Research Article

Mitochondrial genomes infer phylogenetic relationships among the oldest extant winged insects (Palaeoptera)

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Abstract

Background: The relationships among the oldest winged insects (Palaeoptera), including the Ephemeroptera (mayflies) and Odonata (dragonflies and damselflies), remain unclear. The Palaeoptera together with the Neoptera have evolved as result of a rapid divergence from a common ancestor in the distant past. Thus they are thought to be more susceptible to systematic inadequacies, including taxon sampling, choice of outgroup, marker selection, and phylogenetic methods. Here we reconstruct their phylogenetic relationship using newly sequenced mitochondrial genomes in combination with 90 additional insect mitochondrial genomes. In particular, we investigate the impact of the increased mayfly taxon sampling, the effect of rogue taxa, and the used phylogenetic framework (Bayesian inference (BI) vs. maximum likelihood (ML)) approach.

Results: We found support for the clustering of the Odonata as most ancient, extant winged insects, using BI based on an optimized data matrix. Overall, we found no support for the basal Ephemeroptera clustering and the sister relationship between the Ephemeroptera and Odonata. Our newly sequenced mitochondrial genomes of *Baetis rutilocylindratus*, *Cloeon dipterum*, and *Habrophlebiodes zijinensis* showed the complete set of 13 protein coding genes and a conserved gene orientation with the exception of two inverted tRNAs for *H. zijinensis*.

Conclusions: The increase of palaeopteran taxon sampling in combination with a Bayesian phylogenetic framework was crucial 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. It remains to be tested weather an increased taxon sampling might also reveal the elusive phylogenetic positions of other insect orders.

Keywords: Baetidae, Bayesian Inference, Ephemeroptera, Long-branch attraction, Mayfly, Mitochondrial genomics, Odonata, Palaeoptera problem, Polyneoptera, Pterygota, Rogue taxa

Background

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" [1,2]. 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. [3]). The monophyly of the Neoptera, including the three major lineages Polyneoptera, Paraneoptera, and Holometabola, is widely accepted, although relationships among polyneopteran lineages [4] and the monophyly of the Paraneoptera [5] 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)) [3,6,7]. All hypotheses are supported by morphological as well as molecular data to varying degrees (reviewed by Trautwein et al. [3]). Different authors, using the same set of genes and taxa set but different phylogenetic approaches, namely Bayesian inference (BI) vs. maximum likelihood (ML) [2,8,9], and nucleotide vs. amino acid (aa) sequences [10] obtained results supporting distinct hypotheses. The Palaeoptera hypothesis has received support from several molecular studies based on rRNA genes and nuclear DNA [5,11-13], and from nDNA phylogenomic analyses [9,14,15]. Thomas et al. [12] and Kjer et al. [11] included the most comprehensive taxon sampling with 35, respectively seven species from each of the three lineages (i.e., Ephemeroptera, Odonata, Neoptera) analyzing seven, respectively eight genes (rRNAs, nuclear DNA (nDNA), mitochondrial DNA (mtDNA)). The basal Ephemeroptera hypothesis was supported by previous studies using mitochondrial genome data [10,16,17], and a combined analysis of rRNA

and one nuclear gene [2]. Other studies have concluded the relationships are ambiguous [2]. Notably, studies supporting the basal Ephemeroptera hypothesis have included either a limited number of mayfly species (e.g., one [17,18], two [16,19], four [20], or five [10] species) or a limited number of unlinked loci (e.g., one [10,16,17,20], two [18,19,21], or three [2]). The basal Odonata hypothesis has received support from previous studies based on mitochondrial genome data [22,23], rRNA genes [8,24-27], combining rRNA and nDNA [28], and phylogenomic nDNA [9,29]. These studies, with the exceptions of few studies based on *18S* rRNA [24,25,27], included in total between two [8,29] and 13 species [26] from the orders Ephemeroptera and Odonata.

The conflicting phylogenetic signals may result from the ancient radiation of the lineages Ephemeroptera, Odonata, and Neoptera from a common ancestor in the distant past [4]. These three ancient lineages appear to have diverged rapidly, leaving few characteristics to determine their phylogenetic relationships [4]. Evolutionary rate heterogeneity across clades and the representation of old clades by recent extant taxa make ancient relationships such as those of early winged insects the most difficult challenges for phylogenetics [4,7]. Given the weak phylogenetic signal, reconstructions are more susceptible to systematic errors [4,12,30-32]. Other sources of conflicting signal in studies of the relationships among these three lineages may result from differences in taxon sampling, sequence data, alignment methods, and phylogenetic methods used including models of evolution [3,12,33]. The use of mitochondrial protein-coding genes (PCGs) derived from mitochondrial genomes can circumvent some of these difficulties, since they are easier to align and also appropriate models of molecular evolution are well established [34-36]. On the other hand, mitochondrial genes include several drawbacks, most importantly the possible presence of pseudogenes [37-39]. However, data derived from high-throughput sequencing, depending on the coverage bears the potential to distinguish between functional genes and pseudogenes based on higher mutation rates of the later [40].

