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Molecular Entomological Genetics

How reliable is mitochondrial DNA for recovering the phylogeny of Chironomidae (Culicomorpha: Diptera)?

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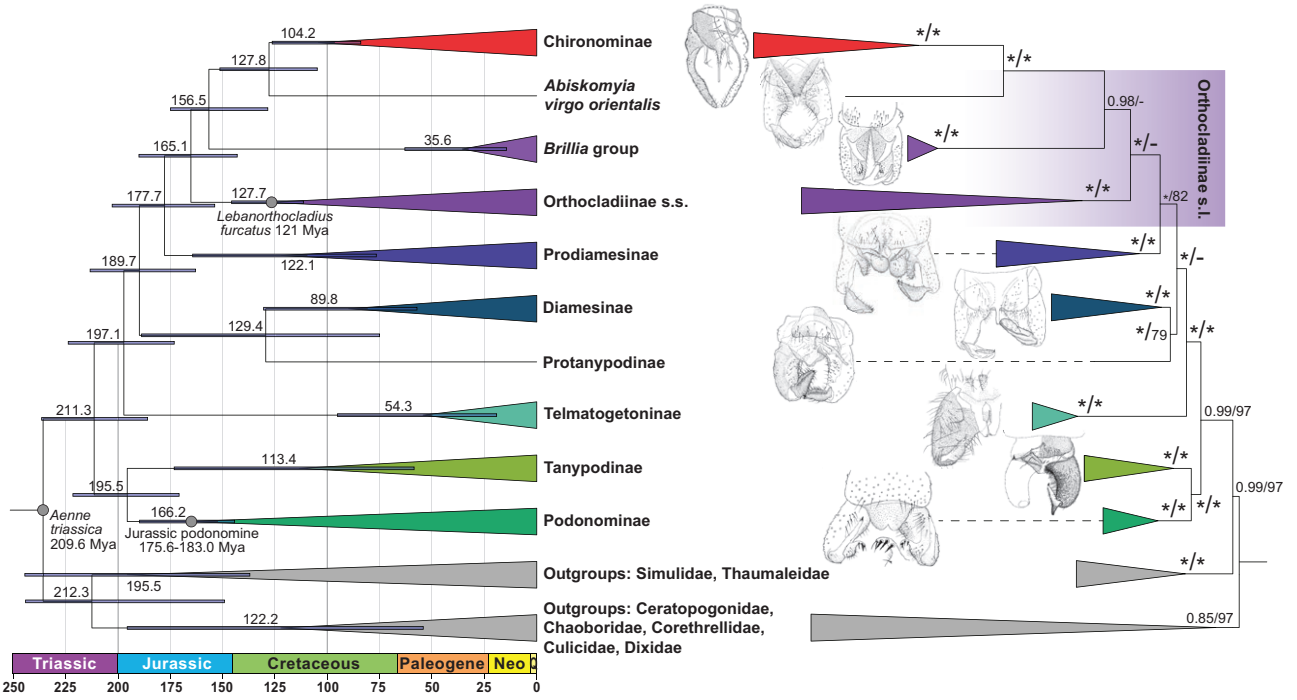
We examine mitochondrial DNA as a source of data to estimate phylogenetic relationships within the Chironomidae (Diptera). Previous studies have shown that mitogenomes often produce ambiguous phylogenetic topologies that are inconsistent with both morphological and multi-locus molecular data. In this study, we sequenced 18 new mitogenomes representing 5 subfamilies, including the first available sequence for Protanypodinae. These were combined with 65 previously annotated chironomids mitogenomes, 8 additional individuals assembled from SRA data, and 8 outgroup taxa. Phylogenetic reconstructions were performed using complete protein-coding genes (PCGs), the first and second codon positions of PCGs (PCG 12), with and without ribosomal genes (12S rDNA and 16S rDNA), and amino acid sequences (AA). Both Bayesian Inference and Maximum Likelihood approaches were implemented. Three alternative outgroup compositions were tested: (i) chironomids only, with restricted rooting on Podonominae; (ii) Ceratopogonidae and Culicidae; and (iii) a diverse selection of Culicomorpha. We found that the AA, PCG12, and PCG 12 + rDNA datasets, when coupled with the third outgroup combination, provide the strongest phylogenetic signal, with the highest effective sample size and log-likelihood scores. In most cases, the resulting tree topologieswere congruent between mitochondrial and multi-locus data. However, some consistent differences in topologies were observed, leading to differences in divergence time estimates. Our phylogenetic study indicates paraphyly of Orthocladiinae due to the positions of *Brillia* Kieffer and *Abiskomyia* Edwards, suggesting that a comprehensive integrative revision of this subfamily is required. We conclude that the reliability of the mitochondrial phylogenetic signal improves with the increased taxon sampling.

Keywords: non-biting midges, molecular evolution, mitochondrial genome, phylogeny, Bayesian inference, maximum likelihood

Graphical Abstract



Introduction

Mitochondrial DNA (mtDNA) is a useful genetic marker for phylogenetic reconstructions and divergence time estimations across various taxa (eg Cameron 2014, Li et al. 2014, Song et al. 2016, Pakrashi et al. 2022), as well as for population-genetic studies (eg Du et al. 2021), DNA barcoding (eg Hebert et al. 2016), spatial genetic distribution and landscape genetics (eg Epp et al. 2018, Wang et al. 2022), ecological adaptation (eg Camus et al. 2017), and adaptive evolution of insects (eg Sanno et al. 2021). The extensive application of mitochondrial markers stems from several characteristics: their relatively small genome size (16 to 20 Kbp), high copy number per cell, increased evolutionary rate compared to nuclear DNA, near-neutral evolution, and minimal recombination (Ballard and Rand 2005, Galtier et al. 2009). However, the use of mtDNA also has drawbacks, including the presence of nuclear mitochondrial pseudogenes (Thalmann et al. 2004), introgression (Mastrantonio et al. 2016), positive selection (Kang and Hu 2023), failure to detect hybrids due to uniparental (maternal) inheritance (Lee et al. 2023), and the spread in symbiotic infections (Frézal and Leblois 2008).

