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Rhapsody in emerald: phylogenetic framework for Lestidae with reference to the systematic position of *Chalcolestes* Kennedy

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Abstract. We reconstruct a phylogenetic framework for the zygopteran family Lestidae based on a molecular dataset comprised of sequence data from the genes COI, 16S, 18S, 28S, and ITS1+2 from 41 ingroup taxa and 8 outgroup taxa with emphasis on the systematic position of the genus *Chalcolestes* Kennedy. We recover Lestidae as monophyletic with good statistical support. The family falls into two subequal clades. One, comprising the genus *Sympecma* Burmeister and *Lestes* Leach sensu lato (including the genus *Archilestes* Selys) is poorly to moderately supported. While the other, comprising the genera *Austrolestes* Tillyard, *Indolestes* Fraser, *Orolestes* McLachlan, and *Chalcolestes* is strongly supported. *Chalcolestes* is recovered as sister to the Oriental genus *Orolestes* with strong support. Our results thus support that *Chalcolestes* is a valid genus not closely related to *Lestes*. Monophyly of *Lestes* requires inclusion of the New World genus *Archilestes*, and our results support the need for a thorough revision of *Lestes*.

Key words. Archilestes, Austrolestes, Lestes, Orolestes, molecular phylogeny

Research Article

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Introduction

Lestidae or emerald damselflies are a small, cosmopolitan zygopteran family comprising nine recognised genera with approximately 150 species (Bridges, 1994; Dijkstra & Kalkman, 2012; Vajda et al., 2018; Paulson & Schorr, 2021). Many—but certainly not all—are metallic green (Figure 1) and often rest with the left and right wings held at approximately 45 degrees to each other providing the basis for the vernacular names 'emerald damselflies' or 'spread-winged damselflies'. More than half of the species are currently placed in the genus *Lestes* Leach, 1815 (Paulson & Schorr, 2021), a heterogeneous genus in dire need of a phylogenetically based revision (Dijkstra & Kalkman, 2012). To date the only higher-level systematic arrangement proposed for the family has been the division of the family into two subfamilies, viz. Lestinae and Sympecmatinae (Fraser, 1951), with the former comprising the genera *Lestes*, *Archilestes* Selys, 1862, *Orolestes* McLachlan, 1895, *Perilestes* Hagen, 1862, *Sinhalestes* Fraser, 1951, and *Chalcolestes* Kennedy, 1920; and the latter comprising *Sympecma* Burmeister, 1839, *Austrolestes* Tillyard, 1913, and *Indolestes* Fraser, 1922. However, many authors (e.g. Rehn, 2003; Bybee et al., 2008; Carle et al., 2008; Dumont et al., 2010; Dijkstra & Kalkman, 2012; Dijkstra et al., 2013; Kim et al., 2014) do not follow this subfamily arrangement, and Djikstra et al. (2014) found that while *Austrolestes* and *Indolestes* came out as sister groups in their analyses, they were not closely related to *Sympecma*. While there has been no

comprehensive phylogenetic analysis of Lestidae, some of the studies above—all focused on higher-level Odonata or Zygoptera relationships—have included several members of Lestidae. Rehn (2003) found that *Sympecma* is the sister group to a clade comprising *Archilestes*, *Lestes*, and *Austrolestes* based on morphology, while Bybee et al. (2008) recovered the conflicting result that *Austrolestes* is the sister to a clade comprising *Sympecma*, *Lestes*, and *Archilestes* based on DNA. In a molecular study, Dumont et al. (2010) found that *Sympecma* is sister to a clade comprising *Indolestes* and *Chalcolestes*, while *Lestes* sensu lato emerged as paraphyletic with respect to all other Lestidae included in the analyses. Kim et al. (2014) were the first to include *Indolestes* in a molecular phylogeny and found it to be sister to *Sympecma*, but they only included *Lestes* as a third representative of Lestidae. Dijkstra et al. (2014) in their molecular phylogeny of Zygoptera included *Indolestes*, *Austrolestes*, *Sympecma*, and six species of *Lestes*. They found that *Sympecma* is the sister to a monophyletic *Lestes*, and that clade is the sister to a clade comprising *Indolestes* and *Austrolestes*. Bybee et al. (2021) in the first comprehensive phylogenomic analysis of Odonata found that *Indolestes* and *Austrolestes* are sisters, and *Orolestes* is the sister to that clade, with *Sympecma* being the sister to *Orolestes* + (*Indolestes* + *Austrolestes*). The sister to that clade is a clade comprising *Lestes* and *Archilestes*.