Overall, mitochondrial markers are well studied and therefore the most widely employed genetic markers in insects, being considered a promising "instrument" for insect systematics [41]. Insect mitochondrial genomes are highly conserved, ranging from 15 to 18 kb in length, containing 37 genes: 13 PCGs, two ribosomal RNAs (rRNAs, *rrnL* and *rrnS*), and 22 transfer RNAs (tRNAs, *trn**) [42]. A non-coding region of variable length, thought to be the origin of initiation of transcription and replication, is typically present [43,44] and referred to as the AT-rich region. The typical ancestral insect mitochondrial genome differs from the ancestral arthropod mitochondrial genome only by the location of *trnL* [45]. Significant differences in structure, gene content, and gene arrangement have been found to be the exception for highly derived taxa.

Importantly there is still an unbalance in the numbers of available molecular markers between the different insect lineages. In particular the early winged insects (i.e., Ephemeroptera and Odonata) are relatively under-represented. This is (partly) because so far most genomic data were obtained for insect orders that are of economical interest (e.g., as pollinators, model species, agricultural pests, and vectors of human diseases, [46]). As of January 2017, there were 39 complete or nearly complete palaeopteran mitochondrial genomes (Ephemeroptera: 18, Odonata: 21) from 22 families (eleven of each) available on GenBank; 22 of which were included in publications (Ephemeroptera: ten, Odonata: twelve). Compared to the number of families that have been described (Ephemeroptera: 42 [47], Odonata: 30 [48]) this is still a relatively low number.

The insect order Ephemeroptera comprises over 3,000 species, comprising as the two most species rich families the Baetidae (833 species) and the Leptophlebiidae (608 species) [47]. Their biogeographic origin is probably Pangean, including a greater diversity and higher endemism rate in the Neotropics and Australasia for the Baetidae, and the Neotropical and Afrotropical regions for the Leptophlebiidae, respectively [47].

We investigated the relationships of the oldest extant winged insects and newly sequenced three mayfly mitochondrial genomes to improve sampling of this under-represented order. New taxa

included one representative of the family Leptophlebiidae, for which no mitochondrial genome data were available, and two representatives of the Baetidae, one from each subfamily. We used BI and ML approaches and created matrices that eliminated taxa that reduced overall tree support (rogue taxa) and that were affected by long-branch attraction (LBA). Our analysis included 29 palaeopteran mitochondrial genomes from 20 families and 64 other insect mitochondrial genomes. We expect the increased taxon sampling to improve the phylogenetic signal of the basal nodes and add support for one of the three competing hypotheses.

Methods

Taxon sampling

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

[**Table 1** should be added here]

Sequencing and assembly

The three mayfly species were sequenced with either 454/FLX pyrosequencing (*C. dipterum*) or Sanger sequencing (*B. rutilocylindratus* and *H. zijinensis*. We extracted DNA from *C. dipterum* from twelve to twenty pooled reared subimago specimens, prepared a shotgun library, and sequenced four lanes on a Roche (454) *GS FLX* machine at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany) (see also [49]). The obtained sequence reads were trimmed and *de novo* assembled using the software Newbler v.2.5.3 (454 Life Science Cooperation) under default settings for large data sets. In order to extract the mitochondrial genome of *C. dipterum*, we performed BLASTN searches [50] using as query all assembled contigs against the NCBI database. We mapped all matching reads back to the mitochondrial genome with BWA [51], using the BWA-SW algorithm [52] with settings suggested for 454 data by CORAL (match score = 2, mismatch penalty = 2, and gap open penalty = 3, [53]).

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*, [54]). Subsequently, based on the previously obtained sequence information we designed six (*B. rutilocylindratus*) respective four (*H. zijinensis*) specific primer pairs (Additional file 1: Table S1; see also Li et al. [55]). 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).

Sequence alignments and data matrices

We prepared two data matrices of aa sequences based on the annotated mitochondrial genomes (see below), 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 [56,57] and showing LBA [58,59] (optimized_taxa matrix; 88 taxa, see Table 1). We used GUIDANCE2 [60,61] to align the sequences, and identify and remove positions detected as ambiguously aligned regions. To run GUIDANCE we applied the default settings, using as multiple sequences alignment program MAFFT v.7.050b (L-INS-I algorithm with default settings, [62]).

The best-fitting partitioning schemes and corresponding aa substitution models were estimated with PartitionFinder v.2 (https://github.com/brettc/partitionfinder, [63]). We used the Bayesian Information Criterion (BIC) to choose the best model, linked branch lengths, and a greedy search [63] with RAxML v.8.2.8 [64]. Thereby each gene was defined as one data block (i.e., possible partition).

