Special Issue: Fossil and Modern Clam Shrimp (Branchiopoda: Spinicaudata, Laevicaudata) Phylogeny and Biogeography of Spinicaudata (Crustacea: Branchiopoda)

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Phylogeny and Biogeography of Spinicaudata (Crustacea: Branchiopoda)

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Spinicaudata (spiny clam shrimp) is a taxon of Branchiopoda occurring since the Devonian and today it occurs nearly globally in temporary water bodies. We present the most species-rich phylogenetic analyses of this taxon based on four molecular loci: *COI*, *16S rRNA*, *EF1α* and *28S rRNA*. Our results support previous findings that Cyzicidae *sensu lato* is paraphyletic. To render Cyzicidae monophyletic we establish a fourth extant spinicaudatan family to accommodate *Eocyzicus*. Within Cyzicidae, none of the genera *Cyzicus*, *Caenestheria* or *Caenestheriella* are monophyletic, and the morphological characters used to define these genera (condyle length and rostrum shape) are not associated with well-delimited clades within Cyzicidae. There is insufficient resolution to elucidate the relationships within Leptestheriidae. However, there is sufficient evidence to show that the leptestheriid genera *Eoleptestheria* and *Leptestheria* are non-monophyletic, and there is no support for the genus *Leptestheriella*. Molecular clock analyses suggest that the wide geographic distribution of many spinicaudatan taxa across multiple continents is largely based on vicariance associated with the break-up of Pangea and Gondwana. Trans-oceanic dispersal has occurred in some taxa (*e.g.*, *Eulimnadia* and within Leptestheriidae) but has been relatively rare. Our results highlight the need to revise the taxonomy of Cyzicidae and Leptestheriidae and provide evidence that the global spinicaudatan diversity may be underestimated due to the presence of numerous cryptic species. We establish Eocyzicidae fam. nov. to accommodate the genus *Eocyzicus*. Consequently, Cyzicidae comprises only two genera – *Cyzicus* and *Ozestheria*. *Ozestheria* occurs also in Africa and Asia and *Ozestheria pilosa* new comb. is assigned to this genus.

Key words: Clam shrimp, *Caenestheria*, *Caenestheriella*, Eocyzicidae, Gondwana, Vicariance.

BACKGROUND

Spinicaudata (spiny clam shrimp) has the most confused taxonomy of any branchiopod group due to the tremendous morphological intraspecific variability, which often overlaps with what has been considered interspecific variation, a high number of hermaphroditic lineages, and poor and inadequate descriptions and type

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material. Daday generated the first monographs for the group and he described and defined most of the primary extant genera and families we recognize today (Daday 1913a b 1914 1915 1923 1925 1926): Limnadiidae Burmeister, 1843, Cyzicidae Stebbing, 1810 (= Caenestheriidae Daday, 1913), and Leptestheriidae Stebbing, 1902. Daday's (1925 1926) concept of Limnadiidae was based primarily upon the form of the head, containing all genera lacking fornices (Fig. 1). This included three genera: *Limnadia* Brongniart, 1820, *Eulimnadia* Packard, 1874, and *Limnadopsis* Spencer and Hall, 1862. In Cyzicidae, Daday (1914) included all genera with a fornix but lacking the "lamina epipoditalis", a triangular lobe on the epipodite of the limbs. In his Cyzicidae concept, Daday recognised *Caenestheria* Daday, 1913, *Eocyzicus* Daday, 1913, *Caenestheriella* Daday, 1913, and *Cyzicus* Audouin, 1837. In Leptestheriidae, Daday (1923) placed all genera bearing the "lamina epipoditalis" and a fornix, which encompassed the genera *Eoleptestheria* Daday, 1913, *Leptestheria* Sars, 1898, and *Leptestheriella* Daday, 1913.

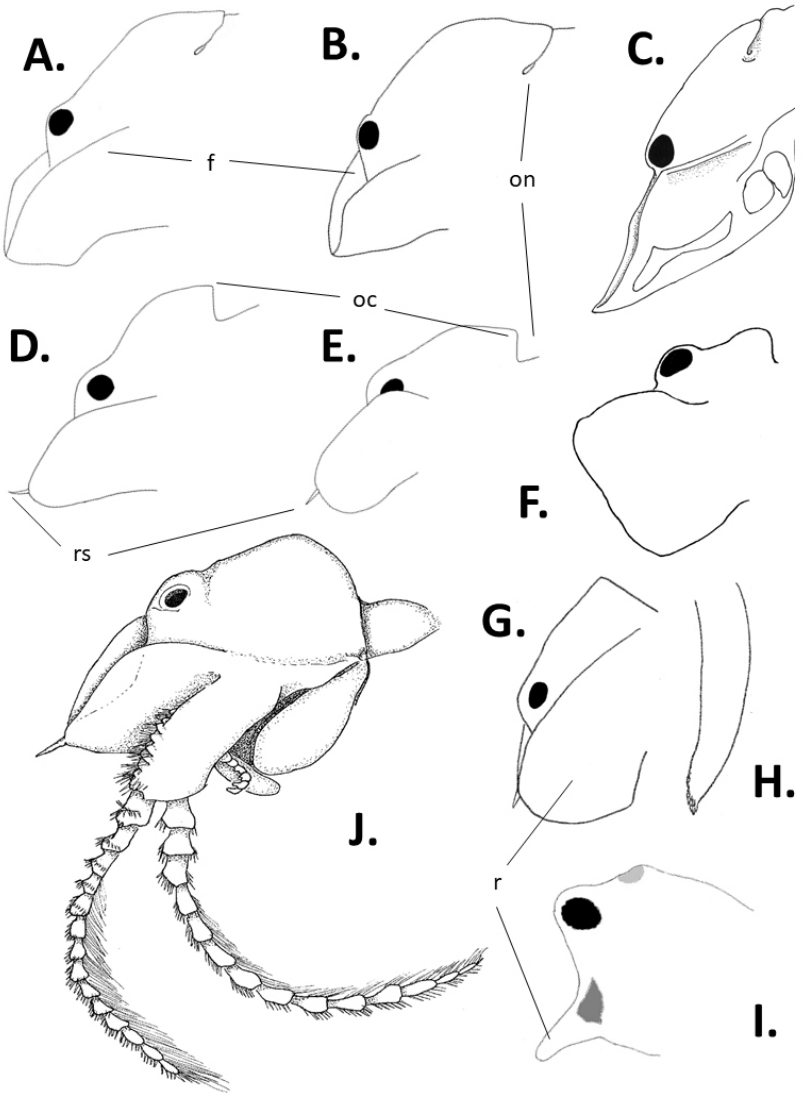

Fig. 1. Figure 1. Representative Spinicaudata and their typical head shapes. A) *Ozestheria altus* Shu et al., 2015, male head left lateral view; B) *Cyzicus californicus* (Packard, 1874), female head left lateral view; C) *Ozestheria pilosa* (Rogers et al., 2013), male head left lateral view; D) *Leptestheria kunmingensis* Shu et al., 2015, male head left lateral view; E) *L. kunmingensis* Shu, et al., 2015, female head left lateral view; F) *Eocyzicus taiwanensis* Rogers et al., 2017, male head left lateral view; G) *Ozestheria* sp. "Mongolia", DCR collection 729, male head left lateral view; H) *Ozestheria* sp. "Mongolia", DCR collection 729, male limb I endopod distal portion, right lateral view; I) *Metalimnadia* sp. DCR collection 853, male head left lateral view; J) *Eoleptestheria* cf. *ticinensis* from Australia, male head left lateral view. Designations: f = fornix; on = occipital notch; oc = occipital condyle; rs = rostral spine; r = rostrum.

Daday's (1913a b 1914 1915 1923 1925 1926) monographs were criticized by many workers (*e.g.*, Uéno 1927; Barnard 1929; Brehm 1933; Gauthier 1933; Linder 1945; Botnariuc 1945 1947; Margalef 1953; Straškraba 1965a 1965b 1966), and many authors (Vecchi 1922; Gauthier 1933; Linder 1945; Botnariuc 1945 1947; Straškraba 1965a 1965b 1966; Wiltshire 1973; Marinček and Petrov 1985; Rogers et al. 2012 2017) demonstrated that certain characters used to define spinicaudatan genera and species were age or food quality dependent.

The genera *Caenestheria* and *Caenestheriella*, created by Daday (1913a), have been particularly contentious. *Caenestheria* and *Caenestheriella* were separated by Daday (1914) from *Eocyzicus* and *Cyzicus*, respectively, by the shape of the rostrum. In the former genera, the rostrum was subacute or triangular in both sexes, in the latter two genera the male rostrum was broadly spatulate. Furthermore, *Cyzicus* and *Caenestheriella* have a long, elongated occipital condyle with a narrow occipital notch, whereas *Eocyzicus* and *Caenestheria* have a short, rounded condyle forming a wide occipital notch. However, several authors synonymised *Caenestheria* into *Eocyzicus* and *Caenestheriella* into *Cyzicus*, based on developmental studies (*e.g.*, Margalef 1953; Straškraba 1965b; Wiltshire 1973; Rogers et al. 2017), demonstrating that the form of the male rostrum changed with the age of the animal. Regardless of the literature demonstrating that the definitions for these genera were problematic, the names *Caenestheria* and *Caenestheriella* continued in use (*e.g.*, Tiwari 1962; Smith and Gola 2001; Timms and Richter 2002; Stoicescu 2004; Olesen and Timms 2005; Richter and Timms 2005; Schmidt and Kiviat 2007). Furthermore, Naganawa (2001) treats *Eocyzicus* as a synonym of *Cyzicus*, with no explanation or citations, an approach not followed by subsequent authors.

In Leptestheriidae, Daday's genus *Leptestheriella* was synonymised with *Leptestheria* by Brtek (1997). based on "… a series of changes between the two groups." However, Brtek (1997) presented no citations, data, or explanation for this conclusion, and listed no examined material or any experiments in support.

Molecular phylogenetic studies of Spinicaudata have consistently rejected the monophyly of Cyzicidae (Schwentner et al. 2009 2018; Sun et al. 2011; Weeks et al. 2009), with *Eocyzicus* (not including species originally assigned to *Caenestheria* or *Ozestheria*) being more closely related to Leptestheriidae and possibly Limnadiidae rather than with the remaining Cyzicidae (Cyzicidae *sensu stricto*). The species included in Cyzicidae s.s. did not cluster into the respective traditional genera but fell into two large clades. One clade included all Australian representatives that morphologically resembled *Caenestheria* and *Caenestheriella* but Schwentner et al. (2015a) showed that the two genera are not monophyletic when including the Australian representatives. The other clade included species that morphologically represented *Caenestheriella* and *Cyzicus* from North America, Japan and Europe (Schwentner et al. 2009; Weeks et al. 2009). These results suggest that apart from *Eocyzicus* none of the traditional cyzicid genera are monophyletic when using molecular data and question the usefulness of the rostral shape and condyle length as genus defining characters, except for the unique combination of a short, rounded condyle and a broadly spatulate rostrum seen in *Eocyzicus*. However, the sampling of Cyzicidae outside of Australia was sparse in these previous studies.

The phylogenetic relationships between *Eocyzicus*, Leptestheriidae and Limnadiidae were not well resolved in most studies (Schwentner et al. 2009; Weeks et al. 2009); however, phylogenomic analyses with up to 864 loci strongly supported a sister group relationship of Leptestheriidae and *Eocyzicus* and Limnadiidae as their closest relative (Schwentner et al. 2018). This contrasts with the morphology based hypothesis that suggested a closer relationship of Leptestheriidae and Cyzicidae (Olesen 1998; Astrop and Hegna 2015), but rather suggests that the ancestor of crown-group Spinicaudata was cyzicid-like.

