia and Fiji; Figure 4), and morphological differences in the posterior part of the pronotum that consistently separate females of *I. rubilio* and *I. aurora* from China from Australo-Pacific *I. aurora* females (see Figure 6).

Overall, the results of our analyses support the split of the Asian and Australo-Pacific forms of *I. aurora* into two well-differentiated taxonomic units and therefore different species, further supporting the specific status of *I. rubilio* [28]. The results of our genetic analyses agree with those of Sánchez-Guillén et al. [18] in placing *I. rubilio* within the *aurora* clade, but contradict those by Dumont [23], who found *I. rubilio* to be either basal to the *Ischnura* group or a member of the *pumilio* clade. The quality control of the DNA sequence data carried out by us here led to the exclusion of several questionable sequences from our datasets, among which were those belonging to *I. rubilio* from Dumont's work. These sequences are likely the product of both specimen misplacement during laboratory work and sequencing of non-orthologous copies of the mitochondrial *COI* gene (see Table 2), which led to the wrong conclusion about the phylogenetic position of this species within the *Ischnura* clade [23]. Similarly questionable, "*COI*-like" sequences were recently identified in Odonata [13], and therefore we encourage research colleagues to carefully examine the sequences they use in their studies in a similar way as we have done here or has been described elsewhere e.g., [50] to avoid the potential amplification of *numts*, which might have important consequences when addressing taxonomical questions [11,13,31].

Although wind-driven dispersal could explain the exchange of individuals between Asia and Oceania (for instance, the individual from Kerala, India with accession no KR149808 that falls within the Australo-Pacific clade; see Figure 2), long-distance dispersal may at the same time be limited by the physical barrier constituted by the sea, which will in turn restrict the contact between the identified clades and lead to the observed genetic differentiation. The observed morphological differences in the shape of the pronotum of both males and females, along with the differences in the S10 dorsal tubercle and male anal appendages found between both Asian and Australo-Pacific species might not be enough to prevent successful hybridization, which could in turn result in a morphological and/or molecular cline along the species' contact zone, with individuals showing intermediate characters between *I. rubilio* and *I. aurora*. There are several examples of hybridization between closely related *Ischnura* species [51,52]. However, as it is assumed that reproduction occurs between mature individuals in *I. rubilio* and *I. aurora* Asian forms but between mature males and teneral females in *I. aurora* from the Australo-Pacific forms [29,53], even if individuals from both species may occasionally come into contact, mating would be restricted due to these behavioural incompatibilities. Without access to the actual specimen from which the DNA sequence was obtained, we cannot establish whether the individual from Kerala that falls within the Australo-Pacific clade is a migrant individual or a hybrid.

The recent discovery of other Asian species in which teneral females mate [16] stresses the need for additional ethological studies to unravel species barriers and species population dynamics. Additionally, the presence of the elaborated mesostigmal protuberances in males but not in females suggests that these do not play a role in male–female assembly (for instance as happens in the European *Ischnura* species; see [54]) but in male–male encounters, which poses an interesting topic for future studies about the function of such structures.

Finally, the results of our genetic analyses point also to the existence of at least a third (or even a fourth) taxonomic unit identified from our *COI* dataset. These correspond to a specimen from India and another specimen from Baliem River in New Guinea, both

labelled as *I. aurora.* Interestingly, Baliem River is the same locality in which the subspecies *I. aurora viduata* was first described [55]. These results stress the need to revise all available material belonging to the numerous *I. aurora* subspecies described as well as to be able to correctly link available DNA sequence data with voucher specimens.

We provide below an identification key for the separation between *I. rubilio* and *I. aurora*:

1. Pale postocular spots present, S8 partially or entirely blue dorsally, S9 blue dorsally, ... **2**

2. ♂: Posterior lobe of prothorax slightly raised laterally; expansion of the same lobe towards the mesothorax wide (approximately half of the width of the prothorax' posterior lobe), reaching the mesostigmal plates in dorsal view (Figure 5a–d); paraproct in ventral view with an accessory medial lobe in addition to the basal one; height of S10 tubercle in posterior view subequal to the width separating each tubercle (Figure 8c,f); black apical stripe in S2 expanding into a mid-dorsal stripe that reaches approximately half of the segment (Figure 7a,b); S8 entirely blue dorsally (Figure 8a,b,d,e);

♀: Posterior lobe of prothorax raised laterally; expansion of the same lobe towards the mesothorax wide (approximately half of the width of the prothorax' posterior lobe) (Figure 5a–d); black humeral stripe narrowing dorsally (Figure 4b); India [and elsewhere] ..... ***rubilio***

2′. ♂: Posterior lobe of prothorax erect in its middle part; expansion of the same lobe towards the mesothorax narrow (approximately one-third of the width of the prothorax' posterior lobe), not reaching the mesostigmal plates in dorsal view (Figure 5e–h); paraproct in ventral view lacking an accessory medial lobe; distance between the bifid tubercles of S10 in posterior view greater than the height of the bifid tubercle (Figure 8i,l); black apical stripe in S2 wide, with the shape of an inverted triangle that may reach the end of S2 in some cases (Figure 7c,d); S8 with only posterior half blue dorsally (Figure 8g,h,j,k);

♀: Posterior lobe of prothorax raised laterally; expansion of the same lobe towards the mesothorax narrow (approximately one-third of the width of the prothorax' posterior lobe) (Figure 5a–d); black humeral stripe of equal width throughout (Figure 4d,f); Polynesia, Australia, New Zealand [and elsewhere]

.....***aurora***

5. Conclusions

- Genetic analyses have showed that specimens currently under the names of *Ischnura rubilio* and *I. delicata* belong to a clade that also includes the *I. aurora* found within the Asian distribution area of this species.
- All the *I. aurora* found within the Australo-Pacific distribution area cluster together in a separate clade. Species delimitation analyses have identified these two clades as different taxonomic units.
- Concordant with the results of the genetic analyses, the morphology of the *I. aurora* collected in China is closer to *I. rubilio* than to *I. aurora* from Australia and Fiji.
- Given these results, we confirm the status of *I. rubilio* as a valid species and provide an identification key for its separation from *I. aurora*.
- Genetic analyses point also to the existence of at least a third taxonomic unit within the *aurora* clade, which stress the need to revise all available material belonging to the numerous subspecies of *I. aurora* that have been described.