Chalcolestes is one of the smallest genera in the family, comprising the two Palaearctic species *C. viridis* (Vander Linden, 1825) and *C. parvidens* (Artobelevski, 1929). It was separated from *Lestes* by Kennedy (1920) based on the shape of the nymphal prementum, which is broad in *Chalcolestes* and narrow in *Lestes* (e.g. Gyulavári et al., 2011). *Chalcolestes* further differs from *Lestes* in having a unique egg-laying behaviour where the female oviposits in branches overhanging waterbodies (e.g. Askew, 2004; Gyulavári et al., 2011). However, *Chalcolestes* is not universally accepted as a separate genus and some works (e.g. Askew, 2004; Smallshire & Swash 2010) still list *viridis* and *parvidens* under *Lestes*. Only Dumont et al. (2010) among the higher-level phylogenetic studies of Zygoptera included *Chalcolestes* in their dataset, and

as mentioned above, they did not find evidence for a close relationship between *Chalcolestes* and *Lestes*. This was confirmed by Gyulavári et al. (2011) based on DNA, and by Vajda et al. (2018) based on adult morphology, although these studies included only European species and were thus restricted to the three genera *Lestes*, *Chalcolestes*, and *Sympecma*.

To clarify the systematic position and taxonomic status of *Chalcolestes* we here analyse the phylogenetic relationships of 39 species of Lestidae representing seven of the recognised nine genera based on DNA sequences from the mitochondrial genes COI and 16S, and the nuclear genes ITS1+2, 5.8S, 18S, 28S, and EF1-α. We further address the phylogenetic position of *Sympecma*, and test the monophyly of *Lestes*.

Material and methods
Taxon sampling

The total dataset is comprised of 41 ingroup and eight outgroup taxa (Supplementary material S1). Our sampling of Lestidae includes representatives of the genera *Archilestes*, *Austrolestes*, *Chalcolestes*, *Indolestes*, *Lestes*, *Orolestes*, and *Sympecma*, with most specimens belonging to *Lestes*. Our outgroup sampling includes the lestoid genera *Megalestes* Selys, 1862, *Nubiolestes* Fraser, 1945, *Perilestes* Hagen, 1862, *Perissolestes* Kennedy, 1941, and *Synlestes* Selys, 1868, as well as *Calopteryx* Leach, 1815, *Enallagma* Charpentier, 1840, and *Platycnemis* Burmeister, 1839 (all Zygoptera). The data set consists of a mixture of sequences from GenBank and BOLD (Barcode of Life Data System) combined with new sequences produced for the present study (see Supplementary material S1 for further details).