The mitochondrial genomes of Chironomidae include 37 genes: 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes (12S and 16S rRNA), along with an AT-rich control region that contains elements for replication and transcription (Brown 1985). As of April 2025, the lengths of validated chironomid mitogenomes range from 18,759 bp (*Stenochironomus tobaduodecimus* Kikuchi and Sasa) to longer sizes, with no clear correlation to subfamily affiliation. The typical mtDNA gene order for most chironomids is: tRNA-Ile—tRNA-Gln—tRNA-Met ND2—tRNA-Trp—tRNA-Cys—tRNA-Tyr—COX1—tRNA-Leu—COX2—tRNA-Lys—tRNA-Asp—ATP8—ATP6—COX3—tRNA-Gly—ND3—tRNA-Ala—tRNA-Arg—tRNA-Asn—tRNA-Ser—tRNA-Glu—tRNA-Phe—ND5—tRNA-His—ND4—ND4L—tRNA-Thr—tRNA-Pro—ND6—CYTB—tRNA-Ser—ND1—tRNA-Leu—16S rDNA—tRNA-Val—12S rDNA—control region (Li et al. 2022, 2024, Lin et al. 2022a, Qi et al. 2023, Zhang et al. 2023). This arrangement corresponds to the ancestral insect mitochondrial genome structure, reflecting the ancestral pancrustacean (Cameron 2014, Tyagi et al. 2020, Pakrashi et al. 2022). Of these genes, 4 PCGs and both rRNA genes are encoded on the light strand, while the remaining genes are encoded on the heavy strand. Minor rearrangements involving tRNA genes have been observed in the genus *Stenochironomus* Kieffer (Zheng et al. 2022).

Although the first chironomid mitogenome was published in 2011, most phylogenetic studies using mtDNA data emerged only after 2021 (Lin et al. 2022a, 2022b, Fang et al. 2023, Zhang et al. 2023). Mitogenomic trees often differ substantially from multi-locus nuclear DNA phylogenies. This incongruence may be caused by incomplete lineage sorting (Kruckenhauser et al. 2014, Montagna et al. 2016, Bryja et al. 2018), mtDNA introgression resulting from interspecific hybridization (Yannic et al. 2010), or rapid (explosive) speciation events (Krause et al. 2008, Chen et al. 2022).

Mitochondrial DNA has been used to reconstruct the phylogenetic relationships of chironomids at both intra- and intergeneric levels (Li et al. 2022, 2024, Lin et al. 2022b, Zheng et al. 2022, Qi et al. 2023, Zhang et al. 2023), within subfamilies (Lin et al. 2022a, 2022b) or between subfamilies (Hiki et al. 2021, Kong et al. 2021, Zheng et al. 2021, Fang et al. 2022, 2023, Jiang et al. 2022). However, in most cases, the reliability of these topologies has not been validated against nuclear DNA or morphological data. A notable exception is the relationship of *Polypedilum* Kieffer and related genera, where mitochondrial results partially agree with a highly sampled multi-locus molecular phylogeny (Tang et al. 2022a, Zhang et al. 2023). Nonetheless, inconsistencies remain: for example, outgroup genera such as *Stictochironomus* Kieffer, *Endochironomus* Kieffer, *Synendotendipes* Grodhaus, and *Polypedilum* Kieffer often

differ in placement between mtDNA and nuclear trees, except for the consistent sister-group relationship between *Stictochironimus* and *Endochironomus*, and between *P. unifascium* (Tokunaga) and *P. masudai* (Tokunaga)

The phylogeny of a relatively species-poor subfamily Prodiamesinae using mtDNA (Lin et al. 2022a) closely matches multi-locus nuclear DNA phylogenies (Cranston et al. 2012), including the assignment of *Propsilocerus* Kieffer to this subfamily. However, the monophyly of the Orthocladiinae has not been consistently supported, largely due to the placement of *Brillia* Kieffer forming a distinct clade from other orthoclads. Within the Palearctic Diamesinae, mitogenomic data suggest that *Potthastia* Kieffer is not sister to *Sympotthastia* Pagast but instead occupies a basal position relative to the remaining Diamesinae. Furthermore, the tribe Boreoheptagyini appears paraphyletic, with *Pagastia* Oliver + *Pseudodiamesa* Goetghebuer forming a sister group to *Diamesa* Meigen, contradicting to morphological (Serra-Tosio 1968, 1973, Rossaro 1995) and robust multi-locus (Semenchenko et al. 2024) trees.

Early mitogenome studies produced unusual internal relationships within Chironomidae. Comparative analysis of 6 mitogenomes, each from a different subfamily, suggested that the Orthocladiinae or Chironominae were sisters to the remaining chironomids (Zheng et al. 2021), contrary to morphological (Brundin 1966, Sæther 1977, Brundin and Sæther 1978, Murray and Ashe 1981, 1985, Andersen and Sæther 1994, Ashe and Connor 2009) and multi-locus or transcriptomic phylogenies (Cranston et al. 2010, Kutty et al. 2018, Tang et al. 2022a, Semenchenko et al. 2024). Depending on the choice of outgroups and gene datasets—37 genes (Fang et al. 2022), 13 PCGs (Kong et al. 2021, Jiang et al. 2022), or 13 PCGs + 2 rRNAs (Hiki et al. 2021)—tree topologies varied significantly. Then most recent study using 25 mitogenomes (13 PCGs + 2 rRNAs) of Chironomidae and extended outgroup (Fang et al. 2023) yielded a topology with high nodal support, largely congruent with morphological and multi-locus phylogenies. In this topology, the earliest branching event separates Podonominae and Tanypodinae as sister groups, consistent with previous studies (Cranston et al. 2010). The monophyly of Chironomoinae was supported by Bayesian inference. The overall topology (Diamesinae + (Prodiamesinae + (Orthocladi inae + Chironominae)) matches both morphological (Sæther 1977, Brundin and Sæther 1978) and molecular phylogenies (Cranston et al. 2010, 2012, Semenchenko et al. 2024). Notably, the problematic genus *Brillia* was excluded, facilitating Orthocladiinae monophyly.