Author Contributions: Conceptualization, M.O.L.-C., I.S.-V. and A.C.-R.; methodology, M.O.L.-C. and I.S.-V.; formal analysis, M.O.L.-C. and I.S.-V.; fieldwork and investigation, M.O.L.-C., I.S.-V. and A.C.-R.; resources, A.C.-R.; data curation, M.O.L.-C. and I.S.-V.; writing—original draft preparation, M.O.L.-C. and I.S.-V.; writing—review and editing, M.O.L.-C., I.S.-V. and A.C.-R.; supervision, M.O.L.-C. and A.C.-R.; project administration, A.C.-R.; funding acquisition, A.C.-R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: DNA sequences generated in this study have all been submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Additional data and methodology are available in Appendices A and B. All the *Ischnura* specimens examined for morphological analyses and/or used for genetics are deposited in the collections of Adolfo Cordero-Rivera and Matjas Bedjanič.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A. Quality Control (QC) and Curation of GenBank Sequence Data

All *COI* and *ITS* sequences deposited in GenBank that were labelled as belonging to the species *Ischnura rubilio*, *Ischnura aurora* and *Ischnura delicata* were downloaded from the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/), through Geneious v. 9.1.8 (https://www.geneious.com). These were added to the sequences obtained by us from samples of *I. aurora* from China, Australia and Fiji and samples of *I. rubilio* from India, as described in the Materials and Methods section of the manuscript. Additionally, sequences belonging to different species within the genus *Ischnura* and other closely related genera (*Aciagrion*, *Ceriagrion*, *Coenagrion*, *Enallagma*, *Erythromma*, *Mortonagrion* and *Pseudagrion*) were also downloaded from GenBank and added to each dataset. Sequences were aligned in MAFFT [32] as implemented in Geneious v. 9.1.8, with a gap open penalty of 3. Alignments were visually inspected, and tails trimmed manually before phylogenetic tree reconstruction. Phylogenetic relationships were reconstructed through maximum likelihood (ML) using FastTree 2.1.11 [56] also implemented in Geneious v. 9.1.8, with the following options: use of GTR model and optimization of the 20 Gamma Likelihood.

Appendix A.1. ITS Dataset

A total of 69 sequences were included in the preliminary *ITS* dataset. After inspection of the alignment, the sequence with accession number MZ809355, belonging to an *Ischnura rubilio* specimen from India, was excluded from further analyses because it corresponded with a partial region of the 18S ribosomal RNA gene, located upstream the region that we sequenced for our study. The tree obtained after removing this sequence and trimming the alignment tails is showed in Figure A1 below. Two of the sequences included in the dataset were placed in the tree far from their expected clades. The first one was the *Ischnura aurora* sequence with GenBank accession number FN356100, which was placed outside of the *aurora* clade. Instead, this sequence appears to be closely related to *I. nursei* within the clade that includes *I. elegans*, *I. senegalensis*, *I. heterosticta*, *I. evansi*, *I. saharensis* and *I. abyssinica* (see Figure A1). The second sequence was that of *Ischnura rubilio* with GenBank accession number MH447434, which falls outside of the *Ischnura* clade, and it is placed as a sister species to *Aciagrion migratum* (see Figure A1). Both sequences were used as queries in a BLAST search against the nr database. Searches were carried out using the MegaBLAST program implemented in Geneious v. 9.1.8 with default options.

The sequence FN356100 resulted in very similar matches with other *Ischnura* ITS sequences in the nr database, but none with *I. aurora.* The sequence MH447434 resulted in matches with *Aciagrion migratum*, *Proischnura subfurcata* and several *Ischnura* species, but none with *I. aurora*. The results of the phylogenetic analysis and the BLAST searches suggest that the specimens from which these sequences were obtained could have been

misidentified and/or misplaced at the time of DNA extraction, and hence these sequences were removed from our datasets and excluded from the final analyses.

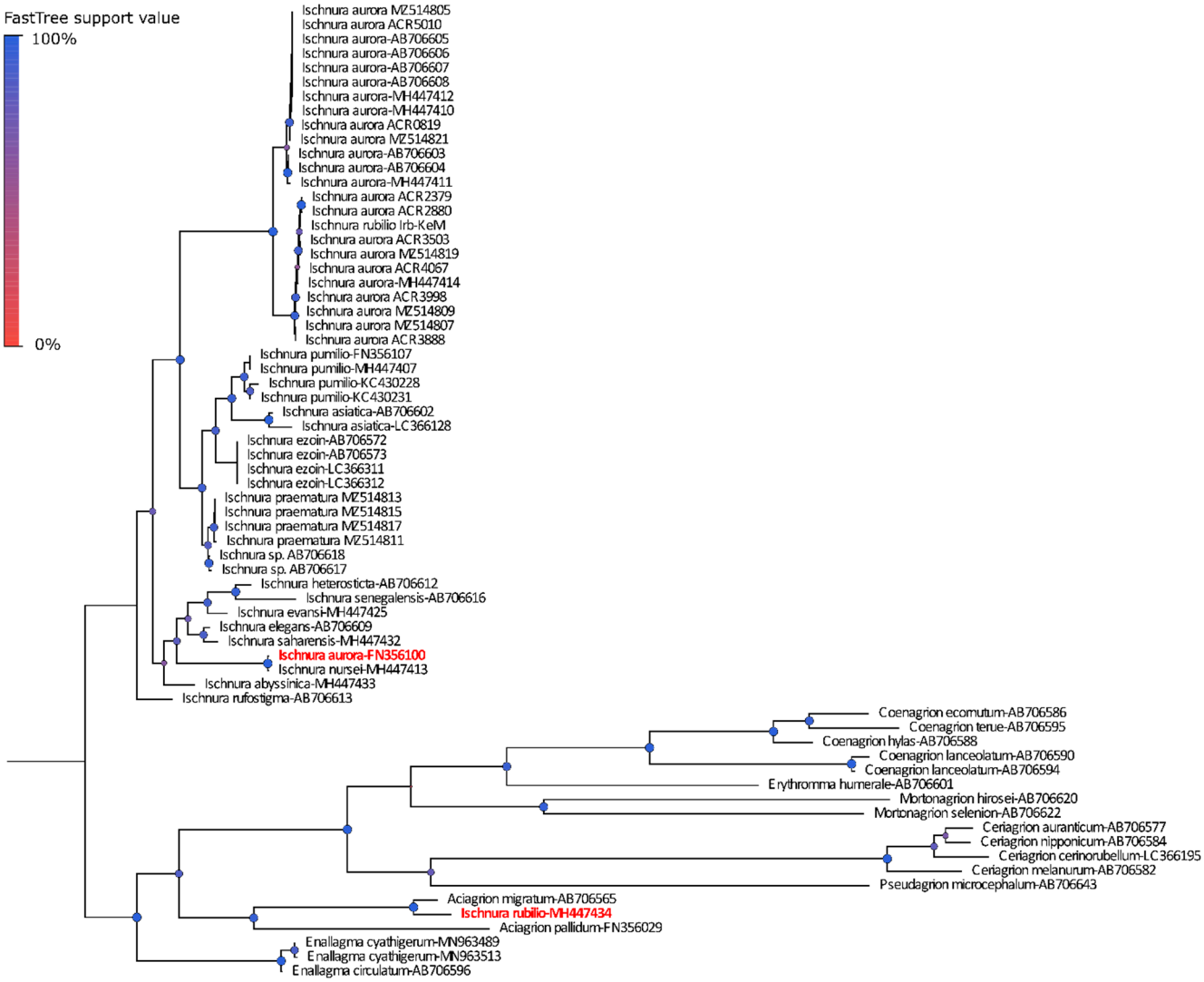


Figure A1. Maximum likelihood tree obtained with FastTree 2.1.11 for the *ITS* dataset (68 sequences; 655 bp-long). Highlighted in red are the two sequences downloaded from GenBank that were finally excluded from our dataset. The coloured dots on the nodes represent FastTree support values according to the legend on the left.