DNA extraction was done as described in Simonsen et al. (2020) as was sequencing of the COI barcode fragment and ITS1+2 (comprised of ITS1, 5.8S and ITS2). The 28S D2 loop was amplified using the PCR protocol of Simonsen et al. (2020) and the primers D2-F2 (with universal tail): **TGTAAAACGACGGCCAGT**GGTTGCTTGAGA-GTGCAGCCC (tail in boldface) and D2-R2 (with universal
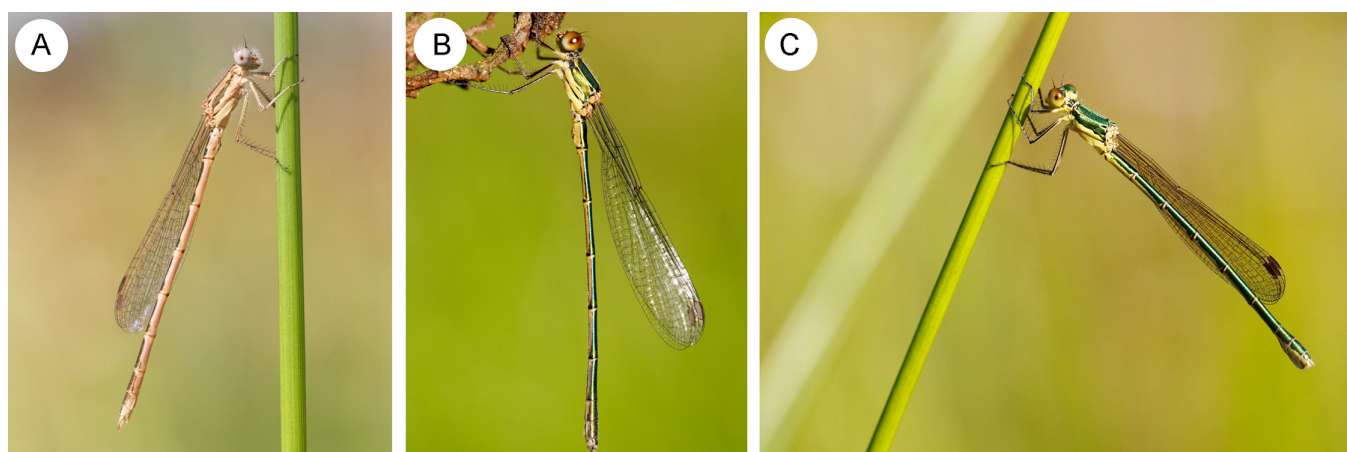
Figure 1. Examples of adult diversity in Lestidae. (A) *Sympecma fusca*; (B) *Chalcolestes viridis*; (C) *Lestes virens* (photo: Kent Olsen).

tail): **CAGGAAACAGCTATGAC**CATGTTAGACTCCTTG-GTCCG (tail in boldface). Both primers were developed for this study. Samples were sequenced at Macrogen Europe using the Sanger method. Contigs and consensus sequences were produced in DNA Baser Sequence Assembler v5.8.0 (Heracle Biosoft, 2018). The identity of all sequences were checked using BLAST on GenBank and/or BOLD Identification System.

Sequences were aligned in MEGA X (Kumar et al., 2018) using the implemented Muscle algorithm. Alignments were subsequently checked by eye in Mesquite v. 3.03 (build 702; Maddison & Maddison, 2015) and the alignment of ITS1+2 was manually corrected.

The combined aligned dataset is comprised of 6430 bp for a maximum of 658 bp COI, 553 bp 16S, 1839 bp 28S, 1979 bp 18S, and 1401 bp ITS1+2.

A full list of species and GenBank numbers for each sequence is provided in Supplementary material S1.

We partitioned the data set by gene with ITS1+2 treated as a single gene, resulting in five partitions. We performed Bayesian Inference analysis in MrBayes 3.2 (Ronquist et al., 2012) using model jumping with a gamma model for variation across sites (nst = mixed rates = gamma). Model jumping is a recommended alternative to *a priori* model testing as it allows MrBayes to sample across the entire GTR model space (Ronquist et al., 2012). The analysis was run for 10 million generations with sampling every 1000 generations and burninfrac set to 0.5.