Over the last few years, a number of molecular genetic studies have been published that explored the diversity and/or phylogeny within certain spinicaudatan genera, often restricted to a particular geographic region (*e.g.*, Bellec and Rabet 2016; Cesari et al. 2007; Reed et al. 2015; Schwentner et al. 2011 2012a 2014 2015a 2015b; Weeks et al. 2009 2014). Particularly for Australia, these studies revealed a much larger species diversity than expected, highlighting the need for integrative approaches to fully assess spinicaudatan diversity. These studies provided crucial insight into the diversity and local evolution of spinicaudatan taxa, but their geographic and taxonomic limitations did not allow for conclusions regarding the large-scale evolutionary or biogeographic history of Spinicaudata. All spinicaudatan families and several genera have a nearly global distribution, which could either be due to vicariance events associated with the break up and movement of continental plates or of multiple independent more recent colonization events via transcontinental and transoceanic dispersal. Only for Limnadiidae have more comprehensive analyses been published (Bellec and Rabet 2016; Weeks et al. 2009 2014), including a revision of its genera based on molecular phylogenetics (Rogers et al. 2012). To reconstruct the evolutionary and biogeographic history of Spinicaudata, we bring the various studies together

into a single comprehensive analysis.

MATERIALS AND METHODS

Samples, DNA extraction, PCR and sequencing

The goal was to obtain an extensive spinicaudatan dataset that brings together published sequences from various studies as well as new sequence data for species from hitherto poorly studied taxa or regions. Available spinicaudatan mitochondrial *COI* (cytochrome *c* oxidase subunit I), mitochondrial *16S rRNA*, nuclear *EF1α* (elongation factor 1-alpha) and nuclear *28S rRNA* sequences were downloaded from GenBank (Table S1). The published data had been analyzed to different extents in the associated publications. In those cases, where the available sequences had been used to delineate species using clearly stated species delineation methods (*e.g.*, Schwentner et al. 2014 2015a) or that explicitly studied intraspecific variability (*e.g.*, Cesari et al. 2007) only one specimen per putative species was selected. Limnadiidae have been comprehensively studied in detail in previous studies (Bellec and Rabet 2016; Reed et al. 2015; Rogers et al. 2012, Schwentner et al. 2009, Weeks et al. 2009 2014) and especially for many *Eulimnadia* species only a single marker was available. Therefore, not all available data were utilized for Limnadiidae, but multiple representatives of each limnadiid genus were included (if available) (Table S1). For Cyzicidae and Leptestheriidae, the goal was also to identify potential cryptic species, therefore, as many representatives as possible were included. However, several individuals with obviously erroneous sequences (*e.g.*, GenBank accession KF966550, which probably is an ostracod sequence) or with only a single locus available were excluded as these were problematic in preliminary analyses (*e.g.*, when individuals of the same species did not share any loci). For Australian *Eoleptestheria ticinensis* and *Eulimnadia* sp. C loci were retrieved from published transcriptomes (GenBank BioSample SAMN06174119 and SAMN06174118; Schwentner et al. 2018) and for *Eulimnadia texana* from the published genome (GenBank BioSample SAMN05965515; Baldwin-Brown et al. 2018) using local BLAST searches. For many published records species or genus names other than the currently recognized names had been used. In all our analyses we applied the currently accepted name following Brtek (1997), Rogers et al. (2012), Schwentner et al. (2015a), Timms and Schwentner (2017), and Rogers (2020) (*e.g.*, for most Cyzicidae s.s. this would be *Cyzicus* rather than *Caenestheriella*). We provide the name applied herein as well as the name under which the specific DNA sequences were published (Table S1).

Fresh material or soil containing viable eggs of species that were previously not studied genetically were collected in a series of field trips and expeditions performed by various authors and sometimes followed by breeding (Table S1). Because not all four markers studied herein had been sequenced in previous studies, voucher specimens or their DNA were obtained if available (*e.g.*, from Schwentner et al. 2009 2014 2015a 2015b) to provide additional loci for these specimens. DNA was extracted using the Qiagen Blood and Tissue or Qiagen QIAamp DNA Micro kits following the manufacturer's instructions. PCR reactions comprised 0.05 µl DreamTaq DNA Polymerase, 1.5 µl DreamTaq

Table 1. List of primers used in this study. For *COI* and *EF1α* different combinations of the available primers were used

Primer name	Marker	Sequence 5'–3'		Reference
LCO1490	*COI*	GGTCAACAAATCATAAAGATATTGG	Forward	Folmer et al. (1994)
LCO2	*COI*	TCNACHAAYCATAAAGAYATTGGAAC	Forward	Krebes and Bastrop (pers. com.)
HCO2198	*COI*	TAAACTTCAGGGTGACCAAAAAATCA	Reverse	Folmer et al. (1994)
HCO-MZ1-rev	*COI*	CTTTVATDCCNGTVGGSACWGCRATAATYAT	Reverse	Krebes et al. (2010)
HCOoutout	*COI*	GTAAATATATGNTGNGCTC	Reverse	Giribet and Edgecombe (2006)
HCO-709	*COI*	AATNAGAATNTANACTTCNGGGTG	Reverse	Blank et al. (2008)
16Sar-L	16S	CGC CTG TTT ATC AAA AAC AT	Forward	Palumbi et al. (1991)
16Sb	16S	CTC CGG TTT GAA CTC AGA TCA	Reverse	Xiong and Kocher (1991)
HaF2For1	*EF1α*	GGGYAAAGGWTCCTTCAARTATGC	Forward	Richter et al. (2007)
M44-1	*EF1α*	GCTGAGCGYGARCGTGGTATCAC	Forward	Cho et al. (1995)
2R53ST	*EF1α*	CAGGAAACAGCTATGACGCGAACTTGCAAGCAATGTGAGC	Reverse	Richter et al. (2007)
EF1αreverse	*EF1α*	GGAAGTCAGAGAAGGACTC	Reverse	Braband et al. (2002)
D1,D2 fw1	28S	AGC GGA GGA AAA GAA ACT A	Forward	Sonnenberg et al. (2007)
D1,D2 rev2	28S	ACG ATC GAT TTG CAC GTC AG	Reverse	Sonnenberg et al. (2007)

Buffer (Thermo Scientific), 0.12 µl dNTPs mix (25 mM each), 1.5 µl of each primer (10 mM each; see Table 1 for list of primers), and 3 µl DNA extract in a total volume of 15 µl. Temperature regimes were: 94°C for 3 min, 37 amplification cycles of 30 s at 94°C, 45 s at 46°C and 1 min at 72°C for *COI*; 35 amplification cycles of 30 s at 94°C, 45 s at 50°C and 1 min at 72°C for *16S rRNA*; 40 amplification cycles of 30 s at 94°C, 30 s at 51°C and 1 min at 72°C for *EF1α*; 40 amplification cycles of 30 s at 94°C, 30s at 52.5°C and 1.5 min at 72°C for *28S rRNA* and a final elongation step of 5 min at 72°C. Success of PCR reactions was assessed on 1.5% TAE gels. PCR products were cleaned-up with FastAP and Exonuclease I (both ThermoFisher Scientific). Sequencing was conducted either on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Carlsbad, USA) at the Bauer Core Facility at Harvard University or with Macrogen.

For *Leptestheria* sp. from Brazil, *L. nobilis* Sars, 1900 and *Ozestheria* sp. from Niger, DNA library preparation was performed using roughly 200 ng of extracted DNA as input. After physical shearing using a bioruptor (15 minutes, HI setting with intervalometer adjusted to 30 s ON, 30 s OFF; Diagenode, Liège, Belgium), we used a standard Ion Xpress library preparation protocol (Ion Xpress n°4471269) with enzyme concentration scaled down to 1:2. We performed the final library size selection with a double SPRI protocol using the following bead/DNA ratios: 0.55 first and then 0.25, to select fragments compatible with the 400 bp sequencing kit of the PGM platform. Sequencing libraries were multiplexed with 12 libraries of other organisms prepared in a similar fashion, using Ion Xpress barcoded adapters (Life Technologies, Carlsbad California). The equimolarity of the library pool was adjusted prior to emulsion PCR (emPCR) via a custom real-time PCR assay (SsoAdvanced supermix; Bio-Rad). No amplification of the libraries was necessary prior to emPCR. An equimolar pool of 20 pM was amplified using the One Touch2 400 bp amplification setup (ion-torrent n°4482002). The sequencing was performed on a 316v2 chip with 850 flows (400 bp sequencing kit; ion-torrent n°4479878).

For *Cyzicus tetracerus* from France, *Ozestheria* from Thailand and Madagascar, *Eocyzicus saharicus* Gauthier, 1937, *Leptestheria cortieri* Daday, 1913, *L. dahalacensis* Rüppel, 1837 from Austria, *L. mayeti* (Simon, 1885) from Tunisia, *Leptestheria* spp. from India and Madagascar and the new limnadiid genus from Bolivia, library preps from DNA were performed with a Nextera XT kit (Illumina): fragmentation and Illumina adapter and index ligation. Equimolar pools of each library were established. Qualification and quantification of the final library was established before sequencing on Illumina Miseq with 2*25 Millions reads cartridge of 300 bases each (30 to 45 libraries per run).

All raw sequences were assembled and sequencing errors corrected using Geneious® 6.1.8 or 11.1.4. All sequences were submitted to GenBank (accession numbers MN553596–MN553672 and MN584937–MN585093; Table S1).

Alignment and phylogenetic analyses

Alignments were performed separately for each marker with MAFFT version 7 (Katoh and Standley 2013) using the slower but more thorough LINS-I option. The cyclestherid *Cyclestheria hislopi* (Baird, 1859) and the laevicaudatan *Lynceus biformis* (Ishikawa, 1895) were included as outgroup taxa (Table S1). The alignments of both ribosomal genes included poorly aligned regions associated with numerous indels. To avoid problems in the phylogenetic analyses due to putative erroneous aligned regions, the alignments of *16S* and *28S rRNAs* were masked with Zorro (Wu et al. 2012) and all positions with scores below 5 were removed. For the two protein-coding genes substitution saturation for codon positions 1 & 2 as well as codon position 3 was tested using the test of Xia et al. (2003) implemented in DAMBE6 (Xia 2017). Translated amino acid sequences were evaluated and assessed for putative stop codons. Aligned sequences for all four genes were concatenated into a single matrix. Best-fitting DNA substitution models were assessed for each gene fragment with MEGA 7 (Kumar et al. 2016) following the Bayesian Information criterion (BIC).

