Using mitochondrial genomes to infer phylogenetic relationships among the oldest extant winged insects (Palaeoptera)

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Abstract

Phylogenetic relationships among the basal orders of winged insects remain unclear, in particular the relationship of the Ephemeroptera (mayflies) and the Odonata (dragonflies and damselflies) with the Neoptera. Insect evolution is thought to have followed rapid divergence in the distant past and phylogenetic reconstruction may therefore be susceptible to problems of taxon sampling, choice of outgroup, marker selection, and tree reconstruction method. Here we newly sequenced three mitochondrial genomes representing the two most diverse families of the Ephemeroptera, one of which is a basal lineage of the order. We then used an additional 90 insect mitochondrial genomes to reconstruct their phylogeny using Bayesian and maximum likelihood approaches. Bayesian analysis supported a basal Odonata hypothesis, with Ephemeroptera as sister group to the remaining insects. This was only supported when using an optimized data matrix from which rogue taxa and terminals affected by long-branch attraction were removed. None of our analyses supported a basal Ephemeroptera hypothesis or Ephemeroptera + Odonata as monophyletic clade sister to other insects (i.e., the Palaeoptera hypothesis). Our newly sequenced mitochondrial genomes of *Baetis rutilocylindratus*, *Cloeon dipterum*, and *Habrophlebiodes zijinensis* had a complete set of protein coding genes and a conserved orientation except for two inverted tRNAs in *H. zijinensis.* Increased mayfly sampling, removal of problematic taxa, and a Bayesian phylogenetic framework were needed to infer phylogenetic relationships within the three ancient insect lineages of Odonata, Ephemeroptera, and Neoptera. Pruning of rogue taxa improved the number of supported nodes in all phylogenetic trees. Our results add to previous evidence for the Odonata hypothesis and indicate that the phylogenetic resolution of the basal insects can be resolved with more data and sampling effort.

Keywords: *Ephemeroptera; Long-branch attraction; Mayfly; Odonata; Palaeoptera problem; Rogue taxa*

1. Introduction

Insects are the most diverse branch of metazoan life, yet the relationships among the basal branches of the winged insects (Pterygota) remains one of the major open questions in the evolution of life. The unresolved relationship of the basal orders Odonata (dragon- and damselflies) and Ephemeroptera (mayflies) to the Neoptera (i.e., to the rest of the Pterygota) has been termed the "Palaeoptera problem" (Hovmöller, 2002; Ogden and Whiting, 2003). The name Paleoptera ("old wings") reflects the inability of the Ephemeroptera and Odonata to fold their wings flat over the abdomen. This has long been considered to be the ancestral condition, in contrast to the more derived Neoptera ("new wings") (reviewed by Trautwein et al. 2012). The monophyly of the Neoptera, including the three major lineages Polyneoptera, Paraneoptera, and Holometabola, is widely accepted, although relationships among polyneopteran lineages (Whitfield and Kjer, 2008) and the monophyly of the Paraneoptera (Ishiwata et al., 2011) remain unresolved.

There are three competing hypotheses relating to the Palaeoptera problem: the Palaeoptera hypothesis ((Ephemeroptera + Odonata) + Neoptera), the basal Ephemeroptera hypothesis (Ephemeroptera + (Odonata + Neoptera)), and the basal Odonata hypothesis (Odonata + (Ephemeroptera + Neoptera)) (Kjer et al., 2016; Trautwein et al., 2012; Yeates et al., 2012). All hypotheses are supported by morphological as well as molecular data to varying degrees (reviewed by Trautwein et al. 2012). Different authors, using the same set of genes and taxa set but different phylogenetic approaches, namely Bayesian inference (BI) vs. maximum likelihood (ML) (Mallatt and Giribet, 2006; Meusemann et al., 2010; Ogden and Whiting, 2003), and nucleotide vs. amino acid (aa) sequences (Song et al., 2016) obtained results supporting distinct hypotheses. The Palaeoptera hypothesis has received support from several molecular studies based on rRNA genes and nuclear DNA (Ishiwata et al., 2011; Kjer et al., 2006; Sasaki et al., 2013; Thomas et al., 2013), and from nDNA phylogenomic analyses (Meusemann et al., 2010; Regier et al., 2010; Reumont et al., 2012). Thomas et al. (2013) and Kjer et al. (2006) included the most comprehensive taxon

67 sampling with 35, respectively seven species from each of the three lineages (i.e., Ephemeroptera,
68 Odonata, Neoptera) analyzing seven, respectively eight genes (rRNAs, nuclear DNA (nDNA),
69 mitochondrial DNA (mtDNA)). The basal Ephemeroptera hypothesis was supported by previous
70 studies using mitochondrial genome data (Ma et al., 2014; Song et al., 2016; Zhang et al., 2008), and
71 a combined analysis of rRNA and one nuclear gene (Ogden and Whiting, 2003). Other studies have
72 concluded the relationships are ambiguous (Ogden and Whiting, 2003). Notably, studies supporting
73 the basal Ephemeroptera hypothesis have included either a limited number of mayfly species (e.g.,
74 one (Whiting et al., 1997; Zhang et al., 2008), two (Giribet and Ribera, 2000; Ma et al., 2014), four
75 (Ma et al., 2015), or five (Song et al., 2016) species) or a limited number of unlinked loci (e.g., one
76 (Ma et al., 2014; Ma et al., 2015; Song et al., 2016; Zhang et al., 2008), two (Giribet and Ribera,
77 2000; Wheeler et al., 2001; Whiting et al., 1997), or three (Ogden and Whiting, 2003)). The basal
78 Odonata hypothesis has received support from previous studies based on mitochondrial genome data
79 (Lin et al., 2010; Wan et al., 2012), rRNA genes (Kjer, 2004; Mallatt and Giribet, 2006; Misof et al.,
80 2007; Reumont et al., 2009; Yoshizawa and Johnson, 2005), combining rRNA and nDNA (Blanke et
81 al., 2013), and phylogenomic nDNA (Meusemann et al., 2010; Simon et al., 2009). These studies,
82 with the exceptions of few studies based on *18S* rRNA (Kjer, 2004; Misof et al., 2007; Yoshizawa
83 and Johnson, 2005), included in total between two (Mallatt and Giribet, 2006; Simon et al., 2009)
84 and 13 species (Reumont et al., 2009) from the orders Ephemeroptera and Odonata.

85 The conflicting phylogenetic signals may result from the ancient radiation of the lineages
86 Ephemeroptera, Odonata, and Neoptera from a common ancestor in the distant past (Whitfield and
87 Kjer, 2008). These three ancient lineages appear to have diverged rapidly, leaving few characteristics
88 to determine their phylogenetic relationships (Whitfield and Kjer, 2008). Evolutionary rate
89 heterogeneity across clades and the representation of old clades by recent extant taxa make ancient
90 relationships such as those of early winged insects the most difficult challenges for phylogenetics
91 (Kjer et al., 2016; Whitfield and Kjer, 2008). Given the weak phylogenetic signal, reconstructions

92 are more susceptible to systematic errors (Baurain et al., 2007; Rokas and Carroll, 2006; Thomas et
93 al., 2013; Whitfield and Kjer, 2008; Whitfield and Lockhart, 2007). Other sources of conflicting
94 signal in studies of the relationships among these three lineages may result from differences in taxon
95 sampling, sequence data, alignment methods, and phylogenetic methods used including models of
96 evolution (Talavera and Vila, 2011; Thomas et al., 2013; Trautwein et al., 2012). The use of
97 mitochondrial protein-coding genes (PCGs) derived from mitochondrial genomes can circumvent
98 some of these difficulties, since they are easier to align and also appropriate models of molecular
99 evolution are well established (Abascal et al., 2006; 2005; Le et al., 2017). On the other hand,
100 mitochondrial genes include several drawbacks, most importantly the possible presence of
101 pseudogenes (Bensasson, 2001; Rogers and Griffiths-Jones, 2012; Song et al., 2008). However, data
102 derived from high-throughput sequencing, depending on the coverage bears the potential to
103 distinguish between functional genes and pseudogenes based on higher mutation rates of the later
104 (Claes and De Leeneer, 2014).