We performed Maximum Likelihood analyses in Garli 2.01 (Zwickl, 2006) on Cipres XCEDE (Miller et al., 2010). We used the GTR submodel with the highest posterior probability from the MrBayes analysis for each partition. Models and the overall rate (subsetspecificrates) were unlinked across partitions. We specified *Calopteryx splendens* (Harris, 1782) as outgroup. Ten independent runs of the analysis were done and the analyses were terminated after 20,000 generations without significant change of topology. A majority rule consensus tree was produced in Mesquite. The Bootstrap analysis was run with 1000 bootstrap repetitions and one search repetition per bootstrap repetition, settings otherwise as above. Bootstrap values were calculated in Mesquite.

The full dataset for the MrBayes analysis is provided in the NEXUS format as Supplementary material S2. The ML majority rule consensus tree and bootstrap tree are provided in Supplementary material S3–S4.

Results

The Bayesian phylogram from MrBayes and the consensus tree from the ten ML analyses in Garli are remarkably similar with only two minor differences, which are discussed below. The phylogram from the MrBayes analysis with posterior probabilities and ML bootstrap values is shown in Figure 2. The ML trees from the Garli analyses are provided in Supplementary materials S3–S4.

Lestidae are monophyletic and well supported (BS = 95, PP = 1) in both sets of analyses. The family may be divided into two monophyletic subgroups, hereafter termed Group 1 and Group 2. Group 1 is strongly supported (BS = 89, PP = 1) and comprised of *Chalcolestes*, *Orolestes*, *Austrolestes*, and *Indolestes*. Group 1 may be further subdivided into two groups, one comprising *Chalcolestes* and *Orolestes*, and one comprising *Austrolestes* and *Indolestes*. The sister-group relationship between *Chalcolestes* and *Orolestes* is strongly supported (BS = 98, PP = 1), and each genus is monophyletic with strong support (BS = 100, PP = 1 for both). The group comprising *Austrolestes* and *Indolestes* is well supported (BS = 91, PP = 1), but the position of *A. colensonis* (White, 1846) differs between the Bayesian and ML analyses. In the Bayesian analysis *A. colensonis* emerges in a trichotomy at the base of *Indolestes*. In the ML analyses *A. colensonis* is the sister to the remaining *Austrolestes*, and *Indolestes* is the sister group to *Austrolestes*. However, both arrangements are poorly supported. Group 2 is comprised of *Sympecma*, *Archilestes*, and *Lestes* sensu lato, with *Sympecma* being the sister group to a large clade comprising *Archilestes* and *Lestes* sensu lato. Group 2 does not receive a BS above 50 and is therefore not recovered in the ML bootstrap tree, but it does receive a moderate support in the Bayesian analysis (PP = 0.88). *Sympecma* is monophyletic and strongly supported (BS = 100, PP = 1). *Lestes* sensu lato is paraphyletic with respect to *Archilestes*, but the group comprising the two genera is moderately to well supported (BS = 74, PP = 0.98). Within this group the two species *L. pallidus* Rambur, 1842 and *L. pinheyi* Fraser, 1955 form a moderately to well supported (BS = 60 PP = 97) sister group to a poorly supported (BS < 50, PP = 0.77) group comprising the remaining species. Within the latter group, the three species *L. dissimulans* Fraser, 1955, *L. praemorsus* Hagen, 1862 and *L. umbrinus* Selys, 1891 form a well-supported (BS = 77, PP = 1) group that is sister to a poorly to moderately supported (BS = 53, PP = 0.87) group comprising *Archilestes* and the remaining *Lestes*, which we term *Lestes* sensu stricto. Finally, *Archilestes*, represented by *A. grandis* (Rambur, 1842), is the sister to *Lestes* sensu stricto, with the latter being poorly to moderately well supported (BS = 53, PP = 0.87). We discuss the internal relationships of *Lestes* sensu stricto below, but note that the two sets of analyses differ only with respect to the relationships between *Lestes numidicus* Samraoui, Weekers & Dumont, 2003 and the two subspecies of *Lestes virens*, viz. *L. virens virens* Charpentier, 1825 and *L. virens vestalis* Rambur, 1842. In the Bayesian analysis *L. virens* is monophyletic, while in the ML analyses *L. virens vestalis* is sister to a clade comprising *L. virens virens* and *L. numidicus*.