Prior to phylogenetic analyses, the separate alignments per gene were concatenated into six different matrices. Matrix 1 included all taxa from all four gene fragments. For Matrix 2, individuals with less than two of the four loci present were excluded (to reduce the impact of missing data) and the 3rd codon position of *COI* removed (to reduce the impact of substitution saturation that was detected for the 3rd codon position of *COI*). Matrix 3 and Matrix 4 were taxon-specific matrices that included only representatives of Cyzicidae s.s. For these matrices, new taxon-specific alignments were computed for *16S* and *28S rRNA*. Because the species are more closely related, these alignments had hardly any indels, which greatly decreased alignment uncertainties and eliminated the need for masking. Thereby particularly variable regions were retained, which might be relevant to resolve the relationships among closely related species. Matrix 3 included all Cyzicidae s.s. and Matrix 4 only those representatives with at least two of the four loci present. Phylogenetic analyses of these two matrices were rooted by the well-supported split between the two main clades within

Cyzicidae s.s. (see below). Matrix 5 and Matrix 6 were constructed similarly as Matrices 3 and 4, but taxon-specific for Leptestheriidae and *Eocyzicus*. Matrix 5 included all representatives of these two taxa and Matrix 6 only those with at least two loci present. The phylogenetic analyses were rooted by the split between *Eocyzicus* and Leptestheriidae. The 3rd codon position of *COI* was not removed in any of the taxon-specific matrices as substitution saturation within these taxa was lower. No Limnadiidae-specific matrix was constructed and analysed, as no well-supported point for rooting was available.

Phylogenetic analyses were conducted with MrBayes 3.2.6 (Ronquist et al. 2012) and RAxML 8.2.12 (Stamatakis 2014). MrBayes analyses consisted of six runs, four chains and 30*106 generations for each of the six matrices. The first 10% of generations were discarded as burn-in, after assessing convergence with Tracer v1.6 (Rambaut et al. 2013), and the majority rule consensus tree calculated for the remaining generations. The matrices were partitioned by gene fragment and the best-fitting evolutionary model (GTR + I + G) was applied to each partition independently. Maximum likelihood analyses with RAxML were run under the GTR + G model (-m GTRGAMMA) as it is suggested to ignore the invariant parameter in RAxML. Tree searching and bootstrapping was performed in a single run (-f a), using 500 bootstrap replicates and partitioned by genes. All MrBayes and RAxML analyses were run on the CIPRES Science Gateway. The resulting phylogenetic trees were visualized with FigTree 1.4.3 Rambaut 2006–2019).

Molecular clock analyses

Despite the overall wealth of spinicaudatan fossils, few can be safely assigned to extant spinicaudatan families let alone genera (Astrop and Hegna 2015). To overcome these shortcomings, we used a two-step approach for the molecular clock analyses. First, we used the transcriptome-based data set with 606 genomic loci and an average taxon occupancy per locus of 84% of Schwentner et al. (2018) with several fossil calibration points across Branchiopoda. This data set includes eleven Spinicaudata and allows an overall overview of the timing of key cladogenetic events within Spinicaudata but not detailed age estimates within families or with regards to single biogeographic events. As a second step, we analyzed our species-rich phylogenetic data set using several of the inferred divergence times within Spinicaudata from the former molecular clock analyses of the amino-acid data set as additional calibration points.

We used the amino-acid matrix (matrix 7 of Schwentner et al. 2018), which focused on Branchiopoda with one remipede (*Xibalbanus tulumensis* (Yager, 1987)) and one cephalocarid (*Lightiella incisa* Gooding, 1963) as outgroups. The topology was fixed to the topology obtained by Schwentner et al. (2018). Node ages were estimated with PhyloBayes 4.1 (Lartillot and Philippe 2004) using the uncorrelated gamma multipliers (-ugam; Drummond et al. 2006) relaxed clock model with free parameters for birth death priors (-bd) on divergence times. The consensus tree was recovered using the bpcomp command. The calibrated trees were visualized with FigTree 1.4.3.

Eleven fossil calibration points were applied in this first molecular clock analysis. For all calibration points, the maximum age was set to 636.1 mya (million years ago) based on the youngest Lagerstätten without known Eumetazoa (following Wolfe et al. 2016). Minimum ages for calibration points were: (1) 497 mya for crown-group Allotriocarida based on *Rehbachiella kinnekullensis* Muller, 1983 (following Wolfe et al. 2016), representing the root of the tree, (2) 495 mya for the split between Branchiopoda and Remipedia based on the oldest stem group branchiopod (following Harvey et al. 2012), (3) 405 mya for crown-group Branchiopoda based on *Lepidocaris rhyniensis* (following Olesen 2009 and Wolfe et al. 2016), (4) 405 mya for crown-group Phyllopoda based on *Castracollis wilsonae* Fayers & Trewin, 2003 (following Olesen 2009), (5) 121.8 mya for crown-group Notostraca based on *Chenops yixianensis* Hegna & Dong, 2010 (following Wolfe et al. 2016), (6) 386.9 mya for crown-group Diplostraca based on *Leaia chinensis*, which was placed by Wolfe et al. (2016) as either crown-group Diplostraca or Onychocaudata (setting this constraint for Diplostraca is more conservative), (7) 250 mya for crown-group Cladoceromorpha based on the oldest known fossil Cyclestherioides (following Raymond 1946 and Negrea et al. 1999), (8) 175 mya for the split between Ctenopoda and all other Cladocera based on fossil ctenopod *Smirnovidaphnia smirnovi* Kotov, 2007 (following van Damme and Kotov 2016 and Wolfe et al. 2016), (9) 145 mya for the split between *Daphnia pulex* Leydig, 1860 and its closest relative in the data set (*Ceriodaphnia quadrangula* (Müller, 1875)) based on the oldest *Daphnia* fossils (following van Damme and Kotov 2016), (10) 145 may for the split between *Simocephalus vetulus* (Müller, 1776) and its closest relative in the data set (*Scapholeberis mucronata* (Müller, 1776)) based on the oldest known *Simocephalus* fossils (following van Damme and Kotov 2016) and (11) 255 mya for the split between Limnadiidae and *Eocyzicus* + Leptestheriidae based on the oldest known Perilimnadiidae fossils (following

Astrop and Hegna 2015), as Perilimnadiidae has been suggested to be ancestral to all Limnadiidae except *Limnadopsis*, though Astrop and Hegna (2015) questioned the exclusion of *Limnadopsis*.

To test the impact of the spinicaudatan fossil calibration point (calibration point (11)), the PhyloBayes analysis was repeated with the split between Limnadiidae and *Eocyzicus* + Leptestheriidae set to a minimum of 380 mya based on the assumption that Vertexioidea (known since the mid Devonian) is ancestral to Limnadiidae, while Eosestherioidea is ancestral to Leptestheriidae and Cyzicidae (Astrop and Hegna 2015).

For the subsequent molecular clock analyses with our species-rich data set, a new matrix was constructed for the four loci that included only individuals with at least three loci present and only one representative per species. This second molecular clock analysis was run in BEAST v2.4.6 (Bouckaert et al. 2014) as here normal distributions for node ages inferred from the previous molecular clock analysis could be more precisely defined than in PhyloBayes. Tree models were linked across all loci and site and clock models were unlinked. The GTR model with invariant sites and a relaxed log normal clock with a Birth Death model was applied to all partitions. Four BEAST molecular clock analyses were run, one run each using the age estimates derived from the two different fossil calibration points for the split between Limnadiidae and *Eocyzicus* + Leptestheriidae (see above, calibration point (11)). Each of these were run once with an additional age constraint on the split between *Eocyzicus* and Leptestheriidae (enforcing monophyly for this node) and once without this constraint (the latter accommodates the possibility that *Eocyzicus* and Leptestheriidae are not sister taxa). The fossil calibration points for Diplostraca (calibration point (6)) as well as the split between Limnadiidae and *Eocyzicus* + Leptestheriidae (calibration point (11)) were applied as described above with a uniform distribution. For all calibration points that were derived from inferred nodes of the first molecular clock analysis priors with a normal distribution were applied. Here the inferred mean values were coupled with a sigma that best modelled the 95% Highest Probability Density (HPD) distribution of the inferred node ages. Applied node ages were for the molecular clock analyses with the 255 mya calibration point for Limnadiidae + Leptestheriidae + *Eocyzicus*: (A) 294.6 mya with a sigma of 25 (403 mya with sigma 10 when 380 mya calibration point for Limnadiidae + Leptestheriidae + *Eocyzicus* was applied) for crown group Spinicaudata (B) 153.5 mya with sigma of 63 (155.6 mya with sigma 70) for the split between the Australian Limnadiidae and *Eulimnadia* clades, (C) 66.8 mya with a sigma of 40 (61.6 mya with sigma 30) for the split between *Limnadopsis* and *Paralimnadia*, (D) 64.8 mya with a sigma of 32 (67 mya with sigma 35) for the split between *O. pilosa* and the Australian *Ozestheria* species and where applicable (E) 146 mya with a sigma of 69 (154 mya with sigma 75) for the split between *Eocyzicus* and Leptestheriidae.

RESULTS

The alignments including all spinicaudatans for *COI*, *16S rRNA*, *EF1α* and *28S rRNA* were 599 bp, 449 bp, 746 bp and 675 bp, respectively. In *EF1α* no substitution saturation was detected, though for *COI*, substitution saturation was high and significant for the 3rd codon position. After removing all 3rd codon positions, the *COI* alignment had a length of only 400 bp. For each gene fragment, the GTR + G + I evolutionary model was inferred. No stop codons were detected in *COI* and *EF1α*.

Phylogenetic analyses recovered monophyly of Limnadiidae and Leptestheriidae, while Cyzicidae was paraphyletic. Exclusion of *Eocyzicus*, renders Cyzicidae monophyletic, with all the members of *Eocyzicus* forming an independent clade (Figs. 2, 3; Figs. S1–S9). We refer to the former as Cyzicidae *sensu stricto*. Leptestheriidae was recovered as sister group to Limnadiidae and *Eocyzicus* as their closest relatives, each with full support (posterior probability [pp] = 1.0) in the Bayesian analyses, and varying poorly supported relationships among families in the RAxML analyses, but never with *Eocyzicus* as sister to Cyzicidae s.s. Internal relationships within families were not always consistently recovered (Figs. 2–4, Figs. S1–S14), and for these we focus predominantly on the results obtained with the taxon-specific analyses.

In the molecular clock analyses of the transcriptome data set with PhyloBayes, we used the topology by Schwentner et al. (2018), which proposed *Eocyzicus* to be sister taxon to Leptestheriidae. Here the two alternative calibration points for the split between Limnadiidae and *Eocyzicus* + Leptestheriidae, despite differing by 125 my, had relatively little influence on the inferred node ages within Spinicaudata. Only for this specific node (mean ages 389 mya vs. 271 mya) and the age of crown-group Spinicaudata (mean ages 403 mya vs. 295 mya) was a larger difference in the age estimates observed (Figs. S10, S11). However, in the subsequent molecular clock analyses with BEAST the age of this calibration point had a stronger effect on the inferred node ages, with differences of ~30% between analyses (Fig. 4, Figs. S12–S14). Constraining the BEAST analyses to force a sister group relationships