To identify rogue taxa, we applied the RogueNaRok algorithm [56,57]. We used the bootstrap replicates and the best supported maximum likelihood tree based on the all_taxa matrix (see below). Identified rogue taxa were removed from the all_taxa matrix, resulting in the optimized_taxa matrix (Table 1). Taxa showing evidence of LBA *sensu* Bergsten [65] were identified using heterogeneities in sequence divergence based on a model of evolution derived from the entire data matrix. For this, the Relative Composition Frequency Variability (RCFV) values of each species were calculated with BaCoCa [66] using the partitioned concatenated data matrix (i.e., all_taxa matrix). Taxa with the highest values were excluded in the optimized_taxa matrix, apart from the Ephemeroptera, which were retained because they are the focus of our study and their monophyly is well-established [1,2].

193 **Phylogenetic reconstruction**

194 Phylogenetic reconstructions were carried out using MrBayes v.3.2.4 [67], and RAxML v.8.1.7 [64].
195 We analyzed both matrices (all_taxa, optimized_taxa) using the best-fitting partitioning scheme and
196 model for each (see Table 2). For BI, we unlinked the frequencies, gamma distributions, substitution
197 rates and the proportion of invariant sites across partitions. For each matrix, we run two independent
198 analyses of four MCMC chains with 10^7 generations, sampling every 10^3 generations and discarding
199 a burn-in of 25%. Maximum likelihood inferences were performed with 200 bootstrap replicates.
200 Trees reconstructed from the all_taxa matrix were rooted with *Tetrodontophora bielanensis*
201 (Collembola). Trees reconstructed from the optimized_taxa matrix were rooted with *Nesomachilis*
202 *australica* (Archaeognatha, according to Song et al. [10]) because *Tetrodontophora bielanensis* was
203 found to be a rogue taxon (see Results). All trees were visualized with the ape v.4.1 [68], phangorn
204 v.2.1.1 [69], and ggtree v.1.4.20 [70] packages for R v.3 [71].

205 **Annotation and characterization**

206 All mitochondrial genomes were annotated using the MITOS webserver (http://mitos.bioinf.uni-
207 leipzig.de/index.py, [72]), DOGMA [73], and MFannot (http://megasun.bch.umontreal.ca/cgi-
208 bin/mfannot/mfannotInterface.pl). The predicted PCGs were checked for stop codons and manually
209 adjusted by comparison with the longest predicted open reading frames as implemented in Geneious
210 R7 v.7.1.3 (Biomatters Ltd.), and by comparison with the respective homologous insect sequence
211 alignment. For the tRNA prediction, we additionally used ARWEN v.1.2.3 [74] and tRNAscan-SE
212 v.1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/, [75]). The annotated mitochondrial genomes were
213 visualized with OrganellarGenomeDRAW (http://ogdraw.mpimp-golm.mpg.de/cgi-bin/ogdraw.pl,
214 [76]) and manually edited.

215 Nucleotide contents were retrieved using Geneious. To correct biases in the AT content due to
216 incomplete mitochondrial genomes mostly missing the AT-rich region, we removed all AT-rich
217 regions and the two rRNAs including the five close-by tRNAs (*trnL1-trnM*) and recalculated the

nucleotide base pair (bp) compositions. The AT and GC composition skewness were calculated as follows: AT-skew = (A - T) / (A + T), and GC-skew = (G - C) / (G + C) [77].

Results

Data matrices

The final matrices had lengths of 3,675 aa (all_taxa matrix, including 93 taxa) and 3,673 aa (optimized_taxa matrix, including 88 taxa, see Table 1). As best-fitting partitioning scheme for the two matrices, we identified either five partitions (all_taxa matrix, Table 2) or four partitions (optimized_taxa, Table 2). As best-fitting models of aa sequence evolution, we identified MtZoa [78] and MtArt [35].

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

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

The RogueNaRok analysis identified the outgroup *T. bielanensis* as well as *Gryllotalpa orientalis* (Orthoptera) and *Phraortes* sp. (Phasmatodea) as taxa with uncertain phylogenetic positions, leading to less accurate phylogenetic reconstructions. *G. orientalis* (Orthoptera) and *Phraortes* sp.

(Phasmatodea) clustered within the corresponding order using the all_taxa matrix, although the nodes containing these species were not supported by ML. In the test for LBA, *Aposthonia japonica* (Embioptera) and *Challia fletcheri* (Dermaptera) had high RCFV values (0.0237 and 0.0307, respectively) suggesting they were affected by LBA. Both species clustered within other orders: *Challia fletcheri* (Dermaptera) within the Ephemeroptera as sister taxon to a clade containing all representatives of the family Baetidae, and *A. japonica* (Embioptera) within the Phasmotodea as sister taxon to a clade containing all species of the order with the exception of *T. californicum* (Table 3, Additional file 2: Figure S1). The mayflies Teloganodidae sp. (0.0305) and the three baetids *A. yixiani* (0.0235), *B. rutilocylindratus* (0.0232), and *C. dipterum* (0.0201) showed relatively high RCFV values.