Discussion
Phylogenetic topology

Our overall results are similar to those of Dijkstra et al. (2014), where a clade comprising *Indolestes* + *Austro-*

lestes was recovered as a sister group to *Sympecma* + *Lestes* sensu lato. In contrast to this Dumont et al. (2010), Kim et al. (2014), and Bybee et al. (2021) all found *Sympecma* to be closely related to a clade comprising *Indolestes* and—depending on taxon sampling—*Austrolestes, Orolestes*, and *Chalcolestes*. While the sister-group relationship between *Sympecma* and *Lestes* sensu lato is well supported in Djikstra et al. (2014), it is only moderately supported in our Bayesian analysis, and not supported at all in our ML analyses. The clade comprising *Chalcolestes*, *Orolestes*, *Austrolestes*, and *Indolestes* is well supported here and in agreement with previous studies. A clade comprising *Indolestes* and *Austrolestes* was also found by Dijkstra et al. (2014) and Bybee et al. (2021). We consistently recover only *Indolestes* as monophyletic, but our taxon and character sampling is too limited to provide a firm conclusion on *Austrolestes*.

We recover *Lestes* sensu lato including *Archilestes* as monophyletic with good support in both the Bayesian and ML analyses. This is in agreement with Djikstra et al. (2014), but in conflict with Dumont et al. (2010), who recovered *Lestes pinheyi* as the sister to the re-

maining Lestidae. Similar to Dijkstra et al. (2014) we recover a clade comprising *L. pinheyi* and *L. pallidus* as sister group to the remaining *Lestes* sensu lato. Unlike previous studies we find that *Lestes* in the traditional circumscription is paraphyletic, because *Archilestes* is the sister taxon to a subset of *Lestes*, the latter is here termed *Lestes* sensu stricto. Although our taxon sampling within *Lestes* sensu lato is limited to less than half the species currently placed in the genus (Dijkstra & Kalkman, 2012; Paulson & Schorr, 2021), the results clearly support the statement by Dijkstra & Kalkman (2012) that *Lestes* is in dire need of a phylogeny-based revision, and we further suggest that *Archilestes* should be included in such a revision.