Overall, previous studies reveal contradictory results. Poor sampling and inappropriate outgroup selection likely contributed to early misleading topologies, casting doubt on mitogenome-only phylogenies. However, increasing sample sizes and expanding outgroup selection significantly improve phylogenetic inference, although nodal support often remains low. Moreover, lack of consensus regarding dataset composition (eg whether to include or exclude rRNA genes or to use first and second codon positions or amino acids) further complicates comparison across studies. The concatenated dataset includes either 13 PCGs including (Hiki et al. 2021, Kong et al. 2021, Li et al. 2022, Lin et al. 2022a, Fang et al. 2023) or excluding (Jiang et al. 2022) rRNA genes, first and second codon positions of PCGs (Qi et al. 2023), amino acids (Zheng et al. 2022, Li et al. 2024), or various combinations of these datasets (Zheng et al. 2021, Lin et al. 2022b, Zhang et al. 2023). Outgroup choice also varies widely, ranging from chironomid-only (Li et al. 2022, Lin et al. 2022a, 2022b, Zhang et al. 2023) to including Ceratopogonidae and Culicidae (Kong et al. 2021, Zheng et al. 2021, Jiang et al. 2022) or broader outgroups incorporating Simuliidae, Sciaridae, and Drosophilidae (Fang et al. 2022).

Here, we examine the utility of the complete mitochondrial genomes for estimating chironomid phylogeny at the genus level, within and between subfamilies. Our goal is to find the causes of discrepancies between mitogenomic and multi-locus phylogenies, whether biological (eg incomplete lineage sorting, introgression, explosive speciation) or methodological (eg poor taxon sampling, inappropriate outgroups, unsuitable concatenated datasets). We apply both Maximum Likelihood and Bayesian Inference approaches using different datasets: 13 PCGs, PCG12 (first and second codon positions only), and amino acid sequences, with and without ribosomal genes. We reconstructed trees without outgroups, using a short outgroup including one sample of Ceratopogonidae and 2 samples of Culicidae, and an extended outgroup, including specimens from families Ceratopogonidae, Chaoboridae, Corethrellidae, Culicidae, Dixidae, Simuliidae and Thaumaleidae. To increase taxon sampling, we sequenced 18 new mitogenomes from 5 subfamilies and assemble 8 additional mitogenomes from SRA data (NCBI), and combined them with 65 annotated chironomid mitogenomes. Once the dataset with the highest branch support and the highest -lnL and ESS values is found, we reconstruct a dated molecular phylogeny using 3 fossil calibrations and compare the results with previous studies.

Material and Methods

Sampling and DNA Extraction

Specimens were collected by conventional field methods such as Malaise trap for imago, hand collection of larvae from the substrate, and preserved in 96% ethanol for DNA analysis and in 70% ethanol for further study of morphology and identification. We included 1 to 4 samples for Protanypodinae, Prodiamesinae, Orthocladiinae, and Chironominae whereas Diamesinae were the most widely represented with 10 samples. When selecting samples, preference was given to larvae or large imago males, since their size was sufficient to extract total DNA for NGS sequencing. In the case of larvae, their species identity was confirmed by DNA barcoding on a wide range of conspecific chironomids, including imago males. Collection information, date, sequence platform, vouchers ID, and accession numbers of the chironomids and outgroups obtained in this study and mined from GenBank are listed in Supplementary Table S1. Total DNA was extracted from the whole body of the larva using a DNeasy Blood and Tissue DNA kit (QIAGEN, Germany) according to protocol with the final elution volume of 100 µl.

Sequencing and Genome Assembly

An Ion Semiconductor platform was used for sequencing 16 samples. The libraries were prepared from total DNA using the Ion Plus Fragment Library Kit with a set of barcodes and sequenced using 540 chips on Ion S5 (Thermo Fisher Scientific, USA). Paired-end sequencing (150 bp) of 2 additional samples (Supplementary Table 1) was carried out on the Illumina NovaSeq X Plus (Illumina, Hayward, CA) in «Novogen» company. The raw reads were checked for quality with FastQC v.0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) cleaned, renamed, and deduplicated with EXONtools 0.3b (https://github.com/vinni-bio/EXONtools, Vinnikov and Cole 2021) and used for de novo assemblies with SPAdes 3.15.0 (Bankevich et al. 2012) with IonHammer option enabled for reads after Ion S5. For the accurately determine of the number of nucleotides in homonucleotide stretches

we manually compared with related annotated chironomids, or resorted to using ORF finer (https://www.ncbi.nlm.nih.gov/orffinder/) for protein-coding regions. MITOS2 webserver (available at http://mitos.bioinf.uni-leipzig.de/index.py) with invertebrate mitochondrial genetic code was used for annotation of PCGs, 22 transfer RNA genes and ribosomal RNA (rRNA) genes. To assess the effect of ambiguously aligned regions of ribosomal genes we aligned the dataset using MAFFT 7 (Katoh and Standley 2013) with the online service MPI Bioinformatics Toolkit (Zimmermann et al. 2018). Obtained mitogenomes were submitted to GenBank (accession numbers: PP761351-PP761368). The mitogenome maps, GC content and GC skew were drawn and calculated by the CG View server V 1.0 (Grant and Stothard 2008).

Phylogenetic Analyses

To date (Apr 2025), mitogenomes in GenBank have been obtained for 288 species of Chironomidae from 64 genera. A reduced dataset, in which most genera were represented by 2 species, was created to reduce computational time. This was done by random excluding of species from species-rich genera; however, we retained 3 to 4 species for some genera of diamesines to which our sequences were added, resulting in an expanded species set (eg *Boreoheptagyia*, *Diamesa*, *Pagastia*, *Potthastia*). In this study, we did not use mitochondrion from recent articles (Liu et al. 2024, Chen et al. 2025 , Xiao et al. 2025) since the final dataset was prepared in mid-2024.