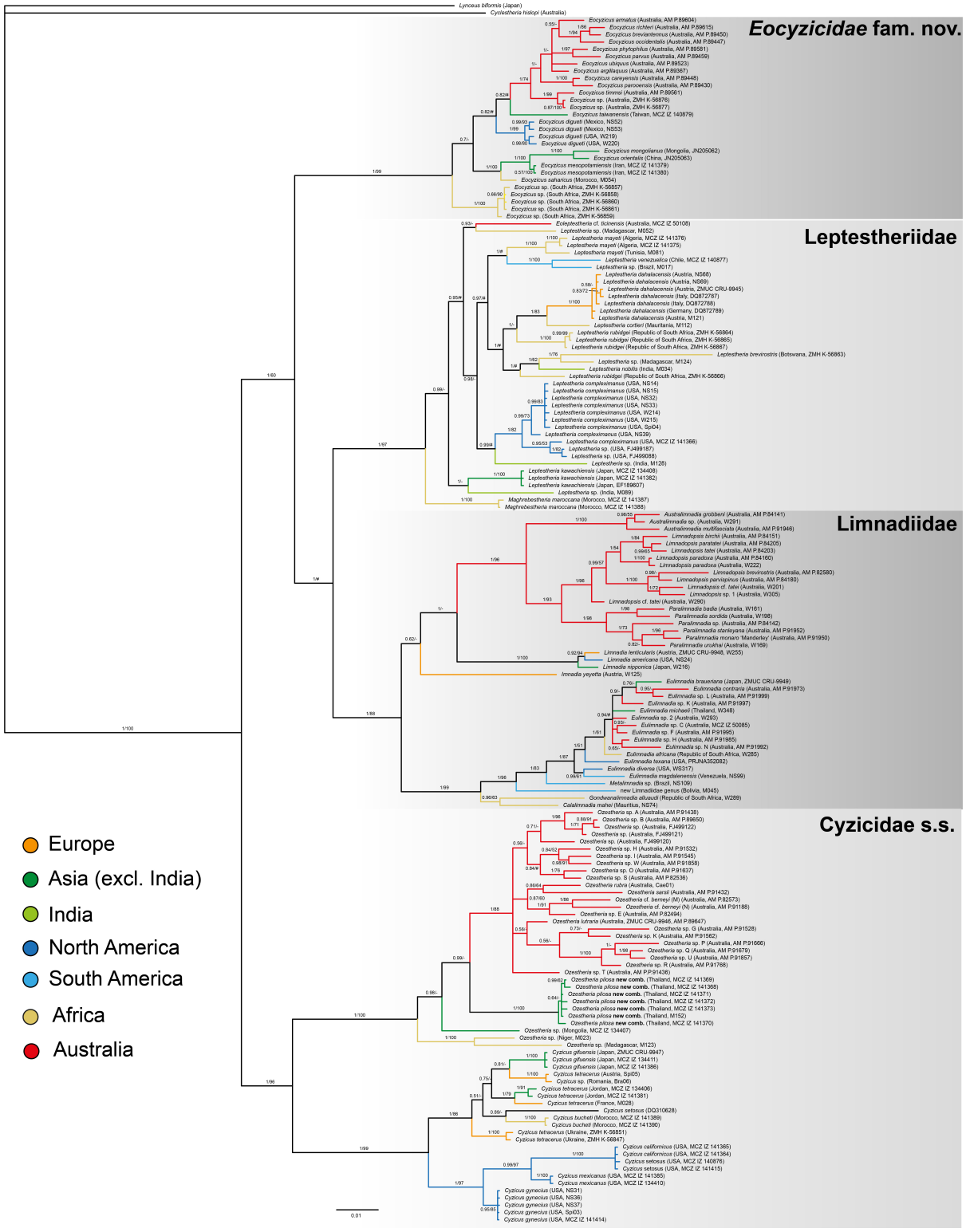

Fig. 2. Phylogenetic relationships of Spinicaudata based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with MrBayes. Only individuals with at least two of the four loci available were included and 3rd codon positions of *COI* were excluded (Matrix 2). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Posterior probabilities and bootstrap support values are provided for each branch. Branches are color-coded according the geographic origin of the specimens. # = node in topology with highest likelihood, but bootstrap support < 50%, - = node not recovered in most topology with highest likelihood.

of Leptestheriidae and *Eocyzicus* did not affect inferred node ages (usually < 1% difference between constrained and unconstrained). In general, older node ages were inferred in the more species-rich BEAST analyses than in the preceding loci-rich PhyloBayes analyses (Fig. 4, Figs. S10–S14). In the following, we focus on the BEAST analyses with the more conservative fossil calibration point (276 mya) (Fig. 4).

Cyzicidae s.s.

Cyzicidae s.s. is divided into two well-supported clades (each pp = 1.0 and bootstrap support [bs] = 100%) that diverged about 239.1 mya (194.9–282.2 mya 95% HPD) (Figs. 2–4, Figs. S4–S6). One clade includes all Australian representatives (*Ozestheria*), which constitute a monophyletic group itself (pp = 1.0; bs = 93 and 94%), as well as species from Southeast Asia (Thailand), Central Asia (Mongolia) and Africa (Niger and Madagascar). *Ozestheria pilosa* (Thailand) or *O. pilosa* together with the Mongolian species constitute the sister group to all Australian *Ozestheria* species and the whole clade was dated to 151.1 mya (120–184.3 mya 95% HPD). The divergence between *O. pilosa* and Australian *Ozestheria* was dated to 119.9 mya (96.3–141.7 mya 95% HPD, Fig. 4) (only 65 mya in the PhyloBayes analyses, Figs. S10, S11), while the oldest divergence in the Australian clade was estimated to about 94.6 mya (76.2–112.6 mya 95% HPD) (36.6 in the PhyloBayes analyses). The two African species clustered together (pp = 1; bs = 100%) and were sister group to all other species of this clade (pp = 0.94–1.0; bs = 33%; Figs. 2, 3, Figs. S4–S6). The two African species, as well as *O. pilosa*, were originally assigned to *Cyzicus*. However, subsequent examinations revealed that they meet the morphological characterization of *Ozestheria*; *i.e.*, the male claspers (= thoracopod I and thoracopod II) having the elongated, spatulate (claw-like) spines or scales in a transverse row at the endopod apex.

The second clade includes Palearctic and Nearctic species from Europe (*e.g.*, France, Austria, Romania and Ukraine), East Asia (Japan), northern Africa (Morocco), Middle East (Jordan) and North America (USA). The North American species constitute a monophyletic group (pp = 1.0; bs = 99–100%) that diverged from all other species of this clade about 116.6 mya (86.2–148.4 mya 95% HPD) (Figs. 2–4). The only possible exception being one specimen identified as *Cyzicus setosus* (Pearse, 1912) from an unknown location (GenBank accessions DQ310628 and DQ310668, deWaard et al. 2006; *C. setosus* is a North American species), which did not cluster with other representatives of the same species but rather with the northern African, Japanese and European species. We strongly suspect that this specimen was mislabelled or misidentified.

Specimens identified as *Cyzicus tetracerus* (Krynicki, 1830) did not cluster together and may constitute up to four species, respectively collected in France, Ukraine, Austria/Romania and Jordan (Figs. 2, 3B, Figs. S4–S6). Uncorrected *p*-distances in *COI* exceeded 13% between each of these putative species. As *C. tetracerus* was first described from Kharkiv in the Ukraine, the Ukrainian population may constitute the "true" *C. tetracerus*. Whether the others represent species new to science or species that have been previously synonymized with *C. tetracerus* is currently unknown. The North American species *Cyzicus setosus* and *Cyzicus californicus* (Packard, 1874) clustered together and share identical *16S* sequences, suggesting that these could be conspecific, while the other two North American species *Cyzicus mexicanus* (Claus, 1860) and *Cyzicus gynecius* (Mattox, 1950) differed by 3.7–5.5% uncorrected *p*-distances in *16S*.

The head morphology (*i.e.*, condyle length and rostrum shape) did not correspond to single clades recovered in the phylogenetic analyses (Fig. 3B). Rather all combinations that defined traditional genera were associated with multiple clades that were not closely related. In several instances groups of closely related species shared identical head morphologies (for example in *Eocyzicus* or several *Ozestheria* species). However, in other instances individuals that were genetically so similar that they probably constitute a single species differed in their head morphology. In *O. pilosa* the length of the condyle varied from short to long, whereas the genetically indistinguishable *C. setosus* and *C. californicus* differed in the shape of their rostrum. In general, specimens with the condyle-rostrum combination that is typical for *Eocyicus* (short condyle, spatulated rostrum) fell into *Eocyzicus* and not into Cyzicidae s.s., the only exception being *Ozestheria* sp. from Mongolia. This specimen had the typical head morphology of *Eocyzicus* but with an additional spine at the tip of the rostrum (Fig. 1).

Eocyzicus

The phylogenetic relationships within *Eocyzicus* were not consistently resolved (Figs. 2, 3A, Figs. S7–S9). In particular, with regards to the positions of the North American *E. digueti* (Richard, 1895) and the South African *Eocyzicus* species. While the analyses with Matrices 1 and 2 mainly suggested the latter to be sister group to all other *Eocyzicus* species (potentially together with Asian, North African and Middle Eastern species) (Fig. 2, Figs. S1–S3), analyses based on Matrices 5 and 6 mainly placed *E. digueti* as sister