105 Overall, mitochondrial markers are well studied and therefore the most widely employed genetic
106 markers in insects, being considered a promising "instrument" for insect systematics (Cameron,
107 2014). Insect mitochondrial genomes are highly conserved, ranging from 15 to 18 kb in length,
108 containing 37 genes: 13 PCGs, two ribosomal RNAs (rRNAs, *rrnL* and *rrnS*), and 22 transfer RNAs
109 (tRNAs, *trn**) (Boore, 1999). A non-coding region of variable length, thought to be the origin of
110 initiation of transcription and replication, is typically present (Saito, 2005; Zhang and Hewitt, 1997)
111 and referred to as the AT-rich region. The typical ancestral insect mitochondrial genome differs from
112 the ancestral arthropod mitochondrial genome only by the location of *trnL* (Boore et al., 1995).
113 Significant differences in structure, gene content, and gene arrangement have been found to be the
114 exception for highly derived taxa.

115 Importantly there is still an unbalance in the numbers of available molecular markers between the
116 different insect lineages. In particular the early winged insects (i.e., Ephemeroptera and Odonata) are

117 relatively under-represented. This is (partly) because so far most genomic data were obtained for
118 insect orders that are of economical interest (e.g., as pollinators, model species, agricultural pests,
119 and vectors of human diseases, Robinson et al., 2011). As of January 2017, there were 39 complete
120 or nearly complete palaeopteran mitochondrial genomes (Ephemeroptera: 18, Odonata: 21) from 22
121 families (eleven of each) available on GenBank; 22 of which were included in publications
122 (Ephemeroptera: ten, Odonata: twelve). Compared to the number of families that have been
123 described (Ephemeroptera: 42 (Barber-James et al., 2008), Odonata: 30 (Dijkstra et al., 2013)) this is
124 still a relatively low number.

125 The insect order Ephemeroptera comprises over 3,000 species, comprising as the two most
126 species rich families the Baetidae (833 species) and the Leptophlebiidae (608 species) (Barber-James
127 et al., 2008). Their biogeographic origin is probably Pangean, including a greater diversity and
128 higher endemism rate in the Neotropics and Australasia for the Baetidae, and the Neotropical and
129 Afrotropical regions for the Leptophlebiidae, respectively (Barber-James et al., 2008).

130 We investigated the relationships of the oldest extant winged insects and newly sequenced three
131 mayfly mitochondrial genomes to improve sampling of this under-represented order. New taxa
132 included one representative of the family Leptophlebiidae, for which no mitochondrial genome data
133 were available, and two representatives of the Baetidae, one from each subfamily. We used BI and
134 ML approaches and created matrices that eliminated taxa that reduced overall tree support (rogue
135 taxa) and that were affected by long-branch attraction (LBA). Our analysis included 29 palaeopteran
136 mitochondrial genomes from 20 families and 64 other insect mitochondrial genomes. We expect the
137 increased taxon sampling to improve the phylogenetic signal of the basal nodes and add support for
138 one of the three competing hypotheses.

139 2. Material and methods

140 *2.1. Taxon sampling*

141 We newly sequenced three mitochondrial genomes of the two most diverse Ephemeroptera
142 families. *Habrophlebiodes zijinensis* GUI, ZHANG & WU, 1996 is the first representative of the family
143 Leptophlebiidae to have its complete mitochondrial genome sequenced. *Baetis rutilocylindratus*
144 WANG, QIN, CHEN & ZHOU, 2011 and *Cloeon dipterum* L. 1761 are both members of the Baetidae
145 and representative each subfamily: *B. rutilocylindratus* from Baetinae, and *C. dipterum* from
146 Cloeninae. There was a mitochondrial genome from one other member of the Baetinae available on
147 GenBank prior to our study (*Alainites yixiani*, GQ502451, Jia & Zhou). Our analysis also included
148 four species from Archaeognatha, three from Zygentoma (formerly Thysanura), ten from Odonata,
149 16 additional species from Ephemeroptera, four from Plecoptera, four from Ensifera of Orthoptera,
150 nine from Caelifera of Orthoptera, 13 from Phasmatodea, two from Mantodea, six from Blattodea, 13
151 from Isoptera, and one each from Collembola, Dermaptera, Grylloblattodea, and Mantophasmatodea
152 (Table 1). Amino acid sequences were obtained from GenBank using a custom Python script
153 (mitogenome_ncbi.py, https://github.com/srutschmann/python_scripts).

154 ***2.2. Sequencing and assembly***

155 The three mayfly species were sequenced with either 454/FLX pyrosequencing (*C. dipterum*) or
156 Sanger sequencing (*B. rutilocylindratus* and *H. zijinensis*). We extracted DNA from *C. dipterum*
157 from twelve to twenty pooled reared subimago specimens, prepared a shotgun library, and sequenced
158 four lanes on a Roche (454) *GS FLX* machine at the Berlin Center for Genomics in Biodiversity
159 Research (BeGenDiv, Berlin, Germany) (see also Rutschmann et al., 2017). The obtained sequence
160 reads were trimmed and *de novo* assembled using the software Newbler v.2.5.3 (454 Life Science
161 Cooperation) under default settings for large data sets. In order to extract the mitochondrial genome
162 of *C. dipterum*, we performed BLASTN searches (Altschul, 1997) using as query all assembled
163 contigs against the NCBI database. We mapped all matching reads back to the mitochondrial genome
164 with BWA (Li and Durbin, 2009), using the BWA-SW algorithm (Li and Durbin, 2010) with settings

suggested for 454 data by CORAL (match score = 2, mismatch penalty = 2, and gap open penalty = 3, Salmela and Schroder, 2011).

Specimens of *B. rutilocylindratus* and *H. zijinensis* were collected in Zijin Hill, Nanjing, China. The DNA was extracted from between two and four larvae using the DNeasy® Blood & Tissue (Qiagen, Leipzig, Germany) kit. Four DNA fragments of each species were amplified with universal primers (*B. rutilocylindratus*: *cob*, *cox1*, *cox3*, *rrnL*; *H. zijinensis*: *cob*, *cox1*, *nad4*, *rrnL*, (Simon et al., 1994)). Subsequently, based on the previously obtained sequence information we designed six (*B. rutilocylindratus*) respective four (*H. zijinensis*) specific primer pairs (Supplementary Table S1; see also Li et al. (2014)). Standard and long polymerase chain reactions (PCRs) were performed on a DNA Engine Peltier Thermal Cycler (Bio-Rad, Shanghai, China). Therefore, we used the rTaq™ DNA polymerase (TaKaRa Bio, Dalian, China) for fragments smaller than two kb and the LA Taq™ polymerase (TaKaRa Bio, Dalian, China) for fragments larger than two kb. All PCR products were purified with the Axygen agarose-out kit. When the PCR amplification signal was too weak to sequence or sequencing resulted in overlap peaks, the products were ligated to pGEM®-T Easy Vector (Promega, Southampton, UK) by *Escherichia coli*, and each resulting clone was sequenced. All purified amplification products were sequenced successively in both directions on an ABI3130xl capillary sequencer. Forward and reverse sequences were assembled and edited using CodonCode Aligner v.3.5.6 (CodonCode Corporation).

2.3. Sequence alignments and data matrices

We prepared two data matrices of aa sequences based on the annotated mitochondrial genomes (see 2.5. Annotation and characterization), including a set with all 93 taxa (all_taxa matrix), and a matrix for which we removed taxa that were either identified as rogue taxa using the RogueNaRok algorithm (Aberer et al., 2012; Wilkinson, 1996) and showing LBA (Felsenstein, 1978; Hedtke et al., 2006) (optimized_taxa matrix; 88 taxa, Table 1). We used GUIDANCE2 (Landan and Graur, 2008;

189 Sela et al., 2015) to align the sequences, and identify and remove positions detected as ambiguously
190 aligned regions. To run GUIDANCE we applied the default settings, using as multiple sequences
191 alignment program MAFFT v.7.050b (L-INS-I algorithm with default settings, Katoh and Standley,
192 2013).