Appendix A.2. COI Dataset

The starting *COI* dataset consisted of 117 sequences and was 457 bp long. All sequences that showed ambiguities (e.g., N, M, K, Y, R . . .) were removed from the dataset: this was the case of sequences with accession numbers KY844428, KY838304, KY832433 (*Ischnura delicata*) and sequences KX053527 and KX053531 (*Ischnura aurora*). Three sequences in the alignment sequences appeared to be quite dissimilar to the rest of the sequences in the dataset, and which introduced a 6 bp-long gap in the alignment (Figure A2a). These were sequences with accession numbers EU219876, EU219877 and LC198680; all of them were labelled as *Ischnura aurora*. Translation into protein yielded nearly identical sequences without stop codons (Figure A2b).

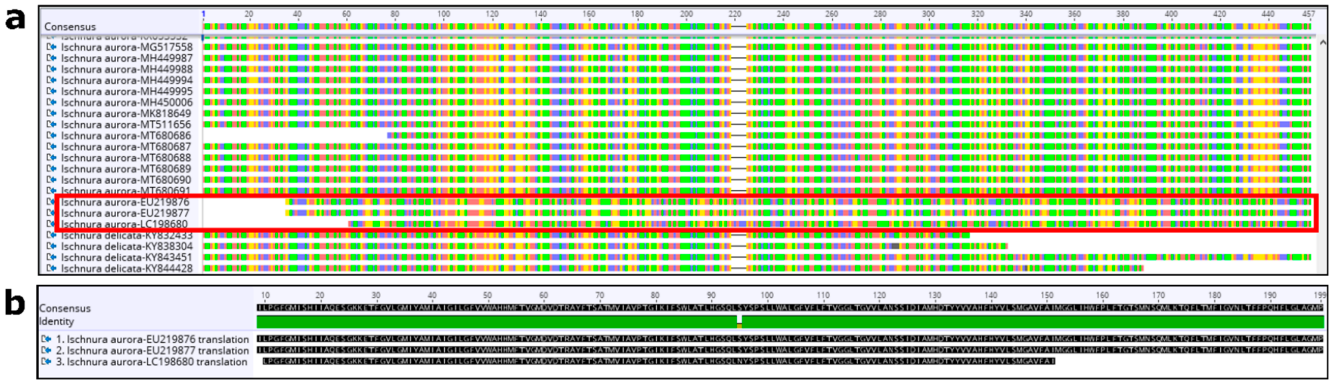


Figure A2. (**a**) Detail of the initial *COI* alignment showing the three dissimilar sequences labelled as *Ischnura aurora* (box in red). (**b**) Alignment of the amino acid sequences corresponding to these same accession number sequences.

BLAST searches were carried out also with MegaBLAST as described above using these three sequences as queries. The searches resulted in nearly the same hits for sequences EU219876 and EU219877, including several *Ceriagrion* and *Ischnura* species. The sequence LC198680 yielded matches with *Ceriagrion* and *Ischnura* species but also with Anisoptera genera such as *Anax* and *Onychogomphus*. Several of the hits obtained in these searches corresponded with sequences of *Ischnura* spp. all belonging to the same study.

The tree obtained from FastTree analysis is shown below. It can be seen how these three sequences are placed together in the same clade, at the end of a very long branch and far from the rest of the sequences included in the analysis (see Figure A3).

The alignment of these sequences with reference *COI* sequences from published complete mitochondrial genomes of *Ischnura* species (*Ischnura elegans* (MK951668), *I. senegalensis* (MT787567) and *I. pumilio* (KC878732)) revealed that they corresponded to the 3′ region of the *COI* gene, located downstream from the Folmer region that we amplified in our study (Figure A4). Therefore, these sequences were excluded from further analyses.

The *COI* sequence from *Ischnura rubilio* with accession number MH449992 falls within the *Aciagrion* clade in the FastTree ML tree (see Figure A3), similarly to the results obtained for the *ITS* sequence from this same specimen (accession number MH447434). The results of a MegaBLAST search using sequence MH449992 as a query resulted in hits with *Aciagrion*, *Ischnura pumilio* or *Ischnura elegans*, among others, which further supports our conclusion that this could be a case of misidentification and/or misplacement of individuals in the laboratory. Hence, this *COI* sequence was also removed from our dataset.

Two sequences downloaded from GenBank with accession numbers MH449981 and MW143324 labelled as *Ischnura rubilio* appear together in the ML tree, in a clade basal to *Ischnura pumilio* (see Figure A3). Both sequences can be translated into an amino acid sequence without stop codons, and both protein sequences were identical. MegaBLAST searches carried out using these two sequences as queries yielded hits with several genera of marine sponges (*Characella*, *Poecillastra* and *Theonella* among others). The sequences obtained by us from the *Ischnura rubilio* specimens from India (MB-IrbKeF, MB-IrbKeM, MB-IrbGir and MB-IrbTam; see Table 1) appear in the tree together in a clade separated from the rest of the sequences included in the analysis (see Figure A3). Translation of these sequences into the protein yielded nearly identical amino acid sequences without stop codons, and BLAST searches using these four sequences as queries returned hits against other Odonata sequences in GenBank, but the closest matches were all sequences belonging to *Nesobasis* spp., a genus in the Coenagrionidae family which is endemic to the Fiji archipelago.