Phylogenetic reconstruction

The Bayesian reconstruction based on the optimized_taxa matrix produced the tree with most support compared to ML or to either analysis of the all_taxa matrix (Figure 1, Table 3, Additional files 2-4: Figures S1-S3). The basal Odonata hypothesis was highly supported in this tree (Bayesian posterior probability (BPP) = 1.00), whereas neither the basal Ephemeroptera hypothesis nor the Palaeoptera hypothesis received support in any analysis (Table 3). The Odonata were monophyletic in all analyses (BPP = 1.00, BS = 100%) whereas the Ephemeroptera were monophyletic only using the optimized_taxa matrix (Figure 1, Table 3, Additional file 3: Figures S2).

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 Figure 1

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

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

Most other nodes of interest were consistently supported across different matrices and analyses (Table 3). The Polyneoptera were moderately supported (BS ≥ 80%) for the optimized_taxa matrix using the ML. Within the Polyneoptera, all orders except Orthoptera and Blattodea were monophyletic (BPP ≥ 0.95, and BS ≥ 90%, Table 3). The two Orthoptera suborders Ensifera and Caelifera were monophyletic (Table 3), whereby the Ensifera clustered together with the Plecoptera for the optimized_taxa matrix using both ML and BI. The Grylloblattodea, Mantophasmatodea, and Phasmatodea were all well supported using the optimized_taxa matrix. For the all_taxa matrix also the Embioptera were recovered in this clade as sister taxa to the Phasmatodea excluding *Timema*

californicum (Figure 1, Additional files 2, 4: Figures S1, S3). The superorder Dictyoptera was monophyletic clade, consisting of the monophyletic Mantodea and the Blattodea including the Isoptera.

Mitochondrial genomes

The mitochondrial genome sequences have been deposited at GenBank with the accession numbers XXXXXXXX, XXXXXXXX, and XXXXXXXX. The pyrosequencing run of *C. dipterum* using the 454 GS FLX system resulted in 651,306 reads, of which 1.14% mapped to the assembled mitochondrial genome. The depth of coverage for the *C. dipterum* mitochondrial genome was 249.2x (± 80.6 SD, Figure 2a). The three mayfly mitochondrial genomes were 15,407 bp (*C. dipterum*), 14,883 bp (*B. rutilocylindratus)*, and 14,355 bp (*H. zijinensis*) long (Table 4). For *B. rutilocylindratus*, the complete mitochondrial genome was sequenced (Figure 2b). The AT-rich regions were incomplete for genomes of *C. dipterum* and *H. zijinensis*. The three tRNAs between the AT-rich region and *nad2* were also missing in *H. zijinensis* due to the incomplete sequencing (Figure 2c). All three sequenced mitochondrial genomes contained the entire set of 13 PCGs, two rRNAs, and either 22 tRNAs (*B. rutilocylindratus*, *C. dipterum*) or 19 for the incomplete mitochondrial genome of *H. zijinensis*, including 23 coded at the (+) strand (21 for *H. zijinensis*) and 14 at the (-) strand (13 for *H. zijinensis*) (Figure 2).

The order of the genes was conserved, with the exceptions of the two inverted tRNAs Arginine and Alanine for *H. zijinensis* (Figure 2c). All PCGs started with the ATN codons (ATT, ATG, ATA), and mostly ended with the complete termination codon (TAA or TAG). Exceptions were an incomplete T termination codon for *nad4* in *B. rutilocylindratus* and *cox1* started with CTC in *C. dipterum*. The AT content of the mitochondrial genomes that were corrected for the missing regions, was lowest in *B. rutilocylindratus* (59.9%, Table 4, Additional file 5: Table S2). The mean AT content for all available mayfly mitochondrial genomes was 65.34%. The average AT-skew was -0.03 (± 0.04 SD) and the average GC-skew was -0.13 (± 0.14 SD).

Table 4 Overview of sequenced ephemeropteran mitochondrial genomes. Total sequence length in base pairs (bp) and individual nucleotide compositions of mayfly mitochondrial genomes calculated based on the whole available sequences and the ones corrected for the incomplete mitochondrial genomes (see Methods; for the complete list of all ephemeropteran mitochondrial genomes see Additional file 5: Table S2)

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

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

Discussion

Phylogenetic relationships of Palaeoptera

Our Bayesian reconstruction based on the optimized_taxa matrix provided strong support for the basal Odonata hypothesis. Previous work based on mitochondrial genome data is (partially) congruent with our findings [10,22,23]. In contrast to our results, a handful of studies based on nucleotide and aa sequences of mitochondrial PCGs and mitochondrial RNA (mtRNAs) found overall strong support for the basal Ephemeroptera hypothesis [10,16,17,20]. Interestingly, Song et al. [10], supporting overall the Ephemeroptera hypothesis, found evidence for the Odonata hypothesis when using aa sequence data. Our results confirm the trend towards a better support for the ancestral position of the Odonata when using an increased mayfly taxon sampling (more than ten species per lineage) [24,25,27]. The exceptions for this are the work by Ogden and Whiting [2] and