Phylogenetic analyses were performed in Bayesian inference (BI) and maximum likelihood (ML) using MrBayes 3.2.7a (Ronquist et al. 2012) and IQTree 2.3.4 (Minh et al. 2020) respectively. We generate 5 datasets (1) 13 protein-coding genes (13 PCGs) (2) only first and second codon positions of 13 protein-coding genes (13 PCGs12) (3) 13 PCGs plus ribosomal rDNA (13 PCGs + rRNAs), (4) 13 PCGs12 + rRNAs, and (5) amino acids (AA) data. Each dataset also reconstructed (i) without outgroups with manually rooting by Podonominae, (ii) using samples from Culicidae and Ceratopogonidae, and (iii) extended outgroup set includes Ceratopogonidae, Chaoboridae, Corethrellidae, Culicidae, Dixidae, Simuliidae, and Thaumaleidae (Table 1). To achieve comparability of results with previous studies, we disregarded calculated evolution rates of various protein-coding genes in chironomids (Zheng et al. 2021, 2022, Lin et al. 2022b, Zhang et al. 2022, Fang et al. 2023, Qi et al. 2023, Li et al. 2024), and we completely excluded transfer RNAs and the control region from the analysis. Generated datasets were concatenated using SequenceMatrix v1.7.8 (Vaidya et al. 2011).

PartitionFinder 2.1.1 (Lanfear et al. 2017) was used to select the best-fit partitioning scheme and models separately for ribosomal RNA and each codon position of protein-coding genes using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. BI analysis was carried out according to the results of PartitionFinder using Markov Chain Monte Carlo (MCMC) randomization. Four Markov chains (3 heated chains, one cold) were run for 10 million generations (1 million for amino acids datasets), with the first 25% of sampled trees discarded as burn-in. Trace files were visually inspected in TRACER 1.7.1 (Rambaut et al. 2018). We carried out ML analysis with 1 million bootstrap replicates (500,000 for amino acids datasets) using ultrafast bootstrapping (UFB; Hoang et al. 2018) and MFP + MERGE to perform the built-in PartitionFinder algorithm. Best models after PartitionFinder 2.1.1 for MrBayes and PartitionFinder implemented in IQTree 2.3.4 are in Supplementary Table S2.

Divergence-time Estimation

Estimation of divergence times among chironomids was calculated by the Bayesian optimized relaxed clock method in BEAST 2.7.7 (Bouckaert et al. 2019). The HKY + G substitution model, estimated base frequencies with the calibrated Yule model, were applied as tree priors (Drummond and Bouckaert 2015). Three fossil records from different lineages were used as calibration points to constrain the analysis. The stem age of Chironomidae was used as log-normal prior calibration with zero offset based on the *Aenne triassica* Krzeminski and Jarzembowski 1999, dated as 209.6 Mya, in accordance with Krosch et al. (2011) and Krosch and Cranston (2013). The second fossil was a Jurassic podonomine dated as 175.6 to 183.0 Mya (Ansorge 1996) according to Cranston et al. (2010, 2012). The third fossil was the oldest known orthoclad *Lebanorthocladius furcatus* Veltz et al. (Veltz et al. 2007) with a mean of 121 Mya (Krosch et al. 2011, Krosch and Cranston 2013). The MCMC run was performed with 50 million generations and sampling every 7,000 generations. Convergence of the analysis was evaluated through the inspection of the effective sample sizes (ESS) using TRACER v.1.7.1. First 25% of trees were discarded from the tree file and a maximum clade credibility tree was obtained using TreeAnnotator 2.7.7. The consensus tree with the confidence interval of divergence time (95% HPD) was visualized in FigTree 1.4.4 (Rambaut 2018).

Results

Of the 18 obtained mitogenomes, only 3 had the complete circular sequence, *Chironomus* sp. (OVO4158), *Diamesa caucasica* Kownacki et Kownacka (EAM1292) and *Pagastia orientalis* (Tshernovskij) (EAM1238). Total length of these mitogenomes was 15,676, 16,020, and 16,133 base pairs, respectively. Two specimens *Abiskomyia virgo orientalis* Makarchenko et Makarchenko (EAM502) and *Orthocladius* sp. (EAM591) had partially missing data in the ribosomal gene 12S rRNA, control region absent (Supplementary Fig. S1). *Hydrobaenus laticaudus* had partially missing data in ND5, ND6, and 16S genes, control region absent. The remaining specimens had missing data of varying lengths in the control region due to decreased coverage after high-throughput sequencing (Semenchenko et al. 2024). Each mitogenome contains 37 typical genes (Supplementary Figs. S1 and S2) in the order and strands usual for all chironomids (Zheng et al. 2021, Lin et al. 2022a, Zhang et al. 2023) with no rearrangements (Zheng et al. 2022). *Tokunagaia rectangularis* (Goetghebuer) has the lowest (73.0%) A + T content across whole mitogenome (without control region) while *Orthocladius* sp. has the highest value—78.4%. We found no relationship between A + T content and subfamily. Most PCGs in each codon positions exhibited negative GT-skew. For the obtained mitogenomes calculated GC content and GC-skew values are in Supplementary Figs. S1 and S2. Start codon of most PCGs was ATN (ATA, ATC, ATG, or ATT) with a few exceptions as TTG in genes ND1, COI, and GTG in some cases of gene ND5. Most genes end with a TAA stop codon, while the TAG triplet occurs in some cases in genes COI, ND2, ND4, and ND5 according to Lin et al. (2022b).

We generated 30 trees (Table 1, Supplementary Figs. S3–S32) using 2 phylogenetic reconstruction methods, 5 dataset variants, and 3 outgroup variants (see Materials and Methods for details). Depending on the alignment due to the presence or absence of external groups, the length of the final datasets changed slightly and amounted 3755–3828 amino acids for AA, 11102–11603 bp for 13 PCG, 12925–14890 bp for 13 PCG + rDNA, 7635–7705 bp for 13 PCG12, and 10344–10633 bp for 13 PCG12 + rDNA datasets. The

Table 1. Outcomes of phylogenetic analyses incorporating Amino Acids (AA), ribosomal RNA (rDNA), 13 protein-coding genes (13 PCG) or only first and second codon positions of 13 PCG (13 PCG12) employing Bayesian inference and Maximum likelihood analysis. Algorithm, effective sample sizes, and log-likelihood scores are given in 2 columns. The combined lines show 3 variants of outgroups in tree reconstruction. The clades with support less than 0.7 BPP or 70% BS are highlighted in red, 0.71 to 0.80 BPP and 71 to 80% BS in green, 0.81 to 0.90 BPP and 81 to 90% BS in blue, and more than 0.91 BPP and 91% BS in black.