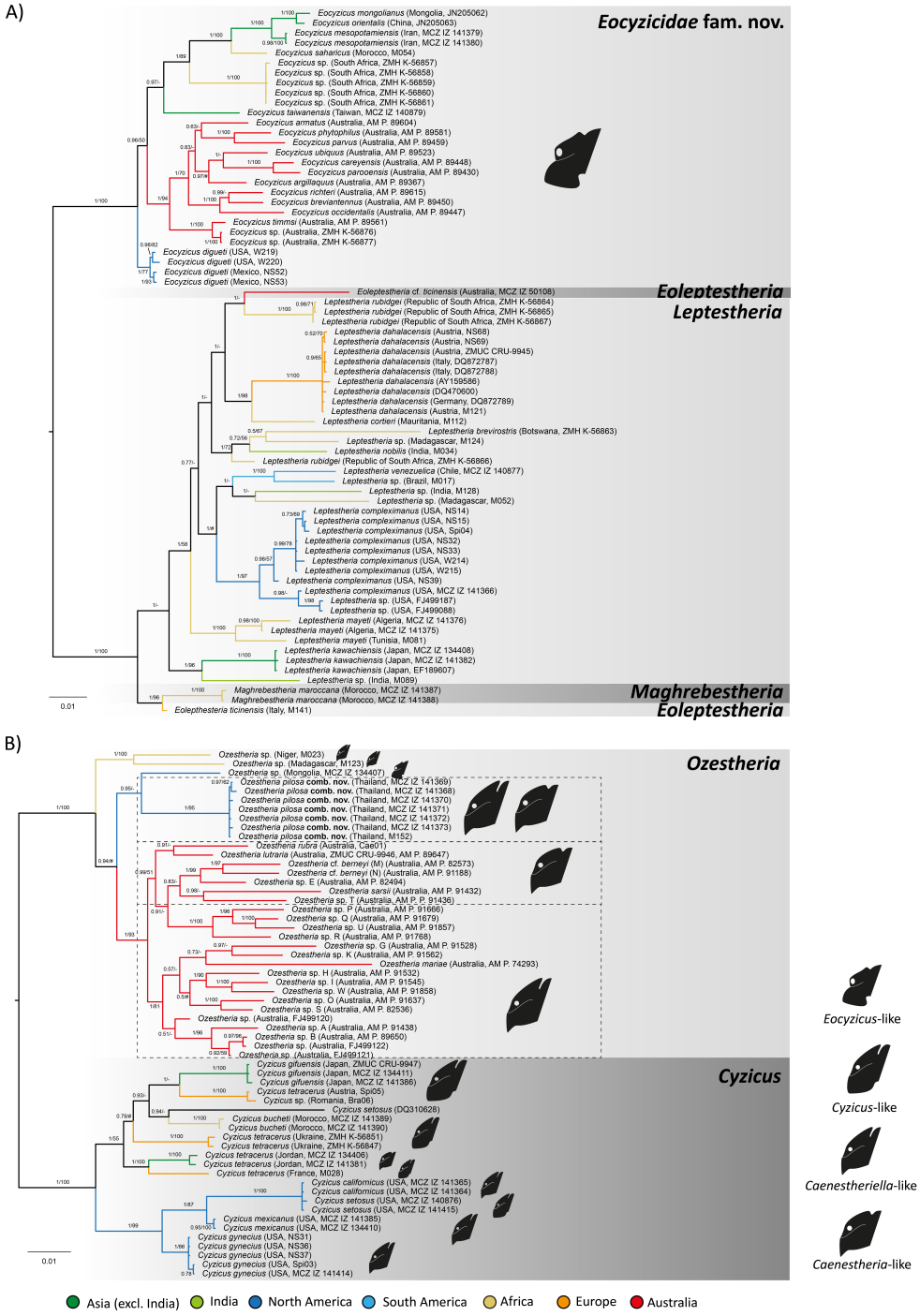

Fig. 3. Phylogenetic relationships of selected spinicaudatan taxa based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with MrBayes using taxon-specific matrices. A) Eocyzicidae fam. nov. and Leptestheriidae using Matrix 5 rooted based in the split between Eocyzicidae and Leptestehriidae and B) Cyzicidae s.s. using Matrix 3 rooted by the deepest split recovered in the analyses of all Spinicaudata. All individuals of the selected taxa and all codon positions were included. Genus affiliations are indicated. For *Eocyzicus* fam. nov. and Cyzicidae s.s. head shapes (rostrum shape and condyle length) corresponding to the four traditional cyzicid genera (*Cyzicus*, *Caenestheria*, *Caenestheriella* and *Eocyzicus*) are mapped based on the observed morphology of studied specimens. Rostrum shapes were differentiated into triangular and spatulated, irrespective of the presence of an additional posterior margin in the latter. Published information on the respective species was not considered to avoid errors for example due to cryptic species or wrong identifications. In some species, including *E. taiwanensis*, changes in rostrum morphology during growth has been observed (*e.g.*, Rogers et al. 2017). Dotted lines indicate groups of specimen with same head shapes. Posterior probabilities and bootstrap support values are provided for each branch. Branches are color-coded according the geographic origin of the specimens. # = node in topology with highest likelihood, but bootstrap support < 50%, - = node not recovered in most topology with highest likelihood.

group to all other *Eocyzicus* species (Fig. 3A, Fig. S7–S9). Below, we focus on analyses of Matrices 5 and 6 as these were tailored specifically to improve the resolution within this taxon. All Australian *Eocyzicus* species constitute a well supported monophyletic group (pp = 1.0; bs = 94%) with an inferred age of 52.8 mya (39.2–67.2 mya 95% HPD) (Fig. 4). This Australian clade is sister group to a clade comprising the African, Asian and Middle Eastern species (pp = 0.96–0.97; bs = 50%; RAxML analysis of Matrix 6 had suggested diverging relationships but with extremely low support). In the latter clade, *E. mongolianus* Uéno, 1927 (Mongolia) and *E. orientalis* Daday, 1913 (China) are nested within a group of Middle Eastern and South African species (pp = 1.0; bs = 89–91%). The Taiwanese species is either sister group to all of the other species of this clade or closer to the Australian clade, but with low support. More taxa are needed to better resolve this clade. The oldest divergence within *Eocyzicus* was dated to 95.6 mya (71.1–119.3 mya 95% HPD) but the molecular clock analyses did not include the North American *E. digueti* and inferred slightly different relationships among species (Fig. 4).

Leptestheriidae

The phylogenetic relationships within Leptestheriidae were not consistently recovered among analyses (Figs. 2–4, Figs. S1–S3, S7–S9, S12–S14). The obtained topologies of the analyses with both Leptestheriidae-specific matrices (Matrices 5 and 6) are rather similar (apart from the taxa not included in Matrix 5), with distinct differences between maximum likelihood and Bayesian analyses. The topologies obtained with the spinicaudatan-wide matrices (Matrices 1 and 2) or in the molecular clock analyses with BEAST differed markedly from these and from each other and had relatively low support for many recovered groups. Also the RAxML analyses had relatively low support values among clades. Below, we focus on analyses of Matrices 5 and 6 as these were tailored specifically to improve the resolution within this taxon. The age of extant Leptestheriidae was estimated at 126 mya (103.3–148.6 mya 95% HPD) (Fig. 4).

The most important difference between Matrices 5 and 6 is the presence of a European representative of *Eoleptestheria ticinensis* (Balsamo-Crivelli, 1859) in Matrix 5. This specimen clustered with *Maghrebestheria maroccana* Thiery, 1988, though it should be kept in mind that only the relatively conserved *28S rRNA* was available for the European *E. ticinensis*. This clade (or only *M. maroccana* in the analysis of Matrix 6) was sister group to all other Leptestheriidae in the Bayesian analyses (pp = 1.0; Fig. 3A, Fig. S9) or most other Leptestheriidae except for *L. kawachiensis* and an Indian species (specimen M089) in the maximum likelihood analyses (Figs. S7, S8). The Australian specimen of *E. ticinensis* (Fig. 1J) does not cluster with the European representative, but is nested deep within *Leptestheria*. The uncorrected *p*-distance for the conservative *28S rRNA* is 3.9% between these two specimens of *Eoleptestheria*. *Leptestheria nobilis*, which was formerly assigned to the now synonymized genus *Leptestheriella*, is also nested within a group of *Leptestheria* species.

The leptestherid species do not group according to their geographic origin (Figs. 2, 3A). Notably, there are several clusters of species from continents that once formed Gondwana, for example *L. brevirostris* Barnard, 1924 (Botswana), one specimen identified as *L. rubridgei* (Baird, 1862) (South Africa), *Leptestheria* sp. (Madagascar; specimen M124) and *Leptestheria nobilis* (India) or *L. venezuelica* Daday, 1913 (South America), *Leptestheria* sp. from Brazil (South America), *Leptestheria* sp. (India; specimen M128) and *Leptestheria* sp. (Madagascar; specimen M052) (age estimated at 94 mya excluding the Madagascan species) or *E. ticinensis* (Australia), *L. rubidgei* (South Africa) and *L. cortieri* (Mauritania) with the European *L. dahalacensis* nested well within this last cluster (each cluster with pp = 1; Fig. 3A, Fig. S3).

There were some instances of putatively cryptic or unrecognized species diversity within Leptestheriidae, in addition to *Eoleptestheria* (see above). Specimens identified as *L. rubidgei* were divided into two strongly separated groups (uncorrected *p*-distance for *16S rRNA*: 4.7–5.0%) that clustered at very different positions within Leptestheriidae (Figs. 2, 3A). The Algerian and Tunisian specimens of *L. mayeti* (Simon, 1885) differed by 12.9% uncorrected *p*-distance in *COI* from each other. In North America, *L. compleximanus* (Packard, 1877) is currently the only recognized species. Within this species uncorrected *p*-distances of up to 4.8% for *COI*, 3.1% for *16S rRNA* and 1.1% for *EF1α* were observed; these increased to 14.5% (*COI*) and 2.4% (*EF1α*) if the unidentified North American *Leptestheria* specimens are included as well.

Limnadiidae

There are two well (pp = 1.0; bs = 96–99%) and consistently supported main clades within Limnadiidae: a clade of Australian endemic genera (*Limnadopsis*, *Paralimnadia* and *Australimnadia*), in which *Paralimnadia* is sister group to *Limnadopsis* and a clade of species from South America (*Metalimnadia* and a putative new genus), Africa (*Gondwanalimnadia* and *Calalimnadia*) as well as the globally distributed

genus *Eulimnadia* (Figs. 2, 4, Figs. S1–S3, S12–S14). The relationships of the Holarctic *Imnadia* and *Limnadia* species, *Imnadia yeyetta* Hertzog, 1935 (Europe), *Limnadia lenticularis* (Linnaeus, 1761) (Europe), *L. nipponica* Ishikawa, 1895 (Japan) and *L. americana* Morse, 1868 (USA), are not consistently resolved (Figs. 2, 4, Figs. S1–S3, S12–S14). Either *Imnadia* and *Limnadia* constituted a monophyletic clade that was sister group to the Australian clade, all other Limnadiidae, or *Limnadia* was the sole sister taxon