Our results support Dijkstra et al. (2013, 2014) in that there is little support for retaining subfamily division within Lestidae, and we find in particular no support for Sympecmatinae comprising *Sympecma*, *Austrolestes*, and *Indolestes*. While there may be support for a subfamily division into three subfamilies, with one comprising *Lestes* sensu lato, one comprising *Sympecma*, and one comprising *Chalcolestes*, *Orolestes*, *Indolestes*, and *Austrolestes*, we consider our taxon sampling too
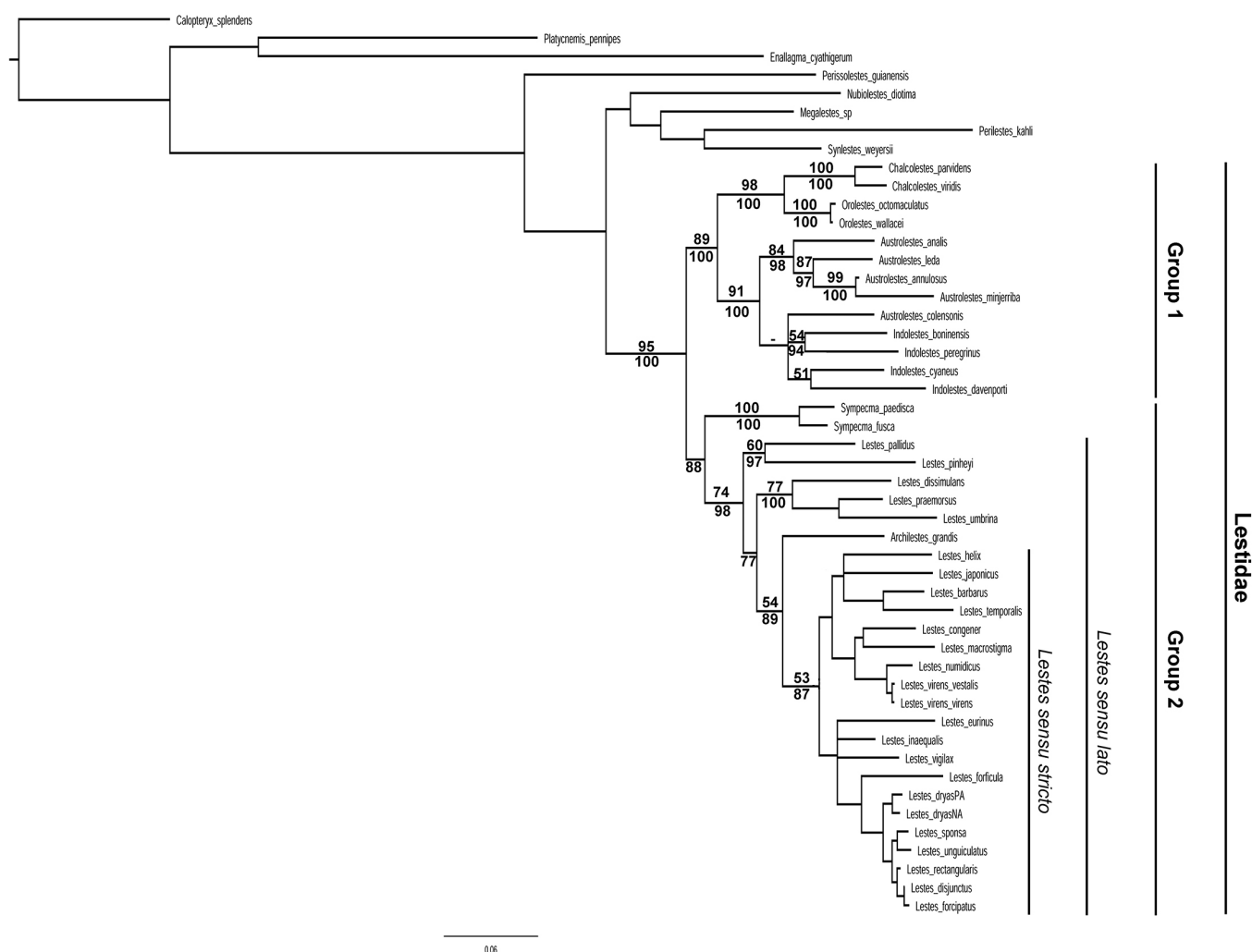

Figure 2. Phylogram from the 10 million generation Bayesian analysis of the combined dataset. Numbers above a line indicate ML bootstrap values ≥ 50, while numbers below a line indicate posterior probabilities ≥ 75. A dash (-) indicates that the group was not recovered in the ML analyses (internal values for *Lestes sensu stricto* not provided).

limited to propose such an arrangement as we have not been able to include the genera *Platylestes* and *Sinhalestes* in our dataset.