Dataset	Algorithm, tree link in Supporting Information, lnL / ESS	Are Protanypodinae sister to Diamesinae?	Sister clade to (*Diamesa* + *Syndiamesa*)	Location of Telmatogetoninae	Location of *Brillia*	Location of *Thienemanniella*
Mitochondrial DNA without outgroup						
AA	MrBayes, S5 ESS: 769 lnL: -119800	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S6 lnL: -115764	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
13 PCG	MrBayes, S7 ESS: 11 lnL: -353400	No	*Potthastia* + *Sympotthastia*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S8 lnL: -352883	No	*Potthastia* + *Sympotthastia*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)
13 PCG + rDNA	MrBayes, S9 ESS: 46 lnL: -388300	Yes	*Potthastia* + *Sympotthastia*	Sister to Prodiamesinae (Orthocladiinae s.l. + Chironominae)	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Sister to *Kiefferophyes* + (*Limnophyes* + *Belgica*)
	IQTree2, S10 lnL: -387783	No	*Pagastia* + *Pseudodiamesa*	Sister to Prodiamesinae + (Orthocladiinae s.l. + Chironominae)	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
13 PCG12	MrBayes, S11 ESS: 5436 lnL: -131700	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
	IQTree2, S12 lnL: -132047	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
13 PCG12 + rDNA	MrBayes, S13 ESS: 4975 lnL: -180400	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Prodiamesinae (Orthocladiinae s.l. + Chironominae)	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
	IQTree2, S14 lnL: -181116	No	*Pagastia* + *Pseudodiamesa*	Sister to Prodiamesinae (Orthocladiinae s.l. + Chironominae)	Sister to Orthocladiinae s.s. (*Abiskomyia* + Chironominae)	Sister to *Kiefferophyes* + (*Limnophyes* + *Belgica*)
Mitochondrial DNA with short outgroup						
AA	MrBayes, S15 ESS: 1936 lnL: -128200	No	*Pagastia* + *Pseudodiamesa*	Sister to Prodiamesinae + ((Diamesinae) + (Protanypodinae (Podonominae + Tanypodinae))	Sister to Orthocladiinae s.s. + (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
	IQTree2, S16 lnL: -123745	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s

Table 1. Continued

Dataset	Algorithm, tree link in Supporting Information, lnL / ESS	Are Protanypodinae sister to Diamesinae?	Sister clade to (*Diamesa* + *Syndiamesa*)	Location of Telmatogetoninae	Location of *Brillia*	Location of *Thienemanniella*
13 PCG	MrBayes, S17 ESS: 163 lnL: -377900	Polytomic with (1) Podonominae + Tanypodinae (2) Prodiamesinae (3) Diamesinae	*Potthastia* + *Sympotthastia*	Polytomic with (1) Diamesinae + Protanypodinae + Prodiamesinae (2) Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S18 lnL: -377159	Sister to Podonominae + Tanypodinae	*Potthastia* + *Sympotthastia*	Basal to Chironomidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
13 PCG + rDNA	MrBayes, S19 ESS: 11 lnL: -429800	Yes	*Potthastia* + *Sympotthastia*	Sister to Prodiamesinae + (Orthocladiinae s.l. + Chironominae)	Sister to *Abiskomyia* + Chironominae	Sister to *Kiefferophyes* (*Limnophyes* + *Belgica*)
	IQTree2, S20 lnL: -429533	Yes	*Pagastia* + *Pseudodiamesa*	Basal to Chironomidae	Sister to Orthocladiinae s.s. (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
13 PCG12	MrBayes, S21 ESS: 5254 lnL: -142600	Sister to Podonominae + Tanypodinae	*Pagastia* + *Pseudodiamesa*	Basal to Chironomidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s
	IQTree2, S22 lnL: -142892	Sister to Tanypodinae + Podonominae	*Pagastia* + *Pseudodiamesa*	Basal to Prodiamesinae + (Diamesinae + (Protanypodinae + (Tanypodinae + Podonominae)))	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s
13 PCG12 + rDNA	MrBayes, S23 ESS: 5489 lnL: -193900	No	*Pagastia* + *Pseudodiamesa*	Basal to Chironomidae	Sister to Orthocladiinae s.s. (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
	IQTree2, S24 lnL: -243797	Yes	*Pagastia* + *Pseudodiamesa*	Basal to Chironomidae	Sister to *Abiskomyia* + Chironominae	Sister to *Kiefferophyes* (*Limnophyes* + *Belgica*)
Mitochondrial DNA with an extended outgroup						
AA	MrBayes, S25 ESS: 476 lnL: -141800	No	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S26 lnL: -136925	No	*Pagastia* + *Pseudodiamesa*	Sister to Chironomoidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
13 PCG	MrBayes, S27 ESS: 300 lnL: -407700	Yes	*Potthastia* + *Sympotthastia*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s
	IQTree2, S28 lnL: -406976	Yes	*Potthastia* + *Sympotthastia*	Sister to Orthocladiinae s.l. + Chironominae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
13 PCG + rDNA	MrBayes, S29 ESS: 971 lnL: -463300	Yes	*Potthastia* + *Sympotthastia*	Sister to Chironomoidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S30 lnL: -463061	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Prodiamesinae (Orthocladiinae s.l. + Chironominae)	Sister to *Abiskomyia* + Chironominae	Sister to *Kiefferophyes* (*Limnophyes* + *Belgica*)