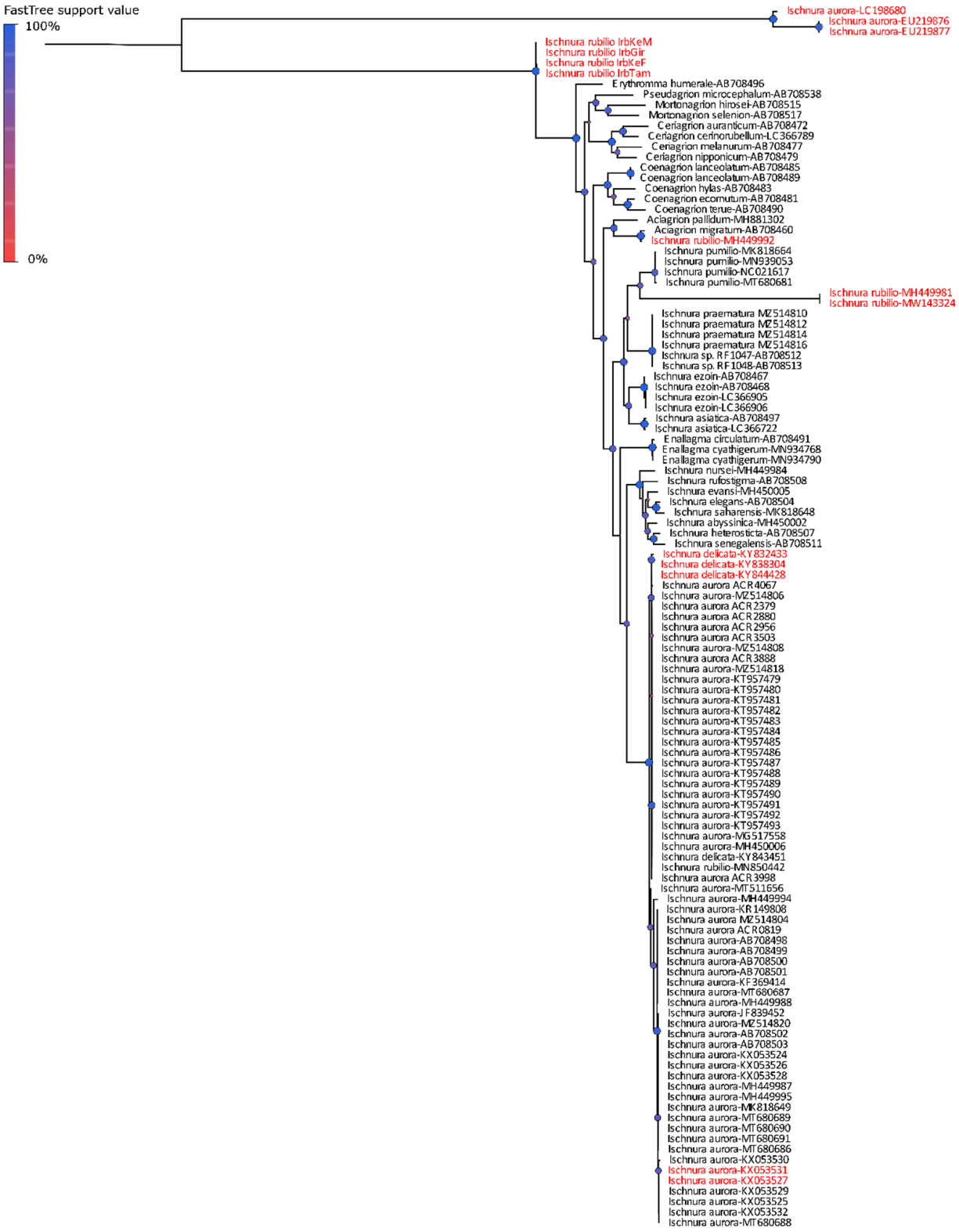


Figure A3. Maximum likelihood tree obtained with FastTree 2.1.11 for the *COI* dataset (117 sequences; 457 bp-long). The tree is rooted by the midpoint. Highlighted in red are the ten sequences downloaded from GenBank and the four sequences obtained in this study that were finally excluded from our dataset. The coloured dots on the nodes represent FastTree support values according to the legend on the left.

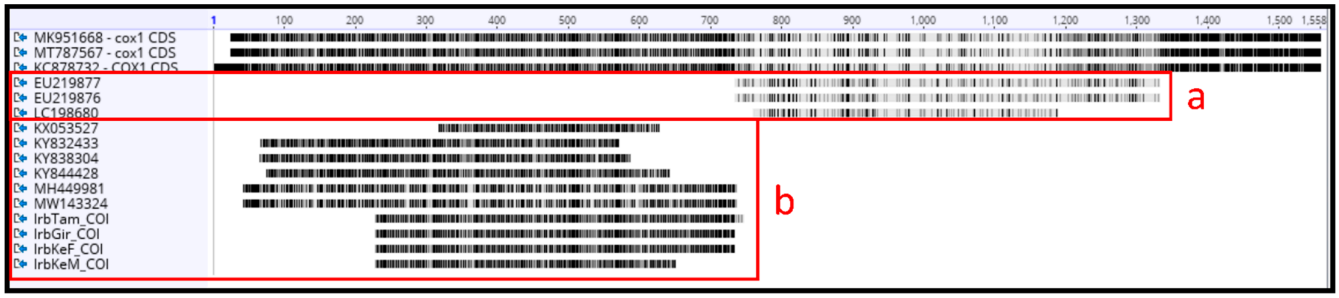


Figure A4. Alignment of sequences EU219876, EU219877 and LC198680 (box **a**) against reference *COI* sequences from *Ischnura* species (first three sequences in the alignment). Box **b** shows sequences that correspond with the Folmer region used in barcode analysis, which was the target region of the primers used in our study.

Even though the results of the BLAST searches for both sets of *I. rubilio* sequences shown mentioned above might initially lead to the conclusion that they could all be cases of sample contamination; this would in fact be quite unlikely. Regarding the *COI* sequences obtained by us from the *I. rubilio* specimens from India, none of the *Nesobasis* spp. sequences to which they are similar were obtained in our laboratory. Furthermore, the *ITS* sequence obtained from the individual MB-IrbKeM falls within the clade of *I. aurora* (see Figure A1), further supporting the fact that this is unlikely a case of contamination. In the case of sequences MH449981 and MW143324, these were produced by two independent laboratories/researchers, even at different times. Sequence MH449981 corresponds to the *I. rubilio* specimen from Kerala (India) included in the work by Dumont [23] and which was submitted to GenBank in 2018. Sequence MW143324 was submitted to GenBank in 2020 and belongs to an unpublished study on Odonata in rice ecosystems of India. Overall, the chances of getting identical (or very similar) sequences because of contamination under these circumstances are very low. Hence, we opted for a more in-depth analysis of these sequences with the aim to determine whether they should be flagged as "*COI*-like" sequences or nuclear mitochondrial copies (*numts*), rather than orthologous copies of the mitochondrial *COI* [11,31]. In fact, *numts* were recently identified in odonates of the genera *Leucorrhinia* and *Calopteryx* [13].

Examination of the chromatograms corresponding to the *I. rubilio* specimens sequenced in this study revealed the presence of some double peaks, which the program interpreted as heterozygous positions, and hence, ambiguity-coded bases in the final sequences. Additionally, the chromatograms of all these individuals showed some messy regions, which were difficult to read, and other regions that were more readable (see Figure A5), which is usually the result of the co-amplification of both *numts* and orthologous copies of the *COI* [29]. This, together with the results of the BLAST searches, suggests that these could be non-orthologous copies of the *COI* gene and, therefore, we labelled these sequences as "*COI*-like" for submission to GenBank, and excluded them from further analyses.

An examination of codon usage comparing the odd *COI* sequences of *I. rubilio* from GenBank (MH449981 and MW143324) with the apparently legitimate *COI* sequences of *I. aurora* (MT680688, MG517558 and MH449988) and the same *COI* region extracted from published complete mitochondrial genomes of other *Ischnura* species (*I. elegans* (KU958378 and MK951668), *I. pumilio* (KC878752) and *I. senegalensis* (MT787567)) is shown in Figure A6.