193 The best-fitting partitioning schemes and corresponding aa substitution models were estimated
194 with PartitionFinder v.2 (https://github.com/brettc/partitionfinder, Lanfear et al., 2012). We used the
195 Bayesian Information Criterion (BIC) to choose the best model, linked branch lengths, and a greedy
196 search (Lanfear et al., 2012) with RAxML v.8.2.8 (Stamatakis, 2014). Thereby each gene was
197 defined as one data block (i.e., possible partition).

198 To identify rogue taxa, we applied the RogueNaRok algorithm (Aberer et al., 2012; Wilkinson,
199 1996). We used the bootstrap replicates and the best supported maximum likelihood tree based on
200 the all_taxa matrix (see 3.1. Data matrices). Identified rogue taxa were removed from the all_taxa
201 matrix, resulting in the optimized_taxa matrix (Table 1). Taxa showing evidence of LBA *sensu*
202 Bergsten (Bergsten, 2005) were identified using heterogeneities in sequence divergence based on a
203 model of evolution derived from the entire data matrix. For this, the Relative Composition Frequency
204 Variability (RCFV) values of each species were calculated with BaCoCa (Kück and Struck, 2014)
205 using the partitioned concatenated data matrix (i.e., all_taxa matrix). Taxa with the highest values
206 were excluded in the optimized_taxa matrix, apart from the Ephemeroptera, which were retained
207 because they are the focus of our study and their monophyly is well-established (Hovmöller, 2002;
208 Ogden and Whiting, 2003).

209 ***2.4. Phylogenetic reconstruction***

210 Phylogenetic reconstructions were carried out using MrBayes v.3.2.4 (Ronquist et al., 2012), and
211 RAxML v.8.1.7 (Stamatakis, 2014). We analyzed both matrices (all_taxa, optimized_taxa) using the
212 best-fitting partitioning scheme and model for each (Table 2). For BI, we unlinked the frequencies,
213 gamma distributions, substitution rates and the proportion of invariant sites across partitions. For

214 each matrix, we run two independent analyses of four MCMC chains with 10^7 generations, sampling
215 every 10^3 generations and discarding a burn-in of 25%. Maximum likelihood inferences were
216 performed with 200 bootstrap replicates. Trees reconstructed from the all_taxa matrix were rooted
217 with *Tetrodontophora bielanensis* (Collembola). Trees reconstructed from the optimized_taxa matrix
218 were rooted with *Nesomachilis australica* (Archaeognatha, according to Song et al. 2016) because
219 *Tetrodontophora bielanensis* was found to be a rogue taxon (see 3. Results). All trees were
220 visualized with the ape v.4.1 (Paradis et al., 2004), phangorn v.2.1.1 (Schliep, 2011), and ggtree
221 v.1.4.20 (Yu et al., 2017) packages for R v.3 (R Development Core Team, 2012).

222 ***2.5. Annotation and characterization***

223 All mitochondrial genomes were annotated using the MITOS webserver (http://mitos.bioinf.uni-
224 leipzig.de/index.py, Bernt et al., 2013), DOGMA (Wyman et al., 2004), and MFannot
225 (http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl). The predicted PCGs were
226 checked for stop codons and manually adjusted by comparison with the longest predicted open
227 reading frames as implemented in Geneious R7 v.7.1.3 (Biomatters Ltd.), and by comparison with
228 the respective homologous insect sequence alignment. For the tRNA prediction, we additionally used
229 ARWEN v.1.2.3 (Laslett and Canback, 2008) and tRNAscan-SE v.1.21
230 (http://lowelab.ucsc.edu/tRNAscan-SE/, Lowe and Eddy, 1997). The annotated mitochondrial
231 genomes were visualized with OrganellarGenomeDRAW (http://ogdraw.mpimp-golm.mpg.de/cgi-
232 bin/ogdraw.pl, Lohse et al., 2013) and manually edited.

233 Nucleotide contents were retrieved using Geneious. To correct biases in the AT content due to
234 incomplete mitochondrial genomes mostly missing the AT-rich region, we removed all AT-rich
235 regions and the two rRNAs including the five close-by tRNAs (*trnL1-trnM*) and recalculated the
236 nucleotide base pair (bp) compositions. The AT and GC composition skewness were calculated as
237 follows: AT-skew = (A - T) / (A + T), and GC-skew = (G - C) / (G + C) (Perna and Kocher, 1995).

238 # 3. Results

239 ### *3.1. Data matrices*

240 The final matrices had lengths of 3,675 aa (all_taxa matrix, including 93 taxa) and 3,673 aa
241 (optimized_taxa matrix, including 88 taxa, Table 1). As best-fitting partitioning scheme for the two
242 matrices, we identified either five partitions (all_taxa matrix, Table 2) or four partitions
243 (optimized_taxa, Table 2). As best-fitting models of aa sequence evolution, we identified MtZoa
244 (Rota-Stabelli et al., 2009) and MtArt (Abascal et al., 2006).

245 The RogueNaRok analysis identified the outgroup *T. bielanensis* as well as *Gryllotalpa orientalis*
246 (Orthoptera) and *Phraortes* sp. (Phasmatodea) as taxa with uncertain phylogenetic positions, leading
247 to less accurate phylogenetic reconstructions. *G. orientalis* (Orthoptera) and *Phraortes* sp.
248 (Phasmatodea) clustered within the corresponding order using the all_taxa matrix, although the
249 nodes containing these species were not supported by ML. In the test for LBA, *Aposthonia japonica*
250 (Embioptera) and *Challia fletcheri* (Dermaptera) had high RCFV values (0.0237 and 0.0307,
251 respectively) suggesting they were affected by LBA. Both species clustered within other orders:
252 *Challia fletcheri* (Dermaptera) within the Ephemeroptera as sister taxon to a clade containing all
253 representatives of the family Baetidae, and *A. japonica* (Embioptera) within the Phasmotodea as
254 sister taxon to a clade containing all species of the order with the exception of *T. californicum* (Table
255 3, Supplementary Fig. S1). The mayflies Teloganodidae sp. (0.0305) and the three baetids *A. yixiani*
256 (0.0235), *B. rutilocylindratus* (0.0232), and *C. dipterum* (0.0201) showed relatively high RCFV
257 values.

258 ### *3.2. Phylogenetic reconstruction*

259 The Bayesian reconstruction based on the optimized_taxa matrix produced the tree with most
260 support compared to ML or to either analysis of the all_taxa matrix (Fig. 1, Table 3, Supplementary
261 Figs S1-S3). The basal Odonata hypothesis was highly supported in this tree (Bayesian posterior

262 probability (BPP) = 1.00), whereas neither the basal Ephemeroptera hypothesis nor the Palaeoptera
263 hypothesis received support in any analysis (Table 3). The Odonata were monophyletic in all
264 analyses (BPP = 1.00, BS = 100%) whereas the Ephemeroptera were monophyletic only using the
265 optimized_taxa matrix (Fig. 1, Table 3, Supplementary Fig. S2).

266 Most other nodes of interest were consistently supported across different matrices and analyses
267 (Table 3). The Polyneoptera were moderately supported (BS ≥ 80%) for the optimized_taxa matrix
268 using the ML. Within the Polyneoptera, all orders except Orthoptera and Blattodea were
269 monophyletic (BPP ≥ 0.95, and BS ≥ 90%, Table 3). The two Orthoptera suborders Ensifera and
270 Caelifera were monophyletic (Table 3), whereby the Ensifera clustered together with the Plecoptera
271 for the optimized_taxa matrix using both ML and BI. The Grylloblattodea, Mantophasmatodea, and
272 Phasmatodea were all well supported using the optimized_taxa matrix. For the all_taxa matrix also
273 the Embioptera were recovered in this clade as sister taxa to the Phasmatodea excluding *Timema*
274 *californicum* (Fig. 1, Supplementary Figs S1, S3). The superorder Dictyoptera was monophyletic
275 clade, consisting of the monophyletic Mantodea and the Blattodea including the Isoptera.