Thomas et al. [12], that overall support the Ephemeroptera respective Palaeoptera hypothesis but contain some data sets in favor of the Odonata hypothesis. Early inconsistencies based on different sequence alignment strategies [2] appear to become less relevant due to the advances of multiple sequence alignment programs. Overall, the vast majority of studies were based on BI and ML. Thomas et al. [12] recovered increased resolution using BI in comparison to ML. Different data partitioning (i.e., gene trees resulting in different tree topologies) by Kjer et al. [11] supported different hypotheses for different genes. Evidently, using rRNAs (nDNA and mtDNA) and the nDNA EF1-α the Odonata hypothesis was recovered whereas the analyses based on the combined and on the mtDNA data matrix resulted in a monophyletic palaeopteran clade. It is questionable whether an increase in analyzed genomic data (i.e., phylogenomic studies) will be able to resolve the Palaeoptera problem. Notably, Misof et al. [79] and Regier et al. [15], using 1,478 PCGs and seven palaeopteran species, respective 62 PCGs and four palaeoptera species, recovered the Ephemeroptera and Odonata as sister clades (i.e., the Palaeoptera hypothesis) but without high support values. Overall, more knowledge about the use and limitation of individual markers are needed. For example Simon et al. [80] found that proteins involved in cellular processes and signaling harbor the most phylogenetic signal.

The increased mayfly taxon sampling highlighted the phylogenetic diversity (as indicated by the branch lengths of the tip taxa, Figure 1, Additional files 2-4: Figures S1-S3) of the Palaeoptera. The overall long-branch lengths and high rate-heterogeneity across sites might be one of the explanations for the "Palaeoptera problem". Especially the family of the Baetidae, which was long thought to be the most ancestral mayfly family [81], showed high base composition heterogeneity possibly evidencing LBA. However, in agreement with other more recent studies we also recovered *S. chinensis* as sister taxa to all other mayflies [55,82]. It remains to be resolved whether an increased taxon sampling may obviate LBA *sensu* Bergsten [65].

Phylogenetic relationships of Polyneoptera

339 Besides the Palaeoptera, a universal consensus on the placement of the Plecoptera, Dermaptera,
340 Embioptera, and Xenoptera (i.e., Grylloblattodea and Mantophasmatodea) remains elusive [10]. The
341 monophyly of the Polyneoptera has become more widely accepted [5,10,23,79,83,84], although
342 several studies and data matrices do not support their monophyly [10,11,80]. This is also reflected by
343 our results, recovering the Polyneoptera as moderately supported monophyletic clade when using the
344 optimized_taxa matrix with ML. The placement of the order Plecoptera also remains elusive. The
345 order often clusters among the more ancestral polyneopteran orders [10,23,79] and in a close
346 relationship to the Dermaptera, although earlier studies using few species and the aa sequences of the
347 mitochondrial PCGs also recovered a sister relationship to the Ephemeroptera [22,85]. Notably, Song
348 et al. [10] was the only study including four dermapteran species. The monophyly of the Orthoptera
349 has been established by previous studies based on mitochondrial genome data and a large number of
350 PCGs [10,79,86,87]. However, in a recent study, the Ensifera also clustered as more ancestral clade
351 [10]. As for this study, Song et al. [10] also used BI and ML based on mitochondrial genome data.
352 Notably, Song et al. [10] explain the more ancestral position of Ensifera by more similar sequence
353 compositional bias in comparison to the outgroups. The relationship between Grylloblattodea and
354 Mantophasmatodea (i.e., Xenoptera superorder) remains elusive. Mostly this is due to the limited
355 amount of available molecular data, including ordinal-level phylogenies based on two [5,10] or three
356 xenopteran species [79]. Using mitochondrial genome data and one species per order, also Song et al.
357 [10] did not recover them as a monophyletic clade. Their monophyly was supported by previous
358 studies using one Mantophasmatodea and one Grylloblattodea species [5], respective one
359 Mantophasmatodea and two Grylloblattodea species [79]). The Phasmatodea have also been found in
360 previous studies as paraphyletic clade due to the outside position of *T. californicum* [10,16]. In
361 contrast, Misof et al. [79], using a large set of nuclear PCGs also found *Timema* as the sister species
362 to all other representatives of the order. The monophyly of the superorder Dictyoptera is generally

363 accepted and the hierarchical clustering (Mantodea + (Blattodea + Isoptera)) has been well supported
364 [5,10,11,16,23,79,88,89] using nucleotide and aa sequences of both mtDNA and nDNA.