Amino acid composition within the selected *COI* region is identical among the reference *Ischnura* spp. included in the analysis (*I. pumilio*, *I. elegans* and *I. senegalensis*), and very similar to that of *I. aurora* (see Figure A6), whereas the two questionable sequences of *I. rubilio* show some differences in the frequency of some amino acids (e.g., F, G, I, M or S; see Figure A6). These differences in amino acid composition are not expected in a gene such as *COI*, even less in the Folmer region sequenced here, which is highly conserved across species and even across genera [11]. Even though the two *I. rubilio* sequences from GenBank showed no stop codons, the observed differences in amino acid composition

between them and other *Ischnura* legitimate *COI* sequences, coupled with the fact that they appear at the end of a long branch (see Figure A3), all constitute "red flags" that make us question these as true mitochondrial *COI* sequences of *I. rubilio*, and therefore we removed them from our dataset.

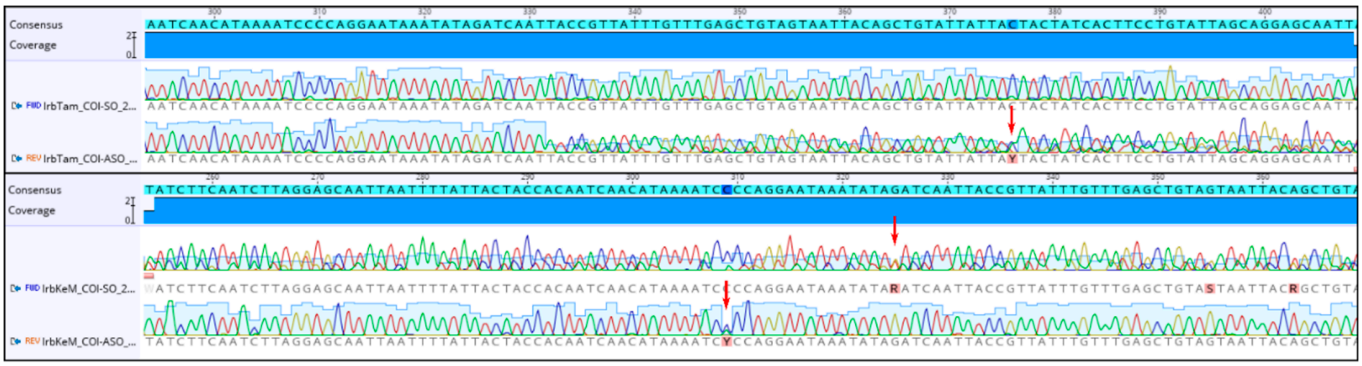
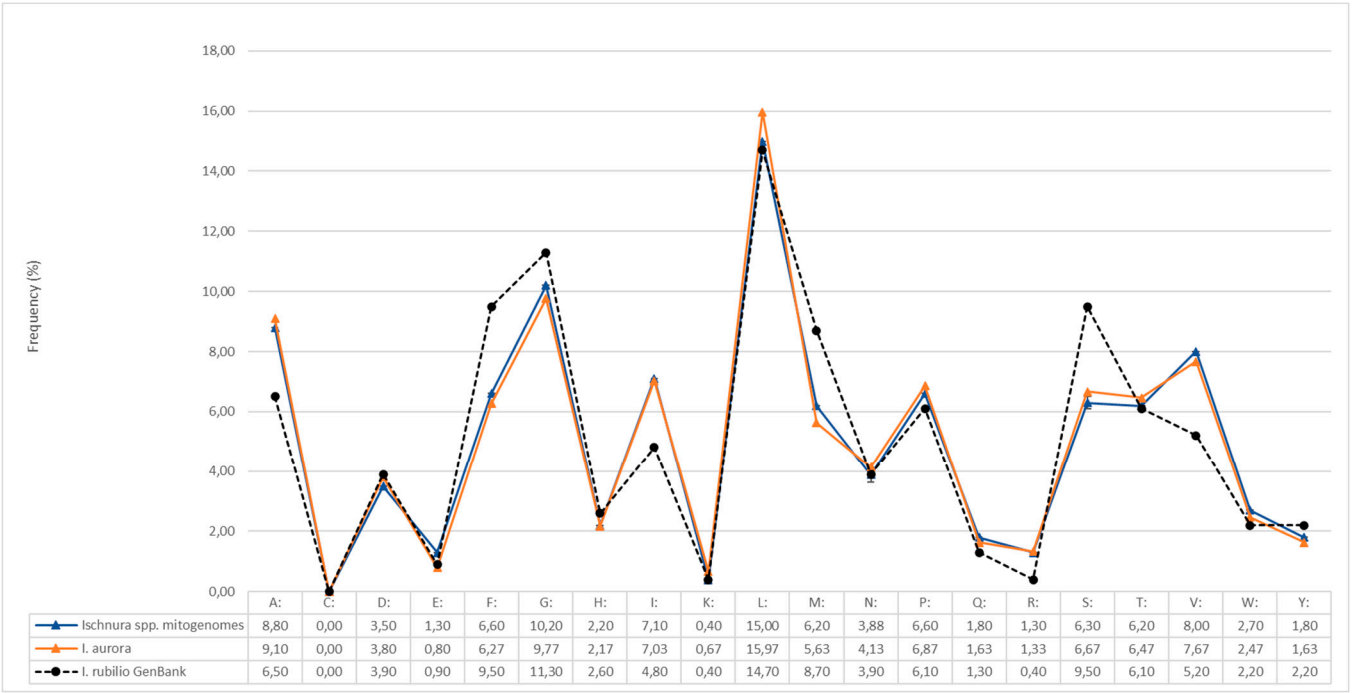


Figure A5. Screenshot of sections of two chromatograms of the *Ischnura rubilio COI* sequences obtained in this study, showing the messy but in some places readable chromatograms. The red arrows point to the double peaks which were interpreted as ambiguity-coded bases by the software.



	A:	C:	D:	E:	F:	G:	H:	I:	K:	L:	M:	N:	P:	Q:	R:	S:	T:	V:	W:	Y:
Ischnura spp. mitogenomes	8,80	0,00	3,50	1,30	6,60	10,20	2,20	7,10	0,40	15,00	6,20	3,88	6,60	1,80	1,30	6,30	6,20	8,00	2,70	1,80
I. aurora	9,10	0,00	3,80	0,80	6,27	9,77	2,17	7,03	0,67	15,97	5,63	4,13	6,87	1,63	1,33	6,67	6,47	7,67	2,47	1,63
I. rubilio GenBank	6,50	0,00	3,90	0,90	9,50	11,30	2,60	4,80	0,40	14,70	8,70	3,90	6,10	1,30	0,40	9,50	6,10	5,20	2,20	2,20

Figure A6. Codon usage analysis for the questionable *COI* sequences of *Ischnura rubilio* downloaded from GenBank (MH449981 and MW143324; black dotted line with circles) and possibly true *COI* sequences of *Ischnura aurora* (orange line with triangles) and reference *COI* sequences extracted from published complete mitogenomes of several *Ischnura* species (blue line with triangles). The codon abbreviations for amino acids on the X axis are standard. The table below the X axis shows the frequency of each amino acid for each set of sequences included in the analysis.