276 ***3.3. Mitochondrial genomes***

277 The mitochondrial genome sequences have been deposited at GenBank with the accession
278 numbers XXXXXXXX, XXXXXXXX, and XXXXXXXX. The pyrosequencing run of *C. dipterum*
279 using the 454 GS FLX system resulted in 651,306 reads, of which 1.14% mapped to the assembled
280 mitochondrial genome. The depth of coverage for the *C. dipterum* mitochondrial genome was 249.2x
281 (± 80.6 SD, Fig. 2a). The three mayfly mitochondrial genomes were 15,407 bp (*C. dipterum*), 14,883
282 bp (*B. rutilocylindratus)*, and 14,355 bp (*H. zijinensis*) long (Table 4). For *B. rutilocylindratus*, the
283 complete mitochondrial genome was sequenced (Fig. 2b). The AT-rich regions were incomplete for
284 genomes of *C. dipterum* and *H. zijinensis*. The three tRNAs between the AT-rich region and *nad2*
285 were also missing in *H. zijinensis* due to the incomplete sequencing (Fig. 2c). All three sequenced
286 mitochondrial genomes contained the entire set of 13 PCGs, two rRNAs, and either 22 tRNAs (*B.*

287 *rutilocylindratus*, *C. dipterum*) or 19 for the incomplete mitochondrial genome of *H. zijinensis*,
288 including 23 coded at the (+) strand (21 for *H. zijinensis*) and 14 at the (-) strand (13 for *H.*
289 *zijinensis*) (Fig. 2).

290 The order of the genes was conserved, with the exceptions of the two inverted tRNAs Arginine
291 and Alanine for *H. zijinensis* (Fig. 2c). All PCGs started with the ATN codons (ATT, ATG, ATA),
292 and mostly ended with the complete termination codon (TAA or TAG). Exceptions were an
293 incomplete T termination codon for *nad4* in *B. rutilocylindratus* and *cox1* started with CTC in *C.*
294 *dipterum*. The AT content of the mitochondrial genomes that were corrected for the missing regions,
295 was lowest in *B. rutilocylindratus* (59.9%, Table 4, Supplementary Table S2). The mean AT content
296 for all available mayfly mitochondrial genomes was 65.34%. The average AT-skew was -0.03 (±
297 0.04 SD) and the average GC-skew was -0.13 (± 0.14 SD).

298 4. Discussion

299 *4.1. Phylogenetic relationships of Palaeoptera*

300 Our Bayesian reconstruction based on the optimized_taxa matrix provided strong support for the
301 basal Odonata hypothesis. Previous work based on mitochondrial genome data is (partially)
302 congruent with our findings (Lin et al., 2010; Song et al., 2016; Wan et al., 2012). In contrast to our
303 results, a handful of studies based on nucleotide and aa sequences of mitochondrial PCGs and
304 mitochondrial RNA (mtRNAs) found overall strong support for the basal Ephemeroptera hypothesis
305 (Ma et al., 2014; Ma et al., 2015; Song et al., 2016; Zhang et al., 2008). Interestingly, Song et al.
306 (2016), supporting overall the Ephemeroptera hypothesis, found evidence for the Odonata hypothesis
307 when using aa sequence data. Our results confirm the trend towards a better support for the ancestral
308 position of the Odonata when using an increased mayfly taxon sampling (more than ten species per
309 lineage) (Kjer, 2004; Misof et al., 2007; Yoshizawa and Johnson, 2005). The exceptions for this are
310 the work by Ogden and Whiting (2003) and Thomas et al. (2013), that overall support the

311 Ephemeroptera respective Palaeoptera hypothesis but contain some data sets in favor of the Odonata
312 hypothesis. Early inconsistencies based on different sequence alignment strategies (Ogden and
313 Whiting, 2003) appear to become less relevant due to the advances of multiple sequence alignment
314 programs. Overall, the vast majority of studies were based on BI and ML. Thomas et al. (2013)
315 recovered increased resolution using BI in comparison to ML. Different data partitioning (i.e., gene
316 trees resulting in different tree topologies) by Kjer et al. (2006) supported different hypotheses for
317 different genes. Evidently, using rRNAs (nDNA and mtDNA) and the nDNA EF1-α the Odonata
318 hypothesis was recovered whereas the analyses based on the combined and on the mtDNA data
319 matrix resulted in a monophyletic palaeopteran clade. It is questionable whether an increase in
320 analyzed genomic data (i.e., phylogenomic studies) will be able to resolve the Palaeoptera problem.
321 Notably, Misof et al. (2014) and Regier et al. (2010), using 1,478 PCGs and seven palaeopteran
322 species, respective 62 PCGs and four palaeoptera species, recovered the Ephemeroptera and Odonata
323 as sister clades (i.e., the Palaeoptera hypothesis) but without high support values. Overall, more
324 knowledge about the use and limitation of individual markers are needed. For example Simon et al.
325 (2012) found that proteins involved in cellular processes and signaling harbor the most phylogenetic
326 signal.

327 The increased mayfly taxon sampling highlighted the phylogenetic diversity (as indicated by the
328 branch lengths of the tip taxa, Fig. 1, Supplementary Figs S1-S3) of the Palaeoptera. The overall
329 long-branch lengths and high rate-heterogeneity across sites might be one of the explanations for the
330 "Palaeoptera problem". Especially the family of the Baetidae, which was long thought to be the most
331 ancestral mayfly family (Ogden and Whiting, 2005), showed high base composition heterogeneity
332 possibly evidencing LBA. However, in agreement with other more recent studies we also recovered
333 *S. chinensis* as sister taxa to all other mayflies (Li et al., 2014; Ogden et al., 2009). It remains to be
334 resolved whether an increased taxon sampling may obviate LBA *sensu* Bergsten (2005).

335 ***4.2. Phylogenetic relationships of Polyneoptera***

336 Besides the Palaeoptera, a universal consensus on the placement of the Plecoptera, Dermaptera,
337 Embioptera, and Xenoptera (i.e., Grylloblattodea and Mantophasmatodea) remains elusive (Song et
338 al., 2016). The monophyly of the Polyneoptera has become more widely accepted (Ishiwata et al.,
339 2011; Letsch and Simon, 2013; Letsch et al., 2012; Misof et al., 2014; Song et al., 2016; Wan et al.,
340 2012), although several studies and data matrices do not support their monophyly (Kjer et al., 2006;
341 Simon et al., 2012; Song et al., 2016). This is also reflected by our results, recovering the
342 Polyneoptera as moderately supported monophyletic clade when using the optimized_taxa matrix
343 with ML. The placement of the order Plecoptera also remains elusive. The order often clusters
344 among the more ancestral polyneopteran orders (Misof et al., 2014; Song et al., 2016; Wan et al.,
345 2012) and in a close relationship to the Dermaptera, although earlier studies using few species and
346 the aa sequences of the mitochondrial PCGs also recovered a sister relationship to the Ephemeroptera
347 (Lin et al., 2010; Zhang et al., 2009). Notably, Song et al. (2016) was the only study including four
348 dermapteran species. The monophyly of the Orthoptera has been established by previous studies
349 based on mitochondrial genome data and a large number of PCGs (Fenn et al., 2008; Ma et al., 2009;
350 Misof et al., 2014; Song et al., 2016). However, in a recent study, the Ensifera also clustered as more
351 ancestral clade (Song et al., 2016). As for this study, Song et al. (2016) also used BI and ML based
352 on mitochondrial genome data. Notably, Song et al. (2016) explain the more ancestral position of
353 Ensifera by more similar sequence compositional bias in comparison to the outgroups. The
354 relationship between Grylloblattodea and Mantophasmatodea (i.e., Xenoptera superorder) remains
355 elusive. Mostly this is due to the limited amount of available molecular data, including ordinal-level
356 phylogenies based on two (Ishiwata et al., 2011; Song et al., 2016) or three xenopteran species
357 (Misof et al., 2014). Using mitochondrial genome data and one species per order, also Song et al.
358 (2016) did not recover them as a monophyletic clade. Their monophyly was supported by previous
359 studies using one Mantophasmatodea and one Grylloblattodea species (Ishiwata et al., 2011),
360 respective one Mantophasmatodea and two Grylloblattodea species (Misof et al., 2014). The