Table 1. Continued

Dataset	Algorithm, tree link in Supporting Information, lnL / ESS	Are Protanypodinae sister to Diamesinae?	Sister clade to (*Diamesa* + *Syndiamesa*)	Location of Telmatogetoninae	Location of *Brillia*	Location of *Thienemanniella*
13 PCG12	MrBayes, S31 ESS: 2658 lnL: -153200	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Chironomoidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S32 lnL: -154651	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Orthocladiinae s.l. + Chironominae	Sister to Orthocladiinae s.s. (*Abiskomyia* + Chironominae)	Basal to Orthocladiinae s.s.
13 PCG12 + rDNA	MrBayes, S33, Figure 1. ESS: 3821 lnL: -209100	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Chironomoidae	Sister to *Abiskomyia* + Chironominae	Basal to Orthocladiinae s.s.
	IQTree2, S34 lnL: -210137	Yes	*Pagastia* + *Pseudodiamesa*	Sister to Chironomoidae	Sister to Orthocladiinae s.s. (*Abiskomyia* + Chironominae)	Sister to *Kiefferophyes* (*Limnophyes* + *Belgica*)

obtained topology, the log-likelihood values (-lnL), Effective Sample Size (ESS) of Bayesian analyses of the resulting trees differed significantly from each other (Table 1, Supplementary Figs. S2–S31). The worst values of -lnL and ESS were found in 13 PCG and 13 PCG + rDNA datasets (Supplementary Figs. S5–S8, S15–S18, S25–S28) with any set of outgroups which relates obviously to the saturation of the third position of the codon in PCG. For this reason, we did not use these trees in further discussion. The amino acid datasets had average tree quality, while trees with highest branch support were AA, 13 PCG12 and 13 PCG12 + rDNA (Supplementary Figs. S3–S4, S9–S14, S19–S24, S29–S32).

The monophyly of Chironomidae and was strongly supported across ML and BI analyses using different databases using outgroups. Also, most subfamilies were supported as monophyletic with the exception of Orthocladiinae which were consistently paraphyletic due to genera *Brillia* and *Abiskomyia*. However, the topology between subfamilies varied significantly, but on the trees with the highest branch support (eg Fig. 1, Supplementary Figs. S23—S32) remained relatively stable and coincided with previous multi-locus molecular studies (Cranston et al. 2012, Semenchenko et al. 2024). Tanypodinae and Podonominae were recovered as sister clades on all trees including outgroups while the first 13 trees were rooted by the podonomines manually. Sister relationships between Diamesinae and Protanypodinae suggested on half of the topologies without a clear correlation with node support, values of -lnL and ESS (Table 1). The relationship of Telmatogetoninae was the most unstable in the trees appeared as sister to Chironominae, sister to Chironomoidae, sister to Orthocladiinae + Chironominae or various combinations of polytomous nodes. Within Diamesinae 2 main topology were obtained: (*Potthastia* + *Sympotthastia*) + ((*Pagastia* + *Pseudodiamesa*) + (*Diamesa* + *Syndiamesa*)) or (*Pagastia* + *Pseudo diamesa*) + ((*Potthastia* + *Sympotthastia*) + (*Diamesa* + *Syndiamesa*). Topologies within Ortocladiinae (except *Abiskomyia* and *Brillia*) and Chironominae remained relatively stable among the most accurate trees.

The dataset 13 PCG12 + rDNA with extended outgroup was used for the divergence time estimation of chironomids (Fig. 2), as this is the highest values of -lnL and ESS and the highest nodal support. The topology of an ultrametric fossil-dated phylogeny almost completely consistent with the tree after Bayesian analysis (Fig. 1), except for minor changes in the Chironominae: *Microtendipes umbrosus* was placed as sister to *Tanytarsus formosanus* and *Kiefferulus tainanus* was placed as sister to clade 1 (see Fig. 1 for details) after branching *Axarus fungorum*. Our fossil-dated phylogeny infers an origin of Chironomidae at around 211 Mya (95% HPD 185.8 to 236.3 Mya) in the Upper Triassic (Fig. 2, Table 2). The subfamilies diversified at Lower Jurassic to Lower Cretaceous. The clade D comprising Chironomoinae originated in the Lower Jurassic however, the diversification of diamesines occurred only in Upper Cretaceous (node F) after divergence with Protanypodinae in Lower Cretaceous (node E). The divergence of the genera *Brillia* (node J) and *Abiskomyia* (node K) from a common stem with Chironominae occurred in Upper Jurassic and Lower Cretaceous, respectively.

Discussion

Relationships Within Culicomorpha

The monophyly of superfamilies Culicoidea and Chironomoidea was strongly confirmed by ML and BI analyses; however, we found the unexpected relationship of *Culicoides arakawae* (Ceratopogonidae) which is belonged to clade Culicoidea (Fig. 1, Supplementary