Appendix B

Table A1. Accession numbers for the *COI* and *ITS* sequences selected for genetic analyses after quality control as described in Appendix A. For *Ischnura aurora*, *I. delicata* and *I. rubilio*, we list the information on the clade to which each individual belongs according to the genetic analyses. Highlighted in bold are the individuals that fall outside their expected clades according to the genetic and ASAP analyses (see main text and Figure A7).

Species	Clade	Collection Locality Data	GenBank Acc. Nos	
			COI	*ITS*
Aciagrion migratum	-	Yamashiro, Kyoto, Japan	AB708460	AB706565
Aciagrion pallidum	-	Thailand	MH881302	FN356029
Ceriagrion auranticum	-	Ibusuki, Kagoshima, Japan	AB708472	AB706577
Ceriagrion cerinorubellum	-	Malaysia	LC366789	LC366195
Ceriagrion melanurum	-	Kugunu, Gifu, Japan	AB708477	AB706582
Ceriagrion nipponicum	-	Suita, Osaka, Japan	AB708479	AB706584
Coenagrion ecornutum	-	Onbetsu, Hokkaido, Japan	AB708481	AB706586
Coenagrion hylas	-	Otofuke, Hokkaido, Japan	AB708483	AB706588
Coenagrion lanceolatum	-	Matsumoto, Nagano, Japan	AB708485	AB706590
C. lanceolatum	-	Abashiri, Hokkaido, Japan	AB708489	AB706594
Coenagrion terue	-	Murakami, Niigata, Japan	AB708490	AB706595
Enallagma circulatum	-	Tobetsu, Hokkaido, Japan	AB708491	AB706596
Enallagma cyathigerum	-	Bastemosen, Bornholm, Germany	MN934768	MN963489
E. cyathigerum	-	Slesvig-Holstein, Germany	MN934790	MN963513
Erythromma humerale	-	Ikeda, Hokkaido, Japan	AB708496	AB706601
Ischnura abyssinica	-	Ambo, Ethiopia	MH450002	MH447433
Ischnura asiatica	-	Fuchu, Toyama, Japan	AB708497	AB706602
I. asiatica	-	Tsukuba, Ibaraki, Japan	LC366722	LC366128
Ischnura elegans	-	Ikeda, Hokkaido, Japan	AB708504	AB706609
Ischnura evansi	-	Sarbaz Gorge, Iran	MH450005	MH447425
Ischnura ezoin	-	Anijima, Ogasawara, Tokyo, Japan	AB708467	AB706572
I. ezoin	-	Otoutojima, Ogasawara, Tokyo, Japan	AB708468	AB706573
I. ezoin	-	Mukojima, Ogasawara, Tokyo, Japan	LC366905	LC366311
I. ezoin	-	Mujkojima, Ogasawara, Tokyo, Japan	LC366906	LC366312
Ischnura heterosticta	-	Fiji	AB708507	MH447432
Ischnura nursei	-	Jaipur, India	MH449984	MH447413
Ischnura praematura	-	Yunnan, China	MZ514810	MZ514811
I. praematura	-	Yunnan, China	MZ514812	MZ514813
I. praematura	-	Yunnan, China	MZ514814	MZ514815
I. praematura	-	Yunnan, China	MZ514816	MZ514817
Ischnura pumilio	-	n.a.	MK818664	FN356107
I. pumilio	-	n.a.	MN939053	KC430228
I. pumilio	-	n.a.	MT680681	KC430231
I. pumilio	-	n.a.	NC021617	MH447407

Table A1. *Cont.*

Species	Clade	Collection Locality Data	GenBank Acc. Nos	
			COI	***ITS***
Ischnura rufostigma	-	Thailand	AB708508	AB706613
Ischnura saharensis	-	n.a.	MK818648	MH447432
Ischnura senegalensis	-	Yonaguni, Okinawa, Japan	AB708511	AB706616
Ischnura sp.	-	Yunnan, China	AB708512	AB706617
Ischnura sp.	-	Yunnan, China	AB708513	AB706618
Mortonagrion hirosei	-	Ishinomaki, Miyagi, Japan	AB708515	AB706620
M. selenion	-	Yamada, Toyama, Japan	AB708517	AB706622
Pseudagrion microcephalum	-	Yonaguni, Okinawa, Japan	AB708538	AB706643
Ischnura aurora	Australo-Pacific	New South Wales, Australia	KF369414	n.a.
Ischnura aurora	Australo-Pacific	Queensland, Australia	JF839452	n.a.
I. aurora	Australo-Pacific	Adelaide, Australia	MH449987	MH447412
I. aurora	Australo-Pacific	Perth, Australia	MH449988	MH447411
I. aurora	Australo-Pacific	Australia	MT680686	n.a.
I. aurora	Australo-Pacific	Japan	MT680687	n.a.
I. aurora	Australo-Pacific	American Samoa	MK818649	n.a.
I. aurora	Australo-Pacific	American Samoa	MT680690	n.a.
I. aurora	Australo-Pacific	Tonga	MT680689	n.a.
I. aurora	Australo-Pacific	French Polynesia	MT680688	n.a.
I. aurora	Australo-Pacific	French Polynesia	MT680691	n.a.
I. aurora	Australo-Pacific	Fiji	AB708502	AB706607
I. aurora	Australo-Pacific	Fiji	AB708503	AB706608
I. aurora	Australo-Pacific	Maroe Bay, Huahine island, French Polynesia	KX053530	n.a.
I. aurora	Australo-Pacific	Afareaitu, Moorea island, French Polynesia	KX053529	n.a.
I. aurora	Australo-Pacific	Paopao river, Moorea island, French Polynesia	KX053532	n.a.
I. aurora	Australo-Pacific	Paopao river, Moorea island, French Polynesia	KX053524	n.a.
I. aurora	Australo-Pacific	Pihaena, Moorea island, French Polynesia	KX053528	n.a.
I. aurora	Australo-Pacific	Mount Mauru, Tahiti island, French Polynesia	KX053526	n.a.
I. aurora	Australo-Pacific	Mount Mauru, Tahiti island, French Polynesia	KX053525	n.a.
I. aurora	Australo-Pacific	Wallis and Futuna	n.a.	MH447410
I. aurora	Australo-Pacific	Guam	AB708500	AB706605
I. aurora	Australo-Pacific	Guam	AB708501	AB706606
I. aurora	Australo-Pacific	Iojima, Ogasawara, Tokyo, Japan	AB708498	AB706603
I. aurora	Australo-Pacific	Iojima, Ogasawara, Tokyo, Japan	AB708499	AB706604
I. aurora	Australo-Pacific	Baliem valley New Guinea	MH449995	n.a.
I. aurora	**Australo-Pacific**	**Malappuram, Kerala, India**	**KR149808**	**n.a.**
I. aurora	Asian	Ugani Sahib, Rajpura, Patial (Punjab), India	MG517558	n.a.
I. aurora	Asian	Nakhon Sawan, Thailand	MH450006	MH447414