361 Phasmatodea have also been found in previous studies as paraphyletic clade due to the outside
362 position of *T. californicum* (Ma et al., 2014; Song et al., 2016). In contrast, Misof et al. (2014), using
363 a large set of nuclear PCGs also found *Timema* as the sister species to all other representatives of the
364 order. The monophyly of the superorder Dictyoptera is generally accepted and the hierarchical
365 clustering (Mantodea + (Blattodea + Isoptera)) has been well supported (Inward et al., 2007;
366 Ishiwata et al., 2011; Kjer et al., 2006; Legendre et al., 2015; Ma et al., 2014; Misof et al., 2014;
367 Song et al., 2016; Wan et al., 2012) using nucleotide and aa sequences of both mtDNA and nDNA.

368 ***4.3. Optimizing data matrix***

369 The optimization of the data matrix overall resulted in a better-supported tree (as measured by the
370 total number of supported nodes and the fact that all nodes supported in the all_taxa matrix were also
371 recovered in the optimized_taxa matrix). In a study on butterfly phylogenetics, the authors also
372 removed rogue taxa and found dramatically increased bootstrap support values (Regier et al., 2013).
373 Comparing the number of supported nodes from the BI and ML approach, the former always resulted
374 in more resolved nodes. This result is consistent with previous work on the "Palaeoptera problem",
375 recovering more support for analyses using BI (Thomas et al., 2013). Generally, the choice of the
376 outgroup species was reported as being crucial for resolving problematic splits in the tree of life such
377 as insects origin (Thomas et al., 2013). Thus, finding the outgroup as a rogue taxon was perhaps not
378 surprising. The close phylogenetic relationship of the Dermaptera and Ephemeroptera
379 (Supplementary Figs S1, S3) might be misleading due to LBA. Li et al. (2014) also found the order
380 Dermaptera as being closely related to the mayflies. On the other hand studies based on
381 phylogenomic data reported the Dermaptera as sister taxa to the orders Plecoptera (Ishiwata et al.,
382 2011; Ma et al., 2014; Simon et al., 2012; Wan et al., 2012; Wu et al., 2014) Zoraptera (Blanke et al.,
383 2013; Misof et al., 2014). Using mitochondrial genome data and an increased taxon sampling (i.e.,
384 four dermapteran species), Song et al. (2016) found the Dermaptera and/or Plecoptera being sister to
385 the remaining taxa within Polyneoptera. Notably, when only using one species of Dermaptera, *C.*

386 *fletcheri* emerged as sister taxa to the Ephemeroptera (Song et al., 2016). This result in agreement
387 with our findings is presumably due to LBA. Phylogenomic data matrices (e.g., expressed sequence
388 tags data: Letsch et al., 2012, 1,478 PCGs: Misof et al., 2014, and nuclear genes Ishiwata et al.,
389 2011) found the Embioptera to be sister clade to the Phasmatodea. Using mitochondrial genomes
390 data and BI, the Embioptera were also found as sister group to the Phasmatodea (excluding *T.*
391 *californicum*) (Song et al., 2016). However, in the same study, they also found support for the sister-
392 relationship of Embioptera-Zoraptera using ML (Song et al., 2016). Overall, more phylogenetic
393 studies will be needed to clarify this issue; mostly also because the use of few taxa from anomalous
394 orders (i.e., Dermaptera and Embioptera) tend to evoke LBA (Song et al., 2016).

395 ***4.4. Characterization of ephemeropteran mitochondrial genomes***

396 Mayfly mitochondrial genomes are widely conserved in gene content and nucleotide composition
397 across eleven families, with only a few differences in the content of their tRNAs. The coverage of
398 the *Cloeon*-mitochondrial genome was in agreement with other studies, using the same sequencing
399 platform (e.g., 59-281x, Pons et al., 2014). The missing of part of the AT-rich region is due to
400 reduced sequencing and assembly efficiency of this low complexity region and common among
401 insect mitochondrial genomes (Li et al., 2014; Tang et al., 2014). The gene order and orientation
402 were with the exception of two shifted tRNAs for *H. zijinensis* identical to the ancestral insect
403 mitochondrial genome (Boore, 1999; Cameron, 2014; Simon et al., 1994). Other mayflies are also
404 reported to miss complete T termination codons in the genes *cox2* and *nad5* (Lee et al., 2009; Li et
405 al., 2014; Tang et al., 2014). The two rRNAs were located between *trnL* and *trnV* (*rrnL*), and
406 between *trnV* and the AT-rich region (*rrnS*), respectively. All ephemeropteran mitochondrial
407 genomes contain an AT-rich region, which is placed between the *rrnS* (- strand) and *trnI* (+ strand).
408 Li et al. (2014) reported two distinct parts within the AT-rich region in *Siphluriscus chinensis,* which
409 also seems to be present in *C. dipterum*. Therein, they described the so called CR_1, which is located
410 close to the *rrnS* and has a high AT content (71.6%), including six identical 140 bp sequences, and

411 the CR_2, which is close to the *trnI* and has a lower AT content (58.1%). *E. orientalis* contains two
412 identical 55 bp long sequences in the AT-rich region (Lee et al., 2009). Few mayfly mitochondrial
413 genomes differ in their gene content from the ancestral insect mitochondrial genome, possessing one
414 additional tRNA. The two heptageniid species *Parafronurus youi* and *Epeorus* sp. encode a second
415 copy of the *trnM* (AUG, *trnM2*) gene located between *trnI* and *trnQ* (Tang et al., 2014; Zhang et al.,
416 2008). For *S. chinensis*, an additional *trnK2* (AAA) gene is described (Li et al., 2014). Overall, Song
417 et al. (2016) found similar genetic distances for the early branching insects lineages. The AT
418 contents for the Ephemeroptera were very similar to the ones of Odonata (62.6%-68.5% for *cox1*,
419 Kim et al., 2014).

420 **5. Conclusions**

421 Our analysis based on an increased mayfly taxon sampling and data matrix optimization
422 supported the Odonata hypothesis and unravelled fine structural changes within the palaeopteran
423 mitochondrial genomes. While both BI and ML overall resulted in highly supported trees, only the
424 BI based on an optimized taxa matrix highly supported the sister-relationship of the Ephemeroptera
425 and Neoptera. The optimized taxa matrix, excluding rogue taxa and taxa with LBA, resulted in an
426 overall better supported tree (as measured by the number of supported nodes) for both BI and ML.
427 Our findings highlight the essential need to increase the taxon sampling of under-represented
428 lineages (such as the Palaeoptera) in order to resolve their phylogenetic position. Here we
429 demonstrated the need for an increased taxon sampling in combination with data matrix
430 optimization, and the use of different phylogenetic approaches in order to resolve an ancient
431 radiation. Establishing general recommendation for data matrix optimization requires additional
432 analyses on a broader range of lineages.

Author contributions

SR and MTM conceived the study; SR sequenced and analyzed the *Cloeon dipterum* genome, and performed all combined analyses; PC and CZ obtained and analyzed the *Baetis rutilocylindratus* and *Habrophlebiodes zijinensis* genomes; SR and MTM wrote the manuscript. All authors made contributions to subsequent revisions and agreed to the final version.