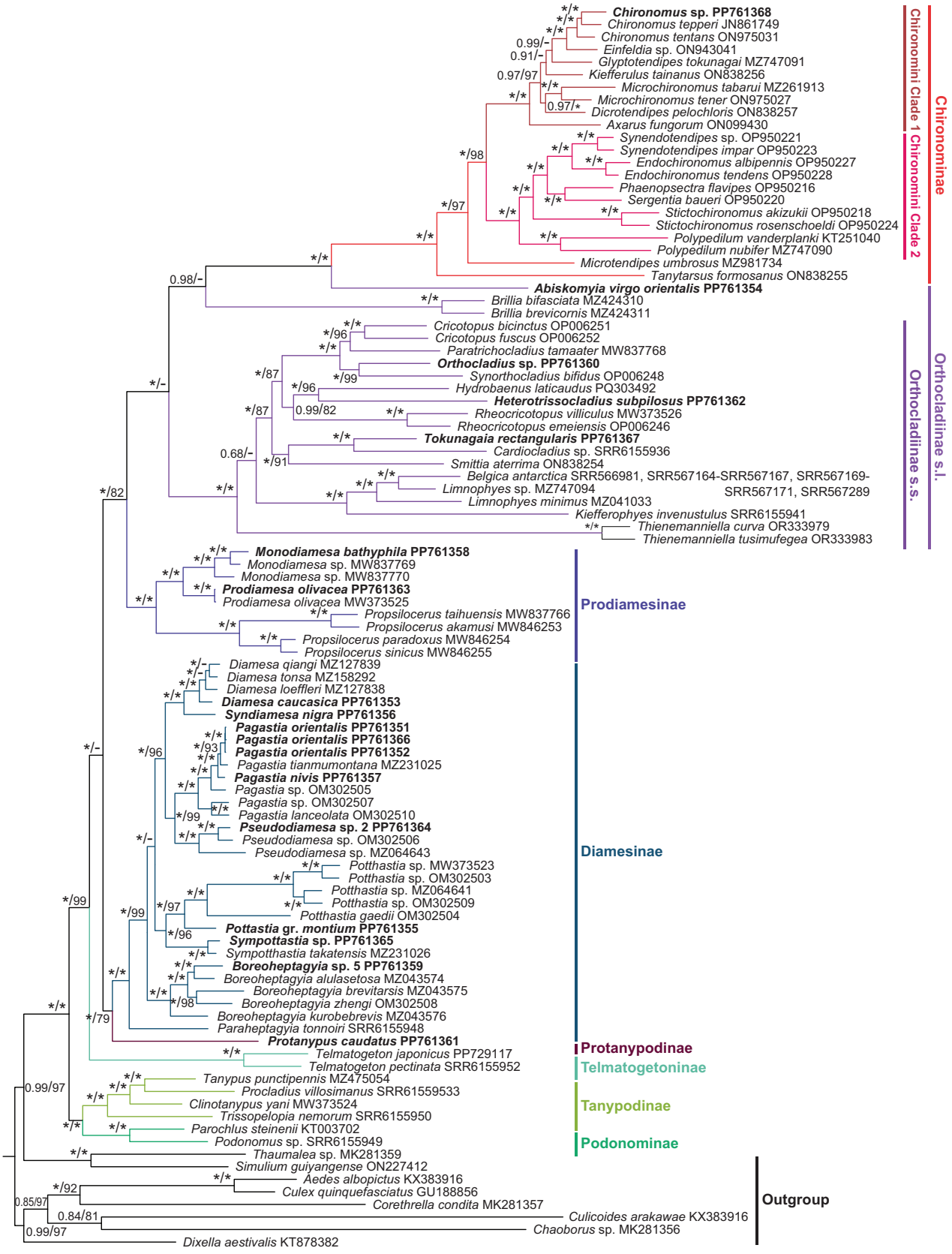


Fig. 1. Bayesian tree of Chironomidae based on first and second codon positions of 13 protein-coding genes and 2 ribosomal genes of mitochondrial DNA. Seven samples of Culicomorpha are used as outgroups. Posterior probability after MrBayes, and bootstrap support of Maximum likelihood (> 70%) are shown and asterisks (*) indicate full node support.

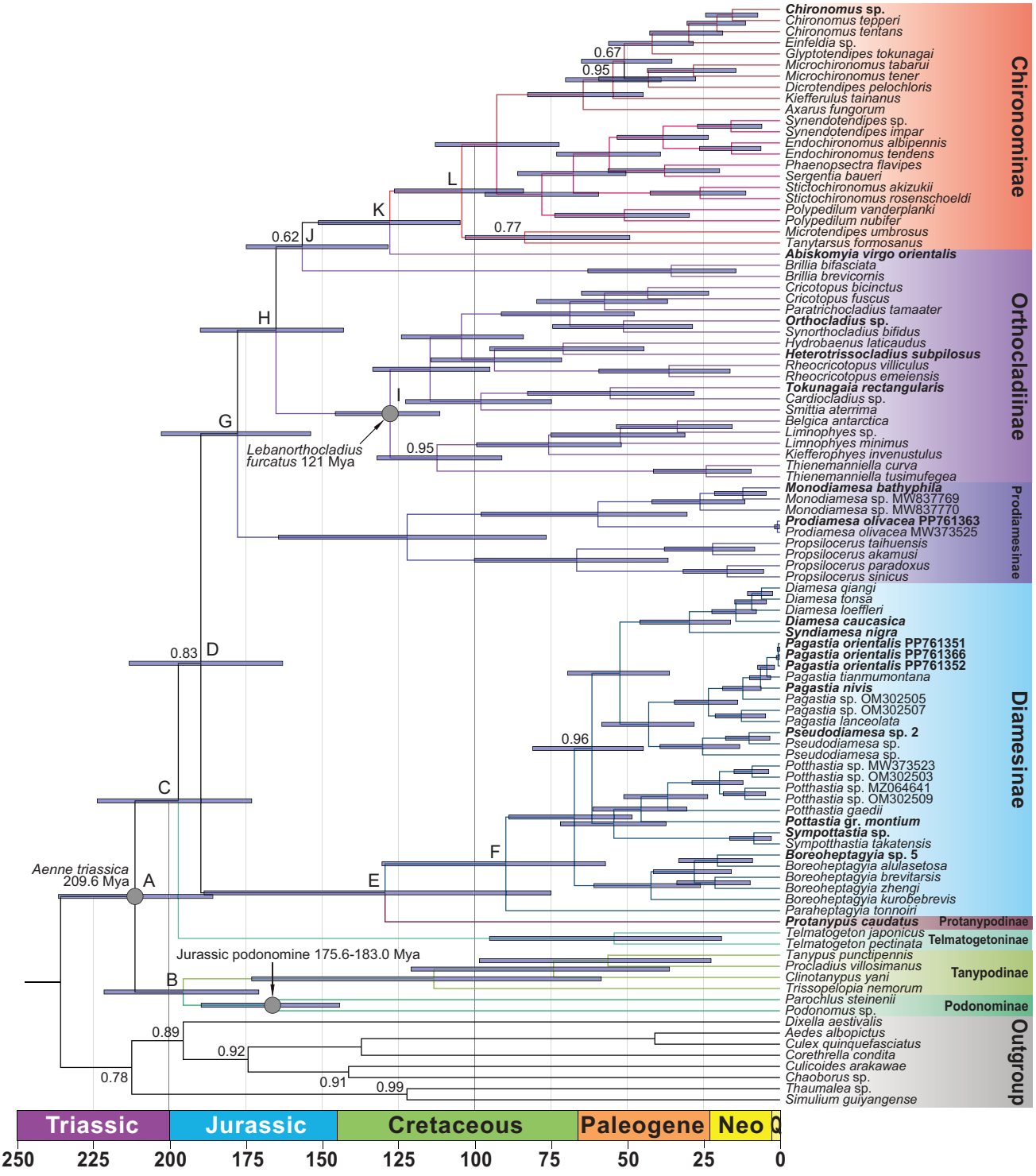


Fig. 2. Fossil-dated BEAST phylogenetic tree of the Chironomidae based on first and second codon positions of 13 protein-coding genes and 2 ribosomal genes of mitochondrial DNA. Posterior probabilities below 1.0 are indicated near the branches while the remaining nodes had probabilities equal to 1.0. The node bar indicates 95% confidence interval of the node age. The geological time axis displays period and epoch. 'L' refers to Lower, 'PC' refers to Paleocene epoch, "P' refers to the Pleistocene epoch and 'Q' refers to the Quaternary period.