Table A1. *Cont.*

Species	Clade	Collection Locality Data	GenBank Acc. Nos	
			COI	*ITS*
I. aurora	Asian	Thailand	KT957479	n.a.
I. aurora	Asian	Thailand	KT957480	n.a.
I. aurora	Asian	Thailand	KT957481	n.a.
I. aurora	Asian	Thailand	KT957482	n.a.
I. aurora	Asian	Thailand	KT957483	n.a.
I. aurora	Asian	Thailand	KT957484	n.a.
I. aurora	Asian	Thailand	KT957485	n.a.
I. aurora	Asian	Thailand	KT957486	n.a.
I. aurora	Asian	Thailand	KT957487	n.a.
I. aurora	Asian	Thailand	KT957488	n.a.
I. aurora	Asian	Thailand	KT957489	n.a.
I. aurora	Asian	Thailand	KT957490	n.a.
I. aurora	Asian	Thailand	KT957491	n.a.
I. aurora	Asian	Thailand	KT957492	n.a.
I. aurora	Asian	Thailand	KT957493	n.a.
Ischnura delicata	Asian	Islamabad, Pakistan	KY843451	n.a.
Ischnura rubilio	Asian	India	MN850442	n.a.
I. aurora	**3rd taxonomic unit in ASAP**	**Baliem Valley New Guinea**	**MH449994**	**n.a.**
I. aurora	**Asian? 4th taxonomic unit in ASAP**	**India**	**MT511656**	**n.a.**

Table A2. List of *Ischnura aurora* material belonging to Adolfo Cordero-Rivera's personal collection used for morphological examination (see main text). Listed are the voucher ID, sex and collection details for each specimen. Collector's names are as follows: Adolfo Cordero-Rivera (ACR), Iago Sanmartín-Villar (ISV) and Haomiao Zhang (HZ).

Voucher ID	Sex	Collection Date	Collection Locality
ACR-00738	male	01/12/2013	Long Swamp; Nelson, Victoria, Australia. ACR leg and det.
ACR-00776	female	02/12/2013	Ming Ming Swamp, Grampians National Park, Victoria, Australia. ACR leg and det.
ACR-00793	female	04/12/2013	Ming Ming Swamp, Grampians National Park, Victoria, Australia. ACR leg and det.
ACR-00794	female	04/12/2013	Ming Ming Swamp, Grampians National Park, Victoria, Australia. ACR leg and det.
ACR-00795	female	04/12/2013	Ming Ming Swamp, Grampians National Park, Victoria, Australia. ACR leg and det.
ACR-00818	male	09/12/2013	pond at Bandiana, Wodonga, Victoria, Australia. ACR leg and det.

Table A2. *Cont.*

Voucher ID	Sex	Collection Date	Collection Locality
ACR-00819	male	09/12/2013	pond at Bandiana, Wodonga, Victoria, Australia. ACR leg and det.
ACR-02338	male	08/05/2015	River at Si Fang Jing, Yunnan, China. ISV leg and det.
ACR-02379	male	13/05/2015	River at Xi Meng, Yunnan, China. ISV leg and det.
ACR-02880	male	19/06/2015	Meng Ding, Yunnan China. ISV leg and det.
ACR-02956	male	26/06/2015	Na Bang, Yunnan, China. ISV leg and det.
ACR-02957	female	26/06/2015	Na Bang, Yunnan, China. ISV leg and det.
ACR-03503	male	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03504	male	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03505	male	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03506	male	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03507	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03508	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03509	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03510	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03511	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03513	female	02/07/2015	Rice field at Huaping. Yunnan, China. ISV leg and det.
ACR-03888	male	10/06/2016	Pond in agricultural area. Mengding, Yunnan, China. ACR leg and det.
ACR-03917	female	11/06/2016	Pond in agricultural area. Mengding, Yunnan, China. HZ leg and det.
ACR-03998	female	19/06/2016	River at Meng Lun, Yunnan, China. ACR leg and det.
ACR-04067	male	24/06/2016	Stream at Meng Lung, Yunnan, China. ACR leg and det.
ACR-05007	male	06/06/2018	Somosomo damm, Chakaudrove, Taveuni, Fiji.
ACR-05008	female	06/06/2018	Somosomo damm, Chakaudrove, Taveuni, Fiji.
ACR-05009	male	06/06/2018	Somosomo damm, Chakaudrove, Taveuni, Fiji.
ACR-05010	male	06/06/2018	Somosomo damm, Chakaudrove, Taveuni, Fiji.
ACR-05091	female	11/06/2018	Korovuli, Seqaqa, Labasa, Vanua Levu, Fiji.

Table A3. Evolutionary divergence (uncorrected p-distances) estimated with MEGA X from nuclear (*ITS*, above) and mitochondrial (*COI*, below) DNA sequence data.