365 **Optimizing data matrix**

366 The optimization of the data matrix overall resulted in a better-supported tree (as measured by the
367 total number of supported nodes and the fact that all nodes supported in the all_taxa matrix were also
368 recovered in the optimized_taxa matrix). In a study on butterfly phylogenetics, the authors also
369 removed rogue taxa and found dramatically increased bootstrap support values [90]. Comparing the
370 number of supported nodes from the BI and ML approach, the former always resulted in more
371 resolved nodes. This result is consistent with previous work on the "Palaeoptera problem",
372 recovering more support for analyses using BI [12]. Generally, the choice of the outgroup species
373 was reported as being crucial for resolving problematic splits in the tree of life such as insects origin
374 [12]. Thus, finding the outgroup as a rogue taxon was perhaps not surprising. The close phylogenetic
375 relationship of the Dermaptera and Ephemeroptera (Additional files 2, 4: Figures S1, S3) might be
376 misleading due to LBA. Li et al. [55] also found the order Dermaptera as being closely related to the
377 mayflies. On the other hand studies based on phylogenomic data reported the Dermaptera as sister
378 taxa to the orders Plecoptera [5,16,23,80,91] Zoraptera [28,79]. Using mitochondrial genome data
379 and an increased taxon sampling (i.e., four dermapteran species), Song et al. [10] found the
380 Dermaptera and/or Plecoptera being sister to the remaining taxa within Polyneoptera. Notably, when
381 only using one species of Dermaptera, *C. fletcheri* emerged as sister taxa to the Ephemeroptera [10].
382 This result in agreement with our findings is presumably due to LBA. Phylogenomic data matrices
383 (e.g., expressed sequence tags data: [83], 1,478 PCGs: [79]) and nuclear genes [5] found the
384 Embioptera to be sister clade to the Phasmatodea. Using mitochondrial genomes data and BI, the
385 Embioptera were also found as sister group to the Phasmatodea (excluding *T. californicum*) [10].
386 However, in the same study, they also found support for the sister-relationship of Embioptera-
387 Zoraptera using ML [10]. Overall, more phylogenetic studies will be needed to clarify this issue;

388 mostly also because the use of few taxa from anomalous orders (i.e., Dermaptera and Embioptera)
389 tend to evoke LBA [10].

390 **Characterization of ephemeropteran mitochondrial genomes**

391 Mayfly mitochondrial genomes are widely conserved in gene content and nucleotide composition
392 across eleven families, with only a few differences in the content of their tRNAs. The coverage of
393 the *Cloeon*-mitochondrial genome was in agreement with other studies, using the same sequencing
394 platform (e.g., 59-281x, [92]). The missing of part of the AT-rich region is due to reduced
395 sequencing and assembly efficiency of this low complexity region and common among insect
396 mitochondrial genomes [55,93]. The gene order and orientation were with the exception of two
397 shifted tRNAs for *H. zijinensis* identical to the ancestral insect mitochondrial genome [41,42,54].
398 Other mayflies are also reported to miss complete T termination codons in the genes *cox2* and *nad5*
399 [55,93,94]. The two rRNAs were located between *trnL* and *trnV* (*rrnL*), and between *trnV* and the
400 AT-rich region (*rrnS*), respectively. All ephemeropteran mitochondrial genomes contain an AT-rich
401 region, which is placed between the *rrnS* (- strand) and *trnI* (+ strand). Li et al. [55] reported two
402 distinct parts within the AT-rich region in *Siphluriscus chinensis,* which also seems to be present in
403 *C. dipterum*. Therein, they described the so called CR_1, which is located close to the *rrnS* and has a
404 high AT content (71.6%), including six identical 140 bp sequences, and the CR_2, which is close to
405 the *trnI* and has a lower AT content (58.1%). *E. orientalis* contains two identical 55 bp long
406 sequences in the AT-rich region [94]. Few mayfly mitochondrial genomes differ in their gene
407 content from the ancestral insect mitochondrial genome, possessing one additional tRNA. The two
408 heptageniid species *Parafronurus youi* and *Epeorus* sp. encode a second copy of the *trnM* (AUG,
409 *trnM2*) gene located between *trnI* and *trnQ* [17,93]. For *S. chinensis*, an additional *trnK2* (AAA)
410 gene is described [55]. Overall, Song et al. [10] found similar genetic distances for the early
411 branching insects lineages. The AT contents for the Ephemeroptera were very similar to the ones of
412 Odonata (62.6%-68.5% for *cox1*, [95]).

Conclusions

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

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Newly generated sequences are available on GenBank (XXXXXXXX, XXXXXXXX, and XXXXXXXX).

Competing interests

The authors declare that they have no competing interests.

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Authors' 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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457 **Tables**

458 **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 Methods).