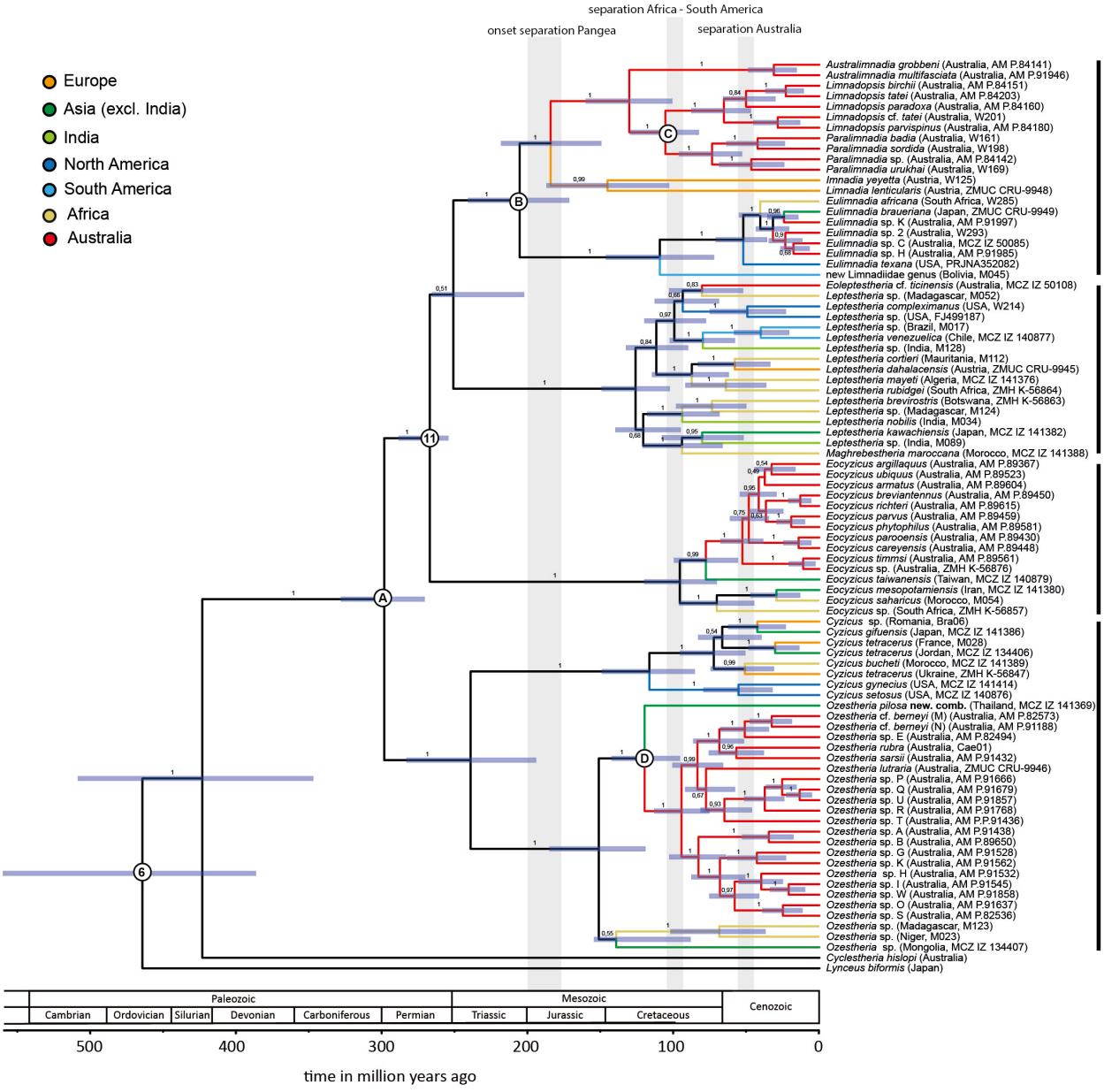

Fig. 4. Molecular clock dated phylogeny. The divergence time estimates are based on the BEAST analyses of *COI*, *16S rRNA*, *EF1α* and *28S rRNA* that included only individuals with at least three of the loci present. Blue bars represent 95% HPD intervals of inferred node ages. The topology was not constrained to enforce a sister group relationship of Leptestheriidae and *Eocyzicus*, thus also no prior was defined for their divergence (Fig. S12 for the constrained analysis). Calibration points (6) and (11) are based on fossils for Diplostraca (minimum age 386.9 mya based on *Leaia chinensis* following Wolfe et al. (2016)) and Limnadiidae + *Eocyzicus* + Leptestheriidae (minimum age 255 mya based on oldest known Perilimnadiidae fossils) following Astrop and Hegna (2015), respectively (Figs. S13 and S14 for an alternative age constraint for Limnadiidae + *Eocyzicus* + Leptestheriidae). Calibrations points (A–D) were inferred from the preceding molecular clock analyses of the amino acid data set of Schwentner et al. (2018) (Figs. S10, S11) and coded as normal distributed priors: (A) 294.6 mya with sigma of 25, (B) 153.5 mya with sigma of 63, (C) 66.8 mya with a sigma of 40 and (D) 64.8 mya with a sigma of 32. Branches are color-coded according the geographic origin of the specimens. Posterior probabilities are provided for each node.

to the Australian clade. However, the support values for these alternative relationships were low and non-significant.

The divergence between the two main clades was dated to 154 mya in the PhyloBayes analyses (Figs. S4, S5; unfortunately, no representative of *Imnadia* or *Limnadia* was available for this molecular clock analyses) and to 205.5 mya (172.2–240.2 mya 95% HPD) in the BEAST analyses (Fig. 4). The age of the Australian clade was estimated to 130.3 mya (101.6–159.4 mya 95% HPD), the divergence between *Paralimnadia* and *Limnadopsis* to 105.6 mya (83.4–129.1 mya 95% HPD) (59 mya in the PhyloBayes analyses) and the oldest divergence within *Limnadopsis* to 65.5 mya (47.7–87 mya 95% HPD) (24 mya in the PhyloBayes analyses) (Fig. 4). The split between the new South American limnadiid genus and *Eulimnadia* was dated to about 109.4 mya (73–145.5 mya 95% HPD) while the split between the North American *Eulimnadia texana* Packard, 1871 and the other *Eulimnadia* species studied herein was dated to 52.2 mya (36.7–70.3 mya 95% HPD).

DISCUSSION

Phylogenetic and Systematic Implications

The taxonomy and systematics of Spinicaudata has always been problematic due the lack of well-defined diagnostic characters and the prevalence of high intraspecific and intrageneric morphological variability (Vecchi 1922; Brehm 1933; Gauthier 1933; Linder 1945; Botnariuc 1945 1947; Margalef 1953; Straškraba 1965a b 1966; Wiltshire 1973; Marinček and Petrov 1985; Rogers et al. 2012 2017). Previous studies had already suggested the non-monophyly of Cyzcidae s.l., with *Eocyzicus* being closer related to Leptestheriidae and/or Leptestheriidae + Limnadiidae (Schwentner et al. 2009 2018; Weeks et al. 2009). Our results strongly support this finding with the most species-rich spinicaudatan molecular data set studied so far. As a consequence, the genus *Eocyzicus* should be placed in its own family and the family definition of Cyzicidae s.s. needs to be adjusted accordingly (see taxonomic section below). Although our phylogenetic analyses suggest a sister group relationship of Leptestheriidae with Limnadiidae, a scenario already proposed by Sars (1898), we think that a sister group relationship of *Eocyzicus* with Leptestheriidae is more likely given the results of the phylogenomic study of Schwentner et al. (2018), which is based on a much larger set of molecular loci.

All four main taxa (= families) within Spinicaudata—Limnadiidae, Leptestheriidae, Eocyzicidae (see below) and Cyzicidae s.s.—are unambiguously and strongly supported as monophyletic. This is particularly notable for Leptestheriidae, which were represented in previous molecular phylogenetic studies solely by species of the genus *Leptestheria* (Schwentner et al. 2009; Weeks et al. 2009). Unfortunately, the phylogenetic relationships within Leptestheriidae were not well resolved, but it appears that *Maghrebestheria* (possibly together with the European *Eoleptestheria*) is the sister group to all other Leptestheriidae. The taxonomic status of *Eoleptestheria* is challenged, with the Australian representative nesting within *Leptestheria* species and the European with *Maghrebestheria*. There is no support for maintaining the previously synonymized genus *Leptestheriella* (Brtek 1997) as the only species in our study that had formerly been assigned to *Leptestheriella* (*L. nobilis*) also nests within *Leptestheria*. Our results suggest that the systematics of Leptestheriidae are in need of revision. However, our results also highlight the need to include more species and to better resolve their overall relationships before such revisions can and should be conducted. Therefore, we did not include Leptestheriidae in the taxonomic section of the present study.

Within Cyzicidae s.s., our results suggest that the distribution of *Ozestheria* is extended from Australia, as proposed by Schwentner et al. (2015a), to Asia and Africa. It is probable that further cyzicids from these continents belong to *Ozestheria* as well. The genera *Caenestheria* and *Caenestheriella* are not supported (as previously suggested by Vecchi 1922; Gauthier 1933; Linder 1945; Botnariuc 1945 1947; Margalef 1953; Straškraba 1965b 1966; Wiltshire 1973; Marinček and Petrov 1985; Rogers et al. 2017; Schwentner et al. 2009 2015a), and a thorough revision of all Cyzicidae s.s. appears necessary. The length of the condyle and especially the shape of the rostrum (Figs. 1, 3) are not suitable taxonomic characters to define and differentiate cyzicid genera, not even among adult specimens. Developmental studies had already shown that rostrum shape can vary with age (Margalef 1953; Straškraba 1965b; Wiltshire 1973; Rogers et al. 2017). Finding new informative characters will be a crucial challenge for future taxonomic revisions of Cyzicidae.

Although the internal relationships of the limnadiid genera are also not fully resolved, in particular of *Imnadia* and *Limnadia*, at least the monophyly of the various genera appears well supported. The monophyletic group comprising the Australian genera *Limnadopsis*, *Paralimnadia and Australimnadia* had been recovered previously (Schwentner et al. 2009; Weeks et al. 2009 2014) as has the clade comprising

Eulimnadia, *Metalimnadia*, *Calalimnadia* and *Gondwanalimnadia* (Weeks et al. 2009 2014; Bellec and Rabet 2016). Apparently *Limnadopsis* and *Paralimnadia* are sister taxa, which is slightly surprising given the fact that most species of *Limnadopsis* and *Australimnadia* are quite large bodied representing the largest extant Spinicaudata. Either their large body size was acquired independently or the species of *Paralimnadia* reduced its size secondarily.

TAXONOMY

Cyzicidae Stebbing, 1910

= Caenestheriidae Daday, 1913: 12 (pro partim)

Diagnosis: Cephalic fornices extending anteriorly to rostral apex. Rostrum variable, blunt to acute, long or short, generally triangular to subquadrate in lateral view. Rostrum with or without an apical spine, usually without. Compound eyes fused medially, sometimes projecting in smoothly arcuate ocular tubercle. Frontal organ sessile. Occipital notch present. Carapace thick, generally rounded. Carapace dorsal margin smooth, lacking carinae, hinge line straight. Carapace with or without pigmentation, growth lines obvious, projecting. Umbone present, projecting well above hinge line. Muscle scar rarely visible. Male first two thoracopods with endopod (= movable finger, *sensu* Olesen 2007) lacking an apical suctorial organ, if modified setae or spines are present these are never with a lateral fringe. Telson without a ventroposterior, posteriorly directed spiniform projection. Eggs 110–170 μm in diameter, spherical and generally lacking ornamentation.

Comments: The Cyzicidae is restricted to two genera: *Cyzicus* and *Ozestheria*. *Cyzicus* is fixed as the type genus for the family (Daday 1913a b).

Cyzicus Audouin, 1837

= *Caenestheriella* Daday, 1914: 106 fide Margalef, 1953, fide Straškraba 1965b
= *Caenestheria* Daday, 1914: 53 pro partim

Diagnosis: Populations composed of males and females (except *C. gynecius* which is only composed of hermaphrodites); amplexus is venter to venter. Rostrum subtriangular (usually females) to subquadrate (usually males), depending on age and gender. Angle between rostrum and frons 160° to 180°. Occipital notch either deep and narrow, often closed or very shallow, or absent. Occipital condyle either conical, subacute, length subequal to basal width or low, rounded, length half or less basal width. Rostral spine generally absent (present in *C. australis*). Carapace valve length ~1.3x valve breadth (umbone to margin). Carapace growth line intervals smooth or ornamented (scarring from algae often mistaken for ornamentation). Carapace typically dark brown, occasionally black, or with yellow markings, often with setae. Clasper endopod (= movable finger) apically unarmed, or with a few setae, apical margin crenulate at most, but never with claw-like seta or scales. Endite IV broadly transverse to cylindrical, bearing a dense, apical field of short spiniform setae. Thoracic segments smooth or with a central dorsoposterior projection and/or set of spines or setae. Eggs attaching to prolonged exopods of thoracopods IX and X. Thoracopod exopods lacking a triangular lamina. Telson posteriolateral spine rows confluent dorsally, with confluence not projecting. Each row has from 10 to 30 spines depending on species. Caudal filament (= telsonic filament) originating between spine rows at fifth, sixth, or seventh spines from confluence. Caudal filament borne or not on projecting mound. Cercopods arcuate, occasionally sinuate, or straight with distal fourth to third bent dorsally. Cercopod with medial longitudinal setal row on proximal 40–60%. Setae plumose and either long or short. Setal row terminates with single spine. Cercopod with subapical, dorsal cirrus, extending from 60–40% of cercopod length. Eggs smooth, unornamented.

Comments: The type species is *Limnadia tetracera* Krynicki, 1830 (now recognized as *C. tetracerus*) fixed by monotypy. Currently, we recognize about 25 species in this genus (Rogers 2020). At this time the genus is morphologically defined based upon the family characters and the clasper endopod apically unarmed, or with apical margin crenulate at most. Further molecular and morphological analyses are needed to clarify relationships among the members of this genus. The genera *Caenestheria* and *Caenestheriella* are not supported in our analysis. Indeed, one species in our analysis that was formerly attributed to *Caenestheriella*, *Cyzicus setosus*, appears to be to be conspecific with *Cyzicus californicus*.