	Australo-Pacific *aurora*	**Asian** *aurora*	***I. delicata***	***I. rubilio***	***I. aurora* MH449994**	***I. aurora* MT511656**	***I. abyssinica***	***I. asiatica***	***I. elegans***	***I. evansi***	***I. ezoin***	***I. heterosticta***	***I. nursei***	***I. praematura***	***I. pumilio***	***I. rufostigma***	***I. saharensis***	***I. senegalensis***
Australo-Pacific *aurora*		0.020	n.a.	0.017	n.a.	n.a.	0.081	0.094	0.088	0.101	0.082	0.095	0.116	0.069	0.083	0.087	0.091	0.109
Asian *aurora*	0.031		n.a.	0.001	n.a.	n.a.	0.084	0.094	0.090	0.104	0.079	0.094	0.110	0.070	0.081	0.081	0.097	0.106
I. delicata	0.031	0.000			n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
I. rubilio	0.031	0.000	0.000		n.a.	n.a.	0.041	0.052	0.047	0.052	0.047	0.052	0.064	0.050	0.052	0.041	0.047	0.058
I. aurora MH449994	0.024	0.027	0.027	0.027		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
I. aurora MT511656	0.022	0.013	0.013	0.013	0.022		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
I. abyssinica	0.135	0.126	0.126	0.126	0.131	0.131		0.079	0.036	0.045	0.062	0.057	0.074	0.054	0.069	0.042	0.042	0.066
I. asiatica	0.132	0.128	0.127	0.127	0.132	0.123	0.150		0.079	0.094	0.044	0.084	0.103	0.042	0.040	0.075	0.083	0.101
I. elegans	0.138	0.129	0.129	0.129	0.133	0.124	0.064	0.145		0.036	0.066	0.043	0.068	0.058	0.065	0.053	0.014	0.055
I. evansi	0.131	0.126	0.126	0.126	0.131	0.124	0.058	0.141	0.058		0.079	0.034	0.073	0.065	0.080	0.051	0.038	0.057
I. ezoin	0.130	0.125	0.125	0.125	0.136	0.116	0.149	0.089	0.147	0.145		0.077	0.086	0.026	0.033	0.064	0.076	0.090
I. heterosticta	0.129	0.120	0.120	0.120	0.122	0.120	0.058	0.145	0.069	0.064	0.132		0.074	0.068	0.079	0.060	0.045	0.040
I. nursei	0.134	0.130	0.130	0.130	0.132	0.128	0.087	0.158	0.078	0.070	0.159	0.083		0.080	0.092	0.076	0.075	0.083
I. praematura	0.148	0.144	0.144	0.144	0.144	0.144	0.140	0.112	0.156	0.142	0.118	0.144	0.153		0.030	0.055	0.064	0.083
I. pumilio	0.150	0.154	0.154	0.154	0.161	0.154	0.160	0.125	0.147	0.148	0.115	0.154	0.148	0.129		0.070	0.075	0.092
I. rufostigma	0.122	0.113	0.113	0.113	0.118	0.109	0.078	0.127	0.069	0.071	0.136	0.078	0.090	0.138	0.154		0.056	0.073
I. saharensis	0.134	0.137	0.137	0.137	0.137	0.137	0.069	0.154	0.035	0.062	0.155	0.080	0.094	0.149	0.147	0.075		0.061
I. senegalensis	0.131	0.133	0.133	0.133	0.129	0.133	0.069	0.145	0.084	0.078	0.154	0.051	0.096	0.136	0.174	0.082	0.086	

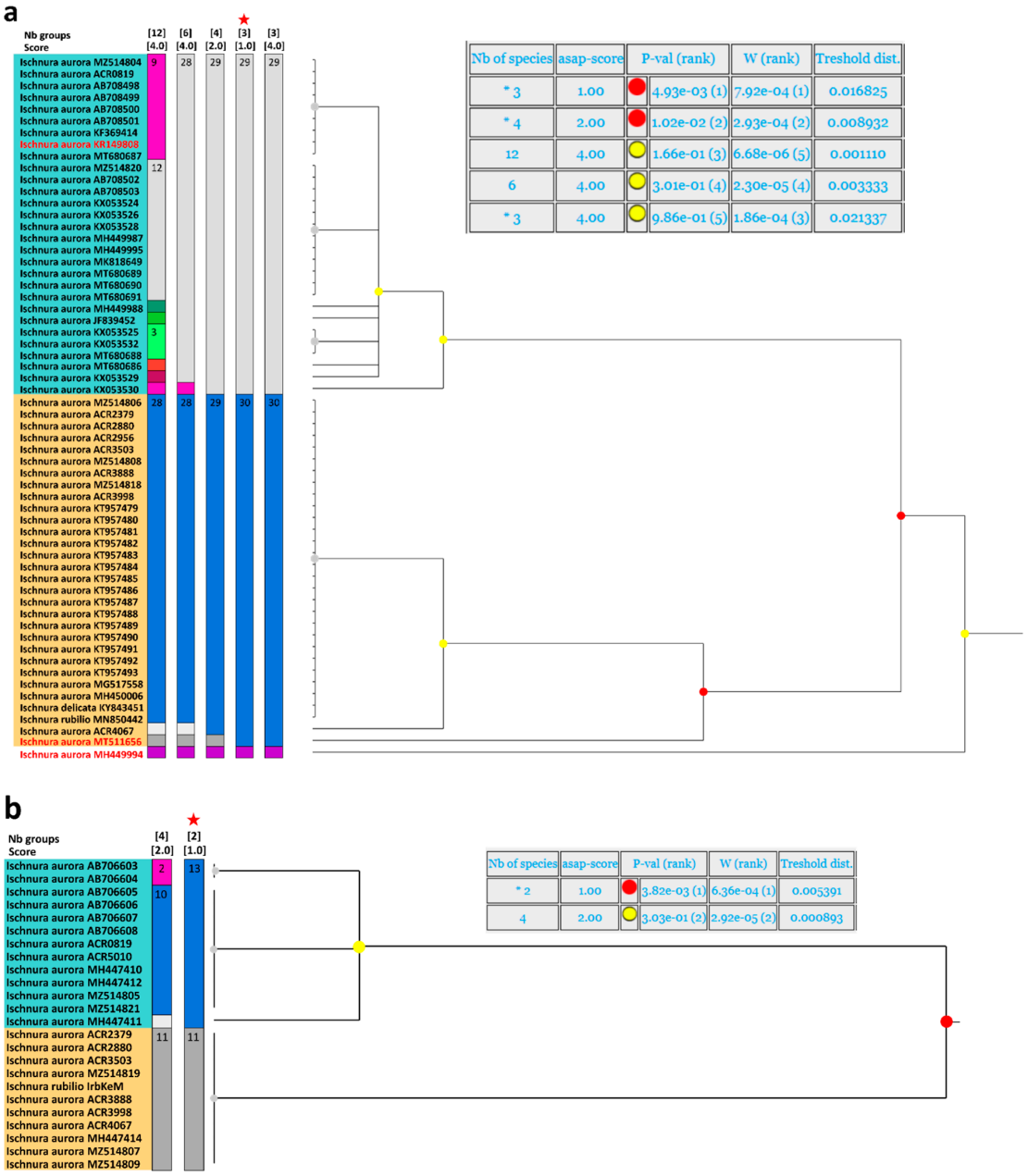


Figure A7. Graphical output of ASAP, showing the results of the species delimitation analysis using the *Ischnura aurora*; *I. delicata* and *I. rubilio* COI sequences and (**b**) the *I. aurora* and *I. rubilio* ITS sequences. Each node of the dendrogram is coloured depending on its probability of being a panmictic species: darker colours for nodes that may be split into smaller groups. The tables show the best partitions found by ASAP in each case, scored and sorted by their *p*-value (the smallest p-value has rank 1) and their rank of relative barcode gap width (the largest gap has rank 1). The asap-score is the average of both ranks: the smaller the asap-score, the better. The predicted number of groups in each partition, together with their asap-score, are also indicated in the dendrograms, with the red star indicating the best partition according to the analysis in each case. Australo-Pacific and Asian clades are highlighted with the same colours used in Figure 2 in the main text. In (**a**), the individual highlighted in red inside the Australo-Pacific clade corresponds to a specimen of *I. aurora* from India. The individuals highlighted in red at the bottom of the dendrogram correspond to the specimens of *I. aurora* from India and New Guinea with COI sequences that are ~2% divergent from the rest of the sequences included in the analyses and identified as belonging to a different species in the ASAP analysis.

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