Position of Chalcolestes

Our results support recent conclusions by Dumont et al. (2010), Gyulavári et al. (2011), Dijkstra and Kalkman (2012), and Vajda et al. (2018) that *Chalcolestes* is a valid genus and should not be considered a synonym of *Lestes*. Gyulavári et al. (2011) analysed COI and ITS sequences from several specimens of *C. viridis* and *C. parvidens*, as well as *Sympecma fusca* and six taxa of European *Lestes* spp. in two separate analyses. In both analyses they found that *Chalcolestes* and *Lestes* are separated by *Sympecma*. However, as they did not analyse a combined dataset and only included European species, their results provide little information on the phylogenetic position of *Chalcolestes*. More importantly, their datasets did not include any non-Lestidae outgroup taxa and if the trees they presented (Gyulavári et al., 2011, fig. 3) are rooted on *Sympecma fusca* (Vander Linden, 1820), *Chalcolestes* and the included representatives of *Lestes* would appear to be sister groups. Even though Vajda et al. (2018) could not conclusively separate *Chalcolestes*, *Lestes*, and *Sympecma* in their statistical morphometric analyses of male morphology, they concluded that differences in the male secondary genitalia structures support full genus status for *Chalcolestes*. Furthermore, they mentioned that female genitalia morphology also supports this, as *Chalcolestes viridis* has two spermathecae, *Sympecma fusca* one spermatheca, and at least *Lestes barberus* (Fabricius, 1798) and *Lestes virens* have no spermatheca (Vajda et al., 2018, p. 254). As mentioned above, Dumont et al. (2010) is the only higher-level phylogenetic study that includes *Chalcolestes*. Their results are similar to ours as *Chalcolestes* is the well-supported sister group to *Indolestes*. Our taxon sampling within Lestidae is broader than that of Dumont et al. (2010) as we include the genera *Orolestes* and *Austrolestes*. We find that *Indolestes* is placed in a strongly supported monophyletic group with *Austrolestes*, and that *Chalcolestes* is placed in a strongly supported monophyletic group with *Orolestes*. As these two groups together form a strongly supported monophyletic group (Group 1), our results are compatible with Dumont et al. (2010), and strongly support full genus status for *Chalcolestes*. Interestingly, *Chalcolestes* is entirely restricted to the Western and Central Palaearctic (Boudot & Duatlova, 2015; Boudot & Willigalla, 2015), while the other three genera are found in Southeast Asia (*Orolestes*), Australia (*Austrolestes*), or from India to Japan and Australia (*Indolestes*) (GBIF.org, 24 September 2021). As our dataset does not include the genera *Platylestes* (India, Southeast Asia) and *Sinhalestes* (Sri Lanka) (GBIF.org, 24 September 2021), we refrain from making any biogeographical conclusions but note that *Chalcolestes* may represent

a dispersal into Central and Western Palaearctic by an otherwise Oriental-Australian group.

Conclusions and further directions

Three main conclusions can be drawn from our results despite the somewhat limited taxon sampling. First, there is no phylogenetic support for dividing Lestidae into the subfamilies Lestinae and Sympecmatinae. Second, *Lestes* as currently defined is almost certainly not a monophyletic group, and we agree with Dijkstra and Kalkman (2012) that a phylogenetic revision of the genus is much needed. Third, *Chalcolestes* is a valid genus that is not closely related to other European genera of Lestidae—neither *Lestes* nor *Sympecma*. Instead, the genus is most likely the sister group to *Orolestes* and placed in a clade with otherwise Oriental-Australian genera.

Other than the much-needed revision of *Lestes* sensu lato, several high profile aspects of Lestidae phylogeny and systematics remain to be solved. The relationship and delimitation of *Austrolestes* and *Indolestes* remain unclear, and the two genera in combination should be the subject of a phylogenetic taxonomic revision. The higher-level phylogeny of Lestidae should be the subject of phylogenomic analyses in the mould of Bybee et al. (2021) and include also *Platylestes* and *Sinhalestes*. Such a study should address the phylogenetic position of *Sympecma*, identify natural divisions that can be used for a subfamily and tribal classification, and resolve the biogeography of the family including the geographical origin of *Chalcolestes*.

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Supplementary material

Supplementary material S1. List of taxa and GenBank accession numbers included in the study.

Supplementary material S2. NEXUS file used in the 10 million generation analysis in MrBayes.

Supplementary material S3. Majority Rule consensus tree from 10 ML analyses in Garli.

Supplementary material S4. Majority Rule bootstrap tree from 1000 random replications in Garli.