Figs. S23–S32). Dixidae forms the sister group to the remaining Culicoidea which contradict with previous studies (Bertone et al. 2008, Wiegmann et al. 2011, Kutty et al. 2018 , Wiegmann and Yeates 2017, etc.) although the topology Chaoboridae + (Corethrel lidae + Culicidae) is observed for the first time. Chironomoidea was monophyletic with a highly supported topology across datasets: Chi ronomidae + (Thaumaleidae + Simuliidae) except for the absence of Ceratopogonidae from this clade. Obtained topology was coincided with Wiegmann et al. (2011), Wiegmann and Yeates (2017), and the sister relationship of Thaumaleidae + Simuliidae corresponded

Table 2. Mean divergence time estimates and 95% HPD (in million years ago, mya) intervals for key chironomid divergences.

Clade	Node Letter	This study		Cranston et al. 2010	Cranston et al. 2012	Krosch et al. 2020	Tang et al. 2022	Semenchenko et al. 2024
		Mean	95% HPD					
Chironomidae	A	211.3	185.8–236.3	211	mid Triassic	199.6	205	230.0
Podonominae + Tanypodinae	B	195.5	170.7–221.4	148				
Chironomidae minus Podonominae, Tanypodinae	C	197.1	173.1–223.6		lower Jurassic		125	
Chironomidae minus Podonominae, Tanypodinae, Telmatogetoninae	D	189.7	162.9–213.2		lower Jurassic			
Diamesinae + Protanypodinae	E	129.4	75.0–188.6					
Diamesinae	F	89.8	57.2–130.4	113	upper Jurassic			163.5
Prodiamesinae + Orthocladiinae s.l.+ Chironominae	G	177.7	153.7–202.7	118			111	146.8
Orthocladiinae s.l. + Chironominae	H	165.1	143.0–189.9		early-mid Jurassic	148.6		133.6
Orthocladiinae s.s.	I	127.7	111.4–145.7					
Chironominae + *Abiskomyia* + *Brillia*	J	156.5	128.4–174.8			148.6		
Chironominae + *Abiskomyia*	K	127.8	104.8–151.2					
Chironominae	L	104.2	84.0–126.3		Lower Cretaceous	108		

also with Pawlowsky et al. (1996), Bertone et al. (2008), Kutty et al. (2016), and Wiegmann and Yeates (2017). The phylogeny of the Culicomorpha was robust and congruent across the most datasets both Bayesian inference and Maximum likelihood. It is surprisingly, the highest posterior probability and bootstrap support values were obtained for datasets including all 3 codon positions of PCGs and amino acids datasets while any datasets with first and second codon positions yielded worse nodal supports.

In this study, we did not aim to reconstruct phylogenetic relationships within Culicomorpha, since this would require including Psychodomorpha (according to Wiegmann and Yeates 2017) in the analysis and increasing the number of samples. Despite this, mitochondrial DNA represents a robust topology at the family level, and the position of the *Culicoides arakawae* requires clarification.

Relationships Among Chironomidae Subfamilies

We provide mitochondrial trees that cannot reflect the real phylogeny of chironomids among subfamilies. This is due to the need to sample the 4 unrepresented subfamilies Aphroteniinae, Buchonomyiinae, Chilenomyiinae, and Usambaromyiinae and more representatives of most of the remaining subfamilies. Despite these acknowledged lacunae, we believe that provided mitogenomic phylogeny can be compared with multi-locus and transcriptomic trees to create a phylogenetic framework for future research.

Clade Podonominae + Tanypodinae were sister to the remaining Chironomidae for stem nodes of the trees. The unification of the subfamilies Podonomidae and Tanypodinae into a single clade forming a semifamily Tanypodoinae is shown in all phylogenies based on mitochondrial data (Hiki et al. 2021, Kong et al. 2021, Fang et al. 2022, 2023, Jiang et al. 2022), as well as in a study with a multi-locus dataset (Cranston et al. 2010) and morphological trees (Brundin 1966, Sæther 1977, 2000, Brundin and Sæther 1978, Murray and Ashe 1981, 1985, Andersen and Sæther 1994). Although subsequent multi-locus (Cranston et al. 2012, Semenchenko et al. 2024) and transcriptome phylogenies (Kutty et al. 2018) using a non-chironomid outgroup suggest that Podonominae diverged from the common chironomid stem before Tanypodinae divergence. However, only in the latest study is this divergence well supported. In the absence of a non-chironomid outgroup, the Tanypodinae are an earlier branching group (Tang et al. 2022b).

It is surprising that some trees with Culicidae and Ceratopogonidae as outgroups (13 PCG12, 13 PCG12 + rDNA, and 13 PCG12 + rDNA) suggested that Telmatogetoninae are the base of the family Chironomidae, which is a new and questionable result according to multi-locus and transcriptomic data despite the high support for this node (Table 1). However, many researchers claimed "plesiomorphic morphological features" in Telmatogetoninae, on the basis of which argued a basal position in trees (Sæther 1977, 1983, 2000, Brundin and Sæther 1978, Murray and Ashe 1981, 1985). Multi-locus data suggest that the Telmatogetoninae branch diverges before (Cranston et al. 2012, Tang et al. 2022b) or after diamesines (Semenchenko et al. 2024), which is confirmed by the 13 PCG12 + rDNA tree without outgroup (both ML and Bayesian analysis) in this study. However, a transcriptome study found the Telmatogetoninae are sister to Diamesinae (Kutty et al. 2018). Another variant of the arrangement of this subfamily as a sister to Orthocladiinae s.l. + Chironominae found support from the remaining unrooted and AA IQTree2 trees in this study. Thus, the position of the Telmatogetoninae in the chironomid tree remains questionable. Mitogenome-based phylogeny probably does not allow its position to be