Ozestheria Schwentner and Richter, in Schwentner, Just, and Richter, 2015

Diagnosis: (modified from Schwentner et al. 2015). Populations composed of males and females; amplexus is venter to venter. Male and female rostrum triangular. Ocular tubercle smoothly arcuate. Angle between rostrum and frons 150° to 170°. Occipital condyle either short and rounded or elongated and subacute. Carapace valve length ~1.5 times valve breadth (hinge to margin). Carapace with or without

sculpturing between growth lines (scarring from algae often mistaken for sculpture). Carapace typically dark brown. Male thoracopod I and II with endopod (= movable finger) bearing one or more transverse apical rows of flattened, broadly subtriangular denticles (claw-like scales) (Fig. 1H). Endite IV broadly transverse to cylindrical, bearing a dense, apical field of short spiniform setae. Eggs attaching to prolonged exopods of thoracopods IX and X. Thoracopod exopods lacking a triangular lamina. Posterior trunk segments with several medial dorsoposterior spines per segment. Telson posterior margin posteriolateral spine rows confluent dorsally, with confluence not projecting. Each row with 10 to 30 spines. Caudal filament originating between spine rows at fifth, sixth, or seventh spines from confluence. Caudal filament borne on projecting mound or not. Cercopods sinuate to curved. Cercopod with medial longitudinal setal row on proximal 40–60%. Setae plumose and either long or short. Setal row terminates with single spine. Cercopod with subapical, dorsal cirrus, extending from 60–40% of cercopod length.

Comments: *Ozestheria lutraria* (Brady, 1886) is the type species (Schwentner et al. 2015). This genus is morphologically defined by the clasper endopod bearing one or more transverse apical rows of flattened, broadly subtriangular denticulae ("claw-like scales"). Several forms previously treated at *Cyzicus* are here reported as *Ozestheria*, with the result that *Ozestheria* is no longer considered to be endemic to Australia. The distribution of this genus apparently extends to Africa and Asia as well suggesting to be primarily Gondwanan, with an extension into Asia. It is currently unclear if further species, which are currently identified as *Cyzicus*, in fact belong to *Ozestheria*. But at this time we can confidently ascribe at least one Asian species to this genus: *Ozestheria pilosa* (Rogers, Thaimuangphol, Saengphan, & Sanoamuang, 2013) **new combination**.

Eocyzicidae, Schwentner, Rabet & Rogers, 2020, fam. nov.

= Caenestheriidae Daday, 1913: 12 (pro partim)

Diagnosis: (Modified from Rogers et al. 2017). Populations composed of males and females; amplexus is venter to venter. Rostrum typically sexually dimorphic. Rostrum subtriangular (usually females) to subquadrate (usually males) or rounded. Adult rostrum not armed with an apical spine (sometimes present in juveniles). Angle between rostrum and frons 170° to 190°. Occipital notch very shallow or absent. Occipital condyle low, rounded or absent, length half or less basal width. Carapace valve length ~1.5 times valve breadth (hinge to margin). Carapace growth line intervals smooth or weakly ornamented (scarring from algae often mistaken for ornamentation). Carapace typically whitish and partly translucent. Clasper endopod apically with one or a few elongated scales, each scale laterally fringed. Endite IV broadly transverse to cylindrical, bearing a dense, apical field of short spiniform setae. Thoracic segments smooth or with a central dorsoposterior projection and/or set of spines or setae. Eggs attaching to prolonged exopods of thoracopods IX and X. Thoracopod epipods lacking a triangular lamella. Telson posterior margin posteriolateral spine rows confluent dorsally, with confluence not or slightly projecting. Each row has from six to 30 spines depending on species and gender. Females typically have more and smaller spines than males. Caudal filament originating between spine rows at fifth, sixth, or seventh spines from confluence. Caudal filament borne on projecting mound. Cercopods arcuate or straight. Cercopod with a dorsomedial longitudinal row of setae or spines on proximal 40–60%. Setae plumose and either long or short. Row terminates with single spine. Cercopod with subapical, dorsal cirrus, extending from 50–40% of cercopod length. Eggs smooth or with surface polygons.

Comments: The type genus is fixed here as *Eocyzicus*. Naganawa (2001) treated *Eocyzicus* as a junior synonym of *Cyzicus*, however. this is not supported by this and previous molecular studies (Schwenter et al. 2009 2018; Weeks et al. 2009).

Eocyzicus Daday, 1914: 193 *sensu* Rogers et al. 2017

= *Caenestheria* Daday, 1914 (pro partim)

Diagnosis: As for the family.

Comments: The type species for the genus is *Eocyzicus orientalis* Daday, 1914, fixed here by designation. The synomyzation of *Cyzicus* with *Eocyzicus* is not supported. Species following Daday's (1913a b) description of *Caenestheria* are now summarized under *Cyzicus* (see above). Relationships within *Eocyzicus* remain unclear, and further sampling across many taxa is required before any meaningful relationships can be determined.

Biogeographic History

To date, little is known about the biogeographic history of extant Spinicaudata. The spinicaudatan fossil record is rich and diverse, but limited almost exclusively to carapaces. Thus the fossil record contributes little information for the evolutionary history of extant taxa

as the fossil relationships are not well resolved and still debated (Astrop and Hegna 2015). It is worth noting that all four spinicaudatan families have a nearly global distribution (the Americas, Eurasia, Africa and Australia) and even genera such as *Leptestheria*, *Eocyzicus*, *Cyzicus* and *Eulimnadia* occur on all or most of these landmasses. Within continents, Spinicaudata have revealed remarkable dispersal potential with species being distributed over > 1000 km and relatively low levels of population differentiation (Cesari et al. 2007; Schwentner et al. 2012a 2014 2015a). Such long-distance dispersal can be mediated via birds or other animal vectors or wind (Bilton et al. 2001) and probably follow the same model as described for the related Anostraca (Rogers 2015 and references cited within).

Our molecular clock analyses suggest that the four extant spinicaudatan families diverged prior to the break-up of Gondwana and Laurasia and possibly of Pangea. Also the divergence between species and species groups inhabiting different continents mostly predate the separation of the respective continental plates. Historic vicariance appears to be the main factor explaining the transcontinental distribution of extant spinicaudatan taxa, though in a few instances more recent transoceanic dispersal events have occurred as well. For other large Branchiopoda, like Anostraca, Laevicaudata, and Cyclestherida, vicariance apparently also played a major role in shaping today's distribution of taxa (Schwentner et al. 2013; Rogers 2015; Sigvardt et al. in press), in particular for southern hemisphere taxa. For Notostraca, previous molecular clock analyses obtained different estimates of their divergence times. While some suggested more recent divergence ages, implying trans-oceanic dispersal events (Mather et al. 2013; Vanschoenwinkel et al. 2012), others suggested divergence times that imply vicariance events (Korn et al. 2013). Our own divergence time estimates within Notostraca (Figs. S10, S11) are similar to the former. One should keep in mind that we predominantly discuss our molecular clock results based on the more conservative calibration point of 255 mya for Limnadiidae + Leptestheriidae + *Eocyzicus*, the divergence ages inferred using the 380 mya calibration point were even older (Figs. S13–S14) and thus even stronger in suggesting vicariance over dispersal.

The divergence between northern and southern hemisphere species of Cyzicidae and Limnadiidae (with the exception of *Eulimnadia*) probably predates the break-up of Pangea in the early to mid-Jurassic, suggesting that geographic distances might have separated these clades already on Pangea. If *Limnadia* and *Imnadia* are indeed nested among the two southern continental clades, a southern hemisphere origin can be hypothesized for Limnadiidae. From there the northern hemisphere could have been colonized when Gondwana and Laurasia were still joined in Pangea. Also for Leptestheriidae, a southern hemisphere origin can be assumed, based on the phylogenetic relationships among its taxa. But here multiple independent colonization events of northern hemisphere continents have to be assumed. The strongly diverging relationships within Leptestheriidae among most analyses do not allow establishing detailed biogeographic hypotheses for this taxon. Most inferred clades within Leptestheriidae do not correspond to geographic regions (*e.g.*, African or Indian species do not form monophyletic groups each) and many deep splits within Leptestheriidae were dated to be 90 mya or older, which roughly coincides with the separation between Africa and South America (~100 mya; McLoughlin 2001) and predates the final break-off of Australia (~50 mya; Beaulieu et al. 2013). Ancestral leptestheriid lineages were probably once widely distributed across Gondwana and when Gondwana broke apart, several of these lineages survived on more than one continent. For example, one leptestheriid clade that was consistently recovered comprised *Leptestheria nobilis* from India, *Leptestheria* sp. (specimen M124) from Madagascar and *L. brevirostris* from Botswana with an estimated clade age of 95 mya. A Madagascar-India-Seychelles block was the first landmass that broke-off from Gondwana around 120 mya (Ali and Aitchinson 2008, McLoughlin 2001) and this leptestheriid clade probably evolved on this landmass and dispersed from Madagascar to continental Africa subsequently. Clades with similar African and Indian distributions were also recovered for the notostracan *Triops* (*e.g.*, clades 18 and 26 in Korn et al. 2013; see also Modak et al. 2018). While some of these distributions might be due to the geological processes (*e.g.*, clade 18 in Korn et al. 2013), others appear to be younger and thus possibly due to more recent dispersal and colonization events (*e.g.*, clade 26 in Korn et al. 2013).

The only spinicaudatan example of repeated transoceanic dispersal is *Eulimnadia*. This genus probably evolved in South America and successfully dispersed to virtually all other continents (there are no extant records from Antarctica, whether Antarctica was historically inhabited is unknown), as well as many oceanic islands (Bellec and Rabet 2016), probably during the last 50 mya. Bellec and Rabet (2016) dated the onset of this global distribution to only 30 mya. *Eulimnadia* is special among Spinicaudata due to its very fast development and short life-cycle, which enables them to survive in short-lived habitats and by which they might escape competition and predation from slower developing taxa, as well as the presence of hermaphrodites instead of females (Bellec and

Rabet 2016). The latter enables the colonization of new habitats from single resting eggs, which greatly improves dispersal effectiveness.

The Australian fauna is noteworthy not only because of its exceptional diversity (Schwentner et al. 2015b), but it apparently evolved independently from other regions even when Australia was still connected to other Gondwanan landmasses. This seems to have been the case for the *Limnadopsis-Paralimnadia-Australimnadia* clade within Limnadiidae and the Australian *Ozestheria* clade. In both cases, age estimates suggest that the Australian clades (134 and 94 mya, respectively) evolved long before the final separation of Australia from the rest of Gondwana (Beaulieu et al. 2013), which occurred around 50 mya. In this relatively long timespan, apparently no exchange between Australia and other continental masses occurred. Such patterns of ancestral regionalization were also found in many other old Gondwanan lineages (*e.g.*, Murienne et al. 2014). Australia was linked to the rest of Gondwana primarily via Antarctica, the only continent without extant Spinicaudata but with a rich fossil record of these animals (Shen 1994). Antarctica may have restricted exchange between Australia and other regions of Gondwana simply by geographic distance (isolation by distance). The age of the Australian *Eocyzicus* clade roughly coincides with the final break-off of Australia, implying that Australia was colonized by *Eocyzicus* around that time. Again, *Eulimnadia* may be the only taxon that colonized Australia after it separated from Gondwana, potentially as Australia drew closer to Asia. It has been suggested that *Eoleptestheria* cf. *ticinensis* invaded Australia recently from China (Timms 2009a); however, our phylogenetic analyses, as well as previous analyses of the genetic diversity within the Australian populations (Schwentner et al. 2015b), suggest a longer presence of this taxon in Australia. Notably, for the large branchiopod taxa Cyclestherida and Notostraca, younger ages have been assumed for the divergence of Australian and non-Australian species (Schwentner et al. 2013; Mathers et al. 2013). In these taxa, dispersal to or from Australia apparently occurred more recently, probably after Australia and Asia moved closer. The case of the notostracan *Triops* is particularly interesting as the putatively closest relatives to the Australian fauna occur in North America (Mathers et al. 2013).