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

Figures

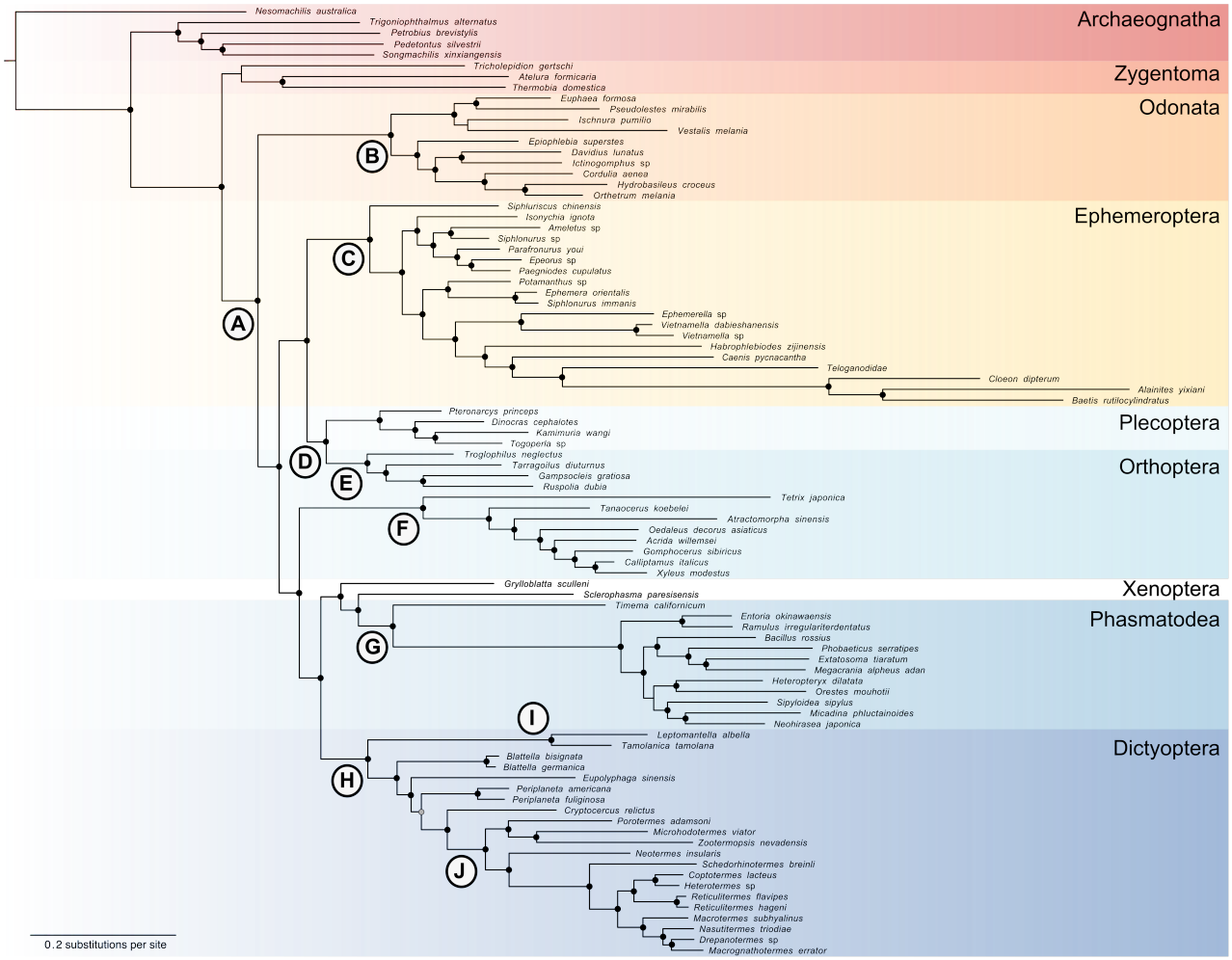
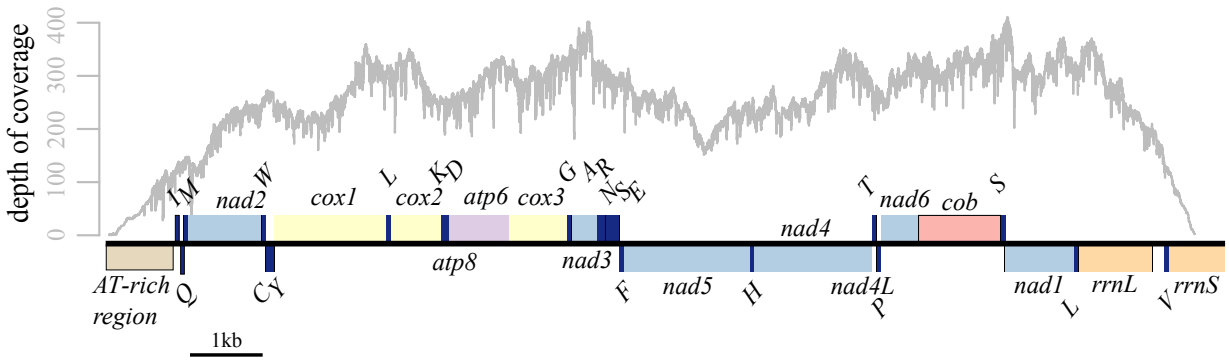
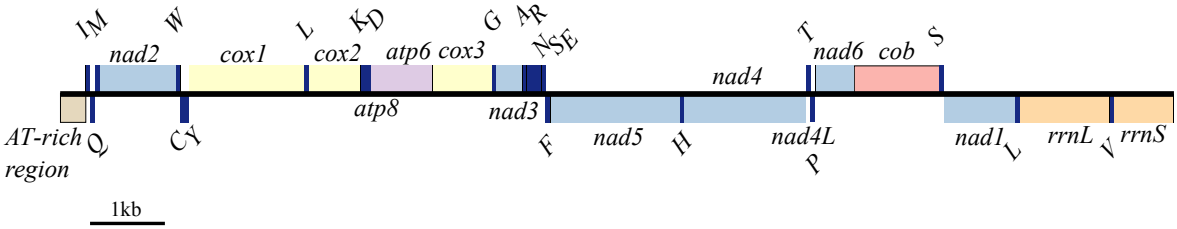