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Data statement

DNA sequences are available on Genbank under accessions no XXXXXXXX- XXXXXXXX. Sequence alignments used for the phylogenetic analyses are available on Mendeley Data https://doi .org/XXX.

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696 ## Figure legends

697 **Fig. 1.** Phylogenetic relationships of insect major orders using Bayesian inference reconstruction
698 based on mitochondrial genome data using the concatenated amino acid sequences of the
699 optimized_taxa matrix (see Table 1). Filled circles indicate supported nodes; whereby black circles
700 represent Bayesian posterior probability (BPP) = 1, and grey circles BPP ≥ 0.95. Letters indicate
701 clades mentioned in the text (see Table 3)

702 **Fig. 2.** Mitochondrial genome maps of three sequenced Ephemeroptera species. The nearly complete
703 mitochondrial genome of (**a**) *Cloeon dipterum*, including coverage depth, the complete
704 mitochondrial genome of (**b**) *Baetis rutilocylindratus,* and the incomplete mitochondrial genome of
705 (**c**) *Habrophlebiodes zijinensis*. Transfer RNA genes are indicated by single-letter IUPAC-IUB
706 abbreviations for their corresponding amino acid. Protein coding genes and ribosomal RNA genes
707 are listed and colored in the following way: *atp6*, *atp8*, ATP synthase subunits 6 and 8 genes (violet);
708 *cob*, cytochrome oxidase *b* gene (red); *cox1-cox3*, cytochrome oxidase *c* subunit 1-3 genes (yellow);
709 *nad1-6, nad4L*, NADH dehydrogenase subunits 1-6 and 4L (light blue); *rrnS, rrnL*, small and large
710 ribosomal RNA subunits (orange); AT-rich region (brown). Genes located at the (+) strand appear
711 above the central line. Genes located on the (-) strand appear below the central line.

712 **Tables**

713 **Table 1.** Set of mitochondrial genomes with according GenBank accession numbers.

Order	Family	Species	Accession
Archaeognatha	Machilidae	*Pedetontus silvestrii*	NC_011717
Archaeognatha	Machilidae	*Petrobius brevistylis*	NC_007688
Archaeognatha	Machilidae	*Songmachilis xinxiangensis*	NC_021384
Archaeognatha	Machilidae	*Trigoniophthalmus alternatus*	NC_010532
Archaeognatha	Meinertellidae	*Nesomachilis australica*	NC_006895
Blattodea	Blattidae	*Periplaneta americana*	NC_016956
Blattodea	Blattidae	*Periplaneta fuliginosa*	NC_006076
Blattodea	Corydiidae	*Eupolyphaga sinensis*	NC_014274
Blattodea	Cryptocercidae	*Cryptocercus relictus*	NC_018132
Blattodea	Ectobiidae	*Blattella bisignata*	NC_018549
Blattodea	Ectobiidae	*Blattella germanica*	NC_012901
Collembola	Tetrodontophorinae	*Tetrodontophora bielanensis*[a]	NC_002735
Dermaptera	Pygidicranidae	*Challia fletcheri*[a]	NC_018538
Embioptera	Oligotomidae	*Aposthonia japonica*[a]	AB639034
Ephemeroptera	Ameletidae	*Ameletus* sp1	KM244682
Ephemeroptera	Baetidae	*Alainites yixiani*	NC_020034
Ephemeroptera	Baetidae	*Baetis rutilocylindratus*	XXXXXXXX
Ephemeroptera	Baetidae	*Cloeon dipterum*	XXXXXXXX
Ephemeroptera	Caenidae	*Caenis pycnacantha*	GQ502451
Ephemeroptera	Ephemerellidae	*Ephemerella* sp.	KM244691
Ephemeroptera	Ephemerellidae	*Vietnamella dabieshanensis*	NC_020036
Ephemeroptera	Ephemerellidae	*Vietnamella* sp.	KM244655
Ephemeroptera	Ephemeridae	*Ephemera orientalis*	NC_012645
Ephemeroptera	Heptageniidae	*Epeorus* sp.	KM244708
Ephemeroptera	Heptageniidae	*Paegniodes cupulatus*	NC_020035
Ephemeroptera	Heptageniidae	*Parafronurus youi*	NC_011359
Ephemeroptera	Isonychiidae	*Isonychia ignota*	NC_020037
Ephemeroptera	Leptophlebiidae	*Habrophlebiodes zijinensis*	XXXXXXXX
Ephemeroptera	Potamanthidae	*Potamanthus* sp.	KM244674
Ephemeroptera	Siphlonuridae	*Siphlonurus immanis*	NC_013822
Ephemeroptera	Siphlonuridae	*Siphlonurus* sp.	KM244684
Ephemeroptera	Siphluriscidae	*Siphluriscus chinensis*	HQ875717
Ephemeroptera	Teloganodidae	sp.	KM244670; KM244703[b]
Grylloblattodea	Grylloblattidae	*Grylloblatta sculleni*	DQ241796
Isoptera	Hodotermitidae	*Microhodotermes viator*	NC_018122
Isoptera	Kalotermitidae	*Neotermes insularis*	NC_018124
Isoptera	Rhinotermitidae	*Coptotermes lacteus*	NC_018125

Isoptera	Rhinotermitidae	*Heterotermes* sp.	NC_018127
Isoptera	Rhinotermitidae	*Reticulitermes flavipes*	NC_009498
Isoptera	Rhinotermitidae	*Reticulitermes hageni*	NC_009501
Isoptera	Rhinotermitidae	*Schedorhinotermes breinli*	NC_018126
Isoptera	Termitidae	*Drepanotermes* sp.	NC_018129
Isoptera	Termitidae	*Macrognathotermes errator*	NC_018130
Isoptera	Termitidae	*Macrotermes subhyalinus*	NC_018128
Isoptera	Termitidae	*Nasutitermes triodiae*	NC_018131
Isoptera	Termopsidae	*Porotermes adamsoni*	NC_018121
Isoptera	Termopsidae	*Zootermopsis nevadensis*	NC_024658
Mantodea	Caliridinae	*Leptomantella albella*	NC_024028
Mantodea	Mantidae	*Tamolanica tamolana*	NC_007702
Mantophasmatodea	Mantophasmatidae	*Sclerophasma paresisensis*	NC_007701
Odonata	Calopterygidae	*Vestalis melania*	NC_023233
Odonata	Coenagrionidae	*Ischnura pumilio*	NC_021617
Odonata	Corduliidae	*Cordulia aenea*	JX963627
Odonata	Epiophlebiidae	*Epiophlebia superstes*	NC_023232
Odonata	Euphaeidae	*Euphaea formosa*	NC_014493
Odonata	Gomphidae	*Davidius lunatus*	NC_012644
Odonata	Gomphidae	*Ictinogomphus* sp.	KM244673
Odonata	Libellulidae	*Hydrobasileus croceus*	KM244659
Odonata	Libellulidae	*Orthetrum triangulare melania*	AB126005
Odonata	Pseudolestidae	*Pseudolestes mirabilis*	NC_020636
Orthoptera	Acrididae	*Acrida willemsei*	NC_011303
Orthoptera	Acrididae	*Calliptamus italicus*	NC_011305
Orthoptera	Acrididae	*Gomphocerus sibiricus*	NC_021103
Orthoptera	Acrididae	*Oedaleus decorus asiaticus*	NC_011115
Orthoptera	Gryllotalpidae	*Gryllotalpa orientalis*[a]	NC_006678
Orthoptera	Pneumoridae	*Tanaocerus koebelei*	NC_020777
Orthoptera	Prophalangopsidae	*Tarragoilus diuturnus*	NC_021397
Orthoptera	Pyrgomorphidae	*Atractomorpha sinensis*	NC_011824
Orthoptera	Rhaphidophoridae	*Troglophilus neglectus*	NC_011306
Orthoptera	Romaleidae	*Xyleus modestus*	NC_014490
Orthoptera	Tetrigidae	*Tetrix japonica*	NC_018543
Orthoptera	Tettigoniidae	*Gampsocleis gratiosa*	NC_011200
Orthoptera	Tettigoniidae	*Ruspolia dubia*	NC_009876
Phasmatodea	Bacillidae	*Bacillus rossius*	GU001956
Phasmatodea	Diapheromeridae	*Micadina phluctainoides*	NC_014673
Phasmatodea	Diapheromeridae	*Sipyloidea sipylus*	AB477470
Phasmatodea	Heteropterygidae	*Heteropteryx dilatata*	NC_014680
Phasmatodea	Heteropterygidae	*Orestes mouhotii*	AB477462
Phasmatodea	Phasmatidae	*Entoria okinawaensis*	NC_014694
Phasmatodea	Phasmatidae	*Extatosoma tiaratum*	NC_017748

Phasmatodea	Phasmatidae	*Megacrania alpheus adan*	NC_014688
Phasmatodea	Phasmatidae	*Neohirasea japonica*	AB477469
Phasmatodea	Phasmatidae	*Phobaeticus serratipes*	NC_014678
Phasmatodea	Phasmatidae	*Phraortes* sp.[a]	NC_014705
Phasmatodea	Phasmatidae	*Ramulus irregulariterdentatus*	NC_014702
Phasmatodea	Timematidae	*Timema californicum*	DQ241799
Plecoptera	Perlidae	*Dinocras cephalotes*	NC_022843
Plecoptera	Perlidae	*Kamimuria wangi*	NC_024033
Plecoptera	Perlidae	*Togoperla* sp.	KM409708
Plecoptera	Pteronarcyidae	*Pteronarcys princeps*	NC_006133
Zygentoma	Lepidotrichidae	*Tricholepidion gertschi*	NC_005437
Zygentoma	Lepismatidae	*Thermobia domestica*	NC_006080
Zygentoma	Nicoletiidae	*Atelura formicaria*	NC_011197

[a], Taxa were excluded in the optimized_taxa matrix (see 2. Material and methods).

[b], Represents sequence consisting of two genomic fragments (contigs) from the same sample.

716 **Table 2.** Overview of data matrices, including the best-fitting partitioning and model schemes, and
717 amino acid (aa) sequence length.
718

Matrix	Model	Length [aa]
all_taxa		
partition_1: *atp6*, *atp8*, *nad2*, *nad3*, *nad6*	MTZOA + Γ + I + F	840
partition_2: *cox1*	MTZOA + Γ + I	528
partition_3: *cob*, *cox2*	MTZOA + Γ + I + F	608
partition_4: *cox3*	MTART + Γ + I	268
partition_5: *nad1*, *nad4*, *nad4L*, *nad5*	MTZOA + Γ + I + F	1431
optimized_taxa		
partition_1: *atp6*, *atp8*, *nad2*, *nad3*, *nad6*	MTZOA+ Γ + I + F	843
partition_2: *cox1*	MTZOA + Γ + I	529
partition_3: *cob*, *cox2*, *cox3*	MTZOA + Γ + I + F	878
partition_4: *nad1*, *nad4*, *nad4L*, *nad5*	MTZOA + Γ + I + F	1423

Table 3. Support for the three hypotheses and node support for clades of interest. Node support is based on Bayesian inference (BI) and maximum likelihood (ML) analyses of two data matrices: optimized_taxa and all_taxa, whereby optimized_taxa had 5 terminal nodes removed based on the occurrence of rogue taxa and long branch attraction. Asterices indicate hypothesis/node support (** = Bayesian posterior probability (BPP) ≥ 0.95 and Bootstrap support (BS) = 90%) * = BPP ≥ 0.90 and BS ≥ 80%). Superorders are underlined, and suborders are italicised. Letters in brackets refer to monophyletic clades in the Fig. 1.

Hypothesis/Clade	BI		ML	
	optimized_taxa	all_taxa	optimized_taxa	all_taxa
Palaeoptera hypothesis				
Ephemeroptera hypothesis				
Odonata hypothesis	**			
Winged insects (A)	**			
<u>Palaeoptera</u>				
Odonata (B)	**	**	**	**
Ephemeroptera (C)	**		**	
<u>Polyneoptera</u>			*	
Plecoptera (D)	**	**	**	**
Orthoptera				
Ensifera (E)	**	**	**	
Caelifera (F)	**	**	**	**
Phasmatodea[a] (G)	**	**	**	**
<u>Dictyoptera (H)</u>	**	**	**	**
Mantodea (I)	**	**	**	**
Isoptera (J)	**	**	**	**

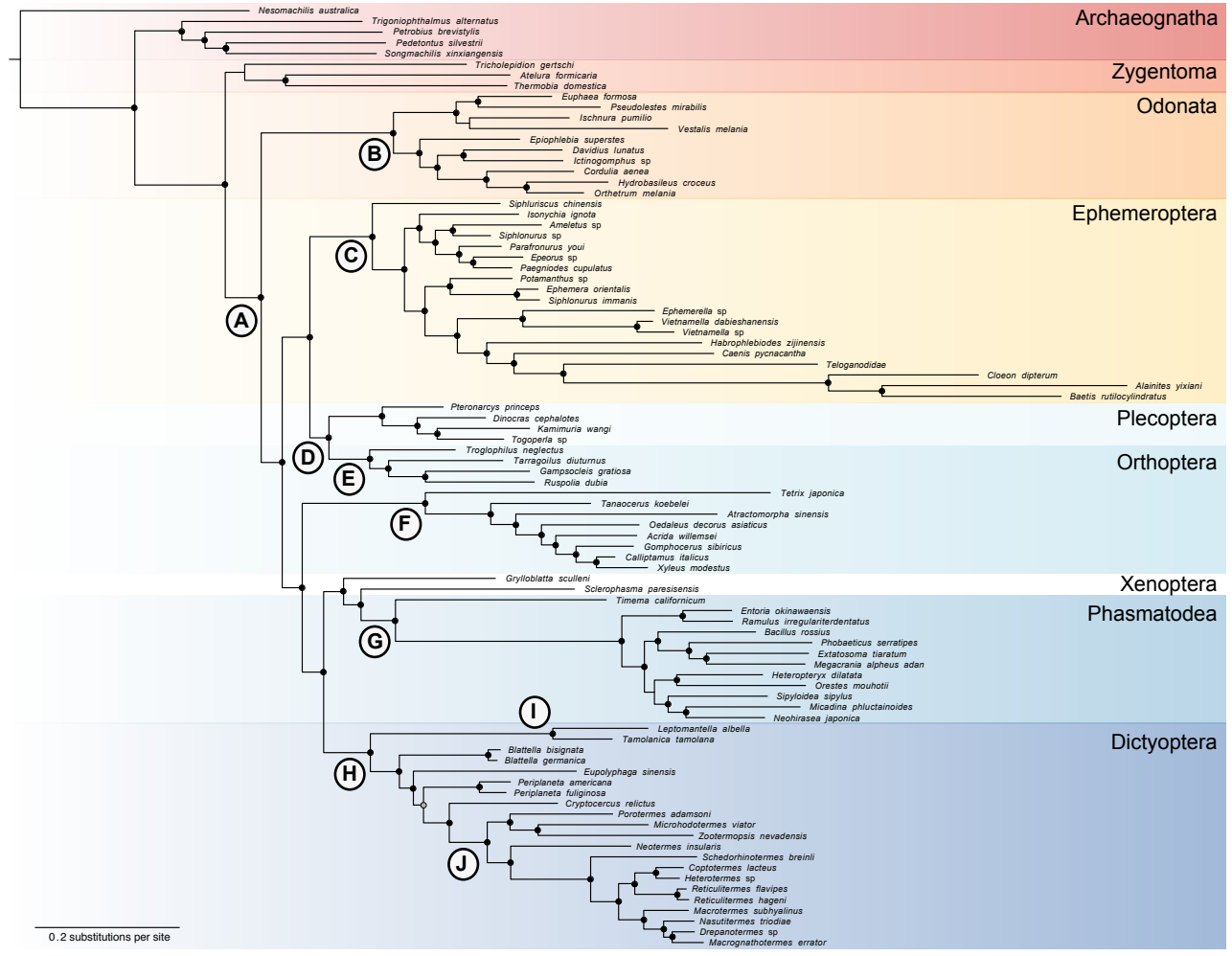
[a], Includes *Aposthonia japonica* (Embioptera) in reconstructions using the all_taxa matrix.

728 **Table 4.** Overview of sequenced ephemeropteran mitochondrial genomes. Total sequence length in
729 base pairs (bp) and individual nucleotide compositions of mayfly mitochondrial genomes calculated
730 based on the whole available sequences and the ones corrected for the incomplete mitochondrial
731 genomes (see 2. Material and methods; for the complete list of all ephemeropteran mitochondrial
732 genomes and Supplementary Table S2).
733

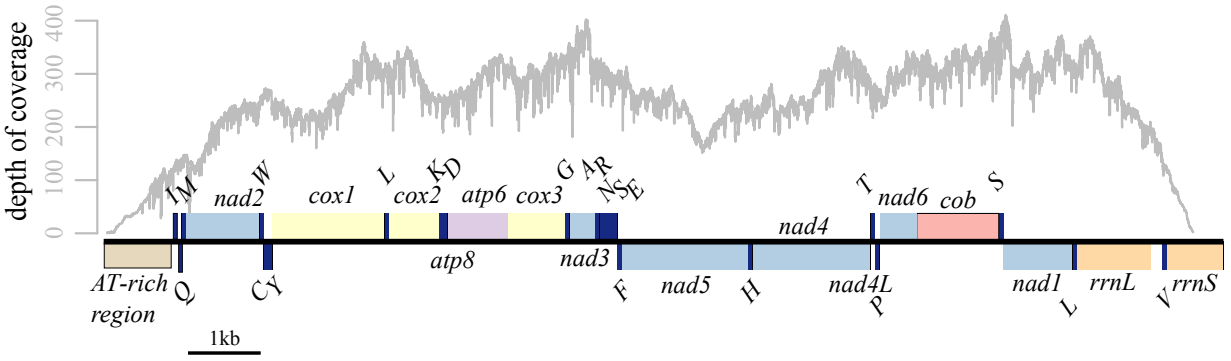
Family	Species	Length [bp]	A%	C%	G%	T%	GC%	AT%
Baetidae	*Baetis rutilocylindratus*	14,883[a]	27.3	19.8	20.1	32.8	39.9	60.1
		12,234	26.7	19.8	20.4	33.2	40.2	59.9
Baetidae	*Cloeon dipterum*	14,355	30.6	14.7	16.3	38.4	30.9	69.0
		12,197	29.9	14.5	16.9	38.7	31.4	68.6
Leptophlebiidae	*Habrophlebiodes zijinensis*	15,407	33.8	20.2	11.0	35.1	31.2	68.9
		12,417	33.6	20.6	11.5	34.3	32.1	67.9

734 [a], Indicates complete mitochondrial genomes; rest are incomplete mitochondrial genomes.

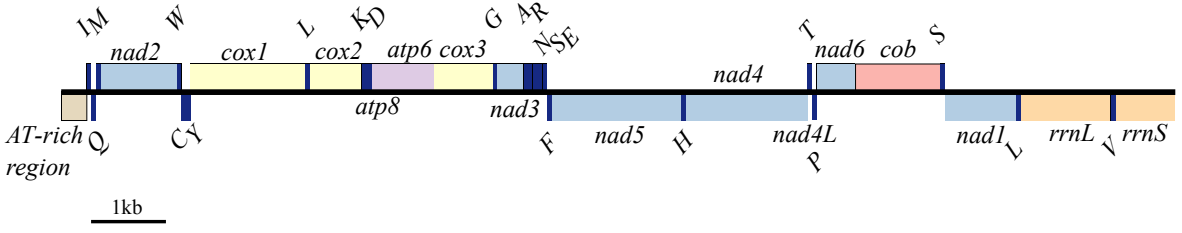
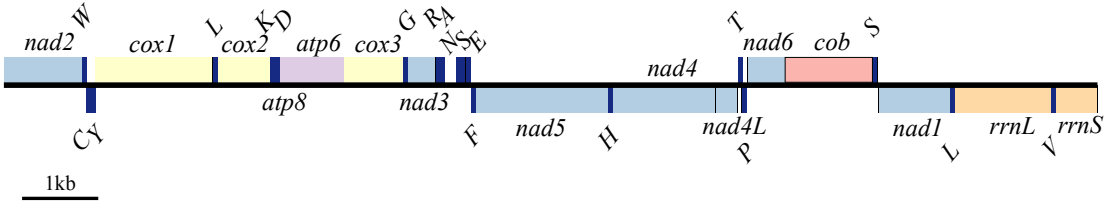
735


Archaeognatha
Nesomachilis australica
Trigoniophthalmus alternatus
Petrobius brevistylis
Pedetontus silvestrii
Songmachilis xinxiangensis
Zygentoma
Tricholepidion gertschi
Atelura formicaria
Thermobia domestica
Odonata
Euphaea formosa
Pseudolestes mirabilis
Ischnura pumilio
Vestalis melania
Epiophlebia superstes
Davidius lunatus
Ictinogomphus sp
Cordulia aenea
Hydrobasileus croceus
Orthetrum melania
B
Ephemeroptera
Siphluriscus chinensis
Isonychia ignota
Ameletus sp
Siphlonurus sp
Parafronurus youi
Epeorus sp
Paegniodes cupulatus
Potamanthus sp
Ephemera orientalis
Siphlonurus immanis
C
A
Ephemerella sp
Vietnamella dabieshanensis
Vietnamella sp
Habrophlebiodes zijinensis
Caenis pycnacantha
Teloganodidae
Cloeon dipterum
Alainites yixiani
Baetis rutilocylindratus
Plecoptera
Pteronarcys princeps
Dinocras cephalotes
Kamimuria wangi
Togoperla sp
Orthoptera
Troglophilus neglectus
Tarragoilus diuturnus
Gampsocleis gratiosa
Ruspolia dubia
D
E
Tetrix japonica
Tanaocerus koebelei
Atractomorpha sinensis
Oedaleus decorus asiaticus
Acrida willemsei
Gomphocerus sibiricus
Calliptamus italicus
Xyleus modestus
F
Xenoptera
Grylloblatta sculleni
Sclerophasma paresisensis
Phasmatodea
Timema californicum
Entoria okinawaensis
Ramulus irregulariterdentatus
Bacillus rossius
Phobaeticus serratipes
Extatosoma tiaratum
Megacrania alpheus adan
Heteropteryx dilatata
Orestes mouhotii
Sipyloidea sipylus
Micadina phluctainoides
Neohirasea japonica
G
I
Dictyoptera
Leptomantella albella
Tamolanica tamolana
Blattella bisignata
Blattella germanica
Eupolyphaga sinensis
Periplaneta americana
Periplaneta fuliginosa
Cryptocercus relictus
H
Porotermes adamsoni
Microhodotermes viator
Zootermopsis nevadensis
Neotermes insularis
Schedorhinotermes breinli
Coptotermes lacteus
Heterotermes sp
Reticulitermes flavipes
Reticulitermes hageni
Macrotermes subhyalinus
Nasutitermes triodiae
Drepanotermes sp
Macrognathotermes errator
J
0.2 substitutions per site

(a) ***Cloeon dipterum*** L. 1761



(b) ***Baetis rutilocylindratus*** Wang, Qin, Cheng & Zhou, 2011



(c) ***Habrophlebiodes zijinensis*** Gui, Zhang & Wu, 1996