The age estimates presented herein may also help to improve our understanding of the relationships between fossil and extant taxa (Astrop and Hegna 2015). We provide the first molecular clock based age estimated for all extant families and many genera. Our results suggest that within each extant family only one to three extant lineages date back more than 150 million years and the main divergence took place within the last 100 million years. Thus the majority of fossil families probably went extinct without any extant representatives; this may be particularly true for the rich Permian and Carboniferous fauna as crown-group Spinicaudata may have only originated around that time. The similar carapace shapes of Cyzicidae and Eocyzicidae might go back to comparable carapace shapes already known from different Euestheriidae fossils since the Permian (Astrop and Hegna 2015). Nevertheless, peculiar similarities in carapace shape between fossil and extant taxa may be generally due to convergence rather than evolutionary stasis. For example, the carapace of the fossil Palaeolimnadiopseidae Defretin-Le Franc, 1965 has large similarity to extant species of *Limnadopsis* and *Australimnadia* (for example, compare Gallego and Breitkreuz 1994 and Gallego 2005 with Timms 2009b or Timms and Schwentner 2012) and it has been suggested that Palaeolimnadiopseidae are the ancestors of *Limnadopsis* (Zhang et al. 1976, but questioned by Astrop and Hegna 2015). Palaeolimnadiopseidae date back as far as the Upper Permian (summarized in Gallego 2005) long before the inferred evolution of Limnadiidae, potentially even before the evolution of crown-group Spinicaudata. Of course, it is possible that some younger species that have been assigned to Palaeolimnadiopseidae belong to the stem lineage of extant Limnadiidae and are not related to the older taxa (Astrop and Hegna 2015). Our divergence time estimates may help to improve the current hypotheses of how such fossil and extant taxa may have been related.

Species Diversity of Spinicaudata

Detailed population-based molecular genetic and integrative taxonomic approaches have revealed much higher species diversities for Spinicaudata (*e.g.*, Schwentner et al. 2011 2014 2015a b; Weeks et al. 2009) and other 'large Branchiopoda' like *Triops* (*e.g.*, Korn et al. 2010; Mathers et al. 2013; Meusel and Schwentner 2017; Vanschoenwinkel et al. 2012). Several species that were assumed to be morphologically variable could be shown to represent an amalgam of multiple, morphologically differentiated, species (*e.g.*, Korn et al. 2010; Schwentner et al. 2012b; Meusel and Schwentner 2017; Tippelt and Schwentner 2018). However, extensive overlaps of intraspecific variability and interspecific variation are prevalent also in these species and their initial delimitation would have been difficult based solely on morphological characters. The majority of these studies have been conducted on the Australian fauna, which now appears to be the continent with the highest extant clam shrimp diversity, harbouring roughly one third of all spinicaudatan

species (Schwentner et al. 2015b). However, the spinicaudatan fauna of other continents have not been studied as extensively. In the analyses presented herein, many species were represented by single individuals or were studied from a few populations only. Despite this relatively sparse intraspecific sampling, instances of putatively cryptic species were revealed in Africa, North America and Europe; in the case of *Leptestheria rubidgei* even within a single population. On the one hand, this suggests that the species diversity of Spinicaudata may be underestimated on local and global scales, on the other hand it shows that the species level taxonomy of many spinicaudata taxa requires revision. Taxonomic revisions that combine detailed morphological and molecular genetic data will become indispensable to assess the true diversity of Spinicaudata and will probably reveal many more currently cryptic species.

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Supplementary Materials

Fig. S1. Phylogenetic relationships of Spinicaudata based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with RAxML. All individuals and codon positions were included (Matrix 1). For each individual, the country of origin and the collection or voucher number are provided (see also Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S2. Phylogenetic relationships of Spinicaudata based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with MrBayes. All individuals and codon positions were included (Matrix 1). For each individual, the country of origin and the collection or voucher number are provided (see also Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Posterior probabilities are provided for each node. (download)

Fig. S3. Phylogenetic relationships of Spinicaudata based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with RAxML. Only individuals with at least two of the four loci available were included and 3rd codon positions of *COI* were excluded (Matrix 2). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S4. Phylogenetic relationships of Cyzicidae s.s. based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* with RAxML. All individuals and codon positions were included (Matrix 3). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S5. Phylogenetic relationships of Cyzicidae s.s. based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with RAxML. Only individuals with at least two of the four loci available were included and 3rd codon positions of *COI* were excluded (Matrix 4). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S6. Phylogenetic relationships of Cyzicidae s.s. based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* with MrBayes. Only individuals with at least two of the four loci available were included and 3rd codon positions of COI were excluded (Matrix 4). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Posterior probabilities are provided for each node. (download)

Fig. S7. Phylogenetic relationships of *Eocyzicus* and Leptestehriidae based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with RAxML. All individuals and codon positions were included (Matrix 5). For each individual, the country of origin and the collection or voucher number are provided (Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S8. Phylogenetic relationships of *Eocyzicus* and Leptestehriidae based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with RAxML. Only individuals with at least two of the four loci available were included and 3rd codon positions of *COI* were excluded (Matrix 6). For each individual, the country of origin and the collection or voucher number are provided (see also Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Bootstrap support values are provided for each node. (download)

Fig. S9. Phylogenetic relationships of *Eocyzicus* and Leptestehriidae based on *COI*, *16S rRNA*, *EF1α* and *28S rRNA* inferred with MrBayes. Only individuals with at least two of the four loci available were included and 3rd codon positions of *COI* were excluded (Matrix 6). For each individual, the country of origin and the collection or voucher number are provided (see also Table S1), if no collection or voucher number was available one of the GenBank numbers is provided. Posterior probabilities are provided for each node. (download)

Fig. S10. Molecular clock dated phylogeny based on the amino acid data set of Schwentner et al. (2018) employing minimum age 255 mya for Limnadiidae + *Eocyzicus* + Leptestheriidae. The topology was fixed to the one obtained by Schwentner et al. (2018). This analysis was performed to obtain node age estimates within Spinicaudata for the subsequent molecular clock analyses using the four gene data set. (download)

Fig. S11. Molecular clock dated phylogeny based on the amino acid data set of Schwentner et al. (2018) employing minimum age 380 mya for Limnadiidae + *Eocyzicus* + Leptestheriidae. The topology was fixed

to the one obtained by Schwentner et al. (2018). This analysis was performed to obtain node age estimates within Spinicaudata for the subsequent molecular clock analyses using the four gene data set. (download)

Fig. S12. Constrained molecular clock dated phylogeny employing minimum age 255 mya for Limnadiidae + *Eocyzicus* + Leptestheriidae. The divergence time estimates are based on the BEAST analyses of *COI*, *16S rRNA*, *EF1α* and *28S rRNA* that included only individuals with at least three of the loci present. The topology was constrained to enforce a sister group relationship of Leptestheriidae and *Eocyzicus* (see Fig. 4 for the unconstrained analysis). Calibration points (6) and (11) are based on fossils for Diplostraca (minimum age 386.9 mya based on *Leaia chinensis* following Wolfe et al. (2016) and Limnadiidae + *Eocyzicus* + Leptestheriidae (minimum age 255 mya based on oldest known Perilimnadiidae fossils following Astrop and Hegna (2015), respectively (Figs. S13 and S14 for an alternative age constraint for Limnadiidae + *Eocyzicus* + Leptestheriidae). Calibrations points (A)–(D) were inferred from the preceding molecular clock analyses of the amino acid data set of Schwentner et al. (2018) (Figs. S10 and S11) and coded as normal distributed priors: (A) 294.6 mya with sigma of 25, (B) 153.5 mya with sigma of 63, (C) 66.8 mya with a sigma of 40, (D) 64.8 mya with a sigma of 32 and (E) 146 mya with a sigma of 69. Branches are color-coded according the geographic origin of the specimens. Posterior probabilities are provided for each branch. (download)

Fig. S13. Unconstrained molecular clock dated phylogeny employing minimum age 380 mya for Limnadiidae + *Eocyzicus* + Leptestheriidae. The divergence time estimates are based on the BEAST analyses of *COI*, *16S rRNA*, *EF1α* and *28S rRNA* that included only individuals with at least three of the loci present. The topology was not constrained to enforce a sister group relationship of Leptestheriidae and *Eocyzicus*, thus also no prior was defined for their divergence (see Fig. S14 for the constrained analysis). Calibration points (6) and (11) are based on fossils for Diplostraca (minimum age 386.9 mya based on *Leaia chinensis* following Wolfe et al. (2016)) and Limnadiidae + *Eocyzicus* + Leptestheriidae (minimum age 380mya based on the assumption that Vertexioidea (known since the mid Devonian) is ancestral to Limnadiidae, while Eosestherioidea is ancestral to Leptestheriidae and Cyzicidae following Astrop and Hegna (2015), respectively (Fig. 4 and Fig. S12 alternative age constraint for Limnadiidae + *Eocyzicus* + Leptestheriidae). Calibrations points (A)–(D) were inferred from the preceding molecular clock analyses of the amino acid data set of Schwentner et al. (2018) (see Figs. S10 and S11) and coded as normal distributed priors: (A) 403 mya with sigma 10, (B) 155.6 mya with sigma 70, (C) 61.6 mya with sigma 30 and (D) 67 mya with sigma 35. Branches are color-coded according the geographic origin of the specimens. Posterior probabilities are provided for each branch. (download)

Fig. S14. Constrained molecular clock dated phylogeny employing minimum age 380 mya for Limnadiidae + *Eocyzicus* + Leptestheriidae. The divergence time estimates are based on the BEAST analyses of *COI*, *16S rRNA*, *EF1α* and *28S rRNA* that included only individuals with at least three of the loci present. The topology was constrained to enforce a sister group relationship of Leptestheriidae and *Eocyzicus*, thus also no prior was defined for their divergence (Fig. S13 for the constrained analysis). Calibration points (6) and (11) are based on fossils for Diplostraca (minimum age 386.9 mya based on *Leaia chinensis* following Wolfe et al. (2016)) and Limnadiidae + *Eocyzicus* + Leptestheriidae (minimum age 380 mya based on the assumption that Vertexioidea (known since the mid Devonian) is ancestral to Limnadiidae, while Eosestherioidea is ancestral to Leptestheriidae and Cyzicidae following Astrop and Hegna (2015), respectively (Fig. 4 and Fig. S12 alternative age constraint for Limnadiidae + *Eocyzicus* + Leptestheriidae). Calibrations points (A)–(D) were inferred from the preceding molecular clock analyses of the amino acid data set of Schwentner et al. (2018) (Figs. S10 and S11) and coded as normal distributed priors: (A) 403 mya with sigma 10, (B) 155.6 mya with sigma 70, (C) 61.6 mya with sigma 30, (D) 67 mya with sigma 35 and (E) 154 mya with sigma 75. Branches are color-coded according the geographic origin of the specimens. Posterior probabilities are provided for each branch. (download)

Table S1. List of all individuals studied. For each individual, we provide species names, the species name under which the respective genetic resources have been deposited in GenBank (only applicable for sequences obtained from earlier studies), details on the collection locality and collection event (for sequences obtained from GenBank only the country is provided), collection or specimen numbers used to identify the specimens, the respective citations for published sequences and the GenBank accession numbers for the four genes. Collection numbers refer to the Australian Museum Sydney (AM P.), Museum of Comparative Zoology, Harvard University (MCZ IZ) and the Center of Natural History in Hamburg (ZMH K). (download)