Fig. 1 Phylogenetic relationships of insect major orders using Bayesian inference reconstruction based on mitochondrial genome data using the concatenated amino acid sequences of the optimized_taxa matrix (see Table 1). Filled circles indicate supported nodes; whereby black circles represent Bayesian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

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



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



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

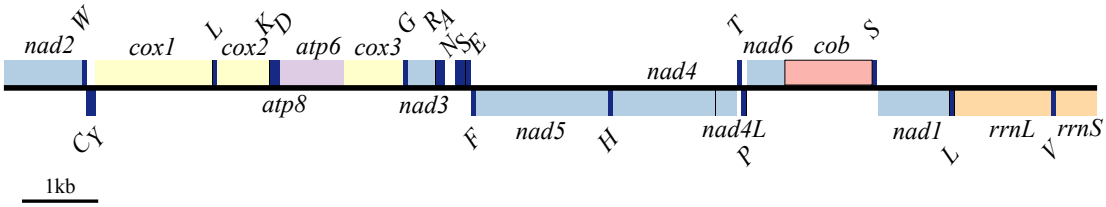


Fig. 2 Mitochondrial genome maps of three sequenced Ephemeroptera species. The nearly complete mitochondrial genome of (**a**) *Cloeon dipterum*, including coverage depth, the complete mitochondrial genome of (**b**) *Baetis rutilocylindratus,* and the incomplete mitochondrial genome of (**c**) *Habrophlebiodes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

Additional files

Additional file 1: Table S1. Universal primers for Sanger sequencing. List of primer pairs used for the PCR amplification and Sanger sequencing of *Baetis rutilocylindratus* (Baetidae) and *Habrophlebiodes zijinensis* (Leptophlebiidae).

Additional file 2: Fig. S1. Phylogenetic relationships of insect major orders using Bayesian inference reconstruction based on mitochondrial genome data using the concatenated amino acid sequences of the all_taxa matrix (see Table 1). Filled circles indicate well-supported nodes; whereby black circles represent Bayesian posterior probability (BPP) = 1, and grey circles BPP ≥ 0.95. Scale bar indicates substitutions per site. Coloured tip labels refer to the orders, whereby light blue = Archaeognatha (AR), dark blue = Blattodea (BL), light green = Ephemeroptera (EP), dark green = Grylloblattodea (GR), red = Isoptera (IS), light orange = Mantodea (MT), dark red = Mantophasmatodea (MP), dark orange = Odonata (OD), light purple = Orthoptera (OR), dark purple = Phasmatodea, yellow = Plecoptera (PL), and brown = Zygentoma (ZY). Five taxa being excluded from the optimized_taxa matrix are coloured in dark grey.

Additional file 3: Fig. S2. Phylogenetic relationships of insect major orders using maximum likelihood reconstruction based on mitochondrial genome data using the concatenated amino acid sequences of the optimized_taxa matrix (see Table 1). Filled circles indicate well-supported nodes; whereby black circles represent Bootstrap Support (BS) ≥ 90, and grey circles BS ≥ 80. Scale bar indicates substitutions per site. Coloured tip labels refer to the orders (for details see Additional file 2: Figure S1).

Additional file 4: Fig. S3. Phylogenetic relationships of insect major orders using maximum likelihood reconstruction based on mitochondrial genome data using the concatenated amino acid sequences of the all_taxa matrix (see Table 1). Filled circles indicate well-supported nodes; whereby black circles represent Bootstrap Support (BS) ≥ 90, and grey circles BS ≥ 80. Scale bar indicates

514 substitutions per site. Coloured tip labels refer to the orders with the exception of taxa being
515 excluded from the optimized_taxa matrix coloured in dark grey (for details see Additional file 2:
516 Figure S1).

517 **Additional file 5: Table S2**. Overview of ephemeropteran mitochondrial genomes. Total sequence
518 length in base pairs (bp) and individual nucleotide compositions of mayfly mitochondrial genomes
519 calculated based on the whole available sequences and the ones corrected for the incomplete
520 mitochondrial genomes (see Methods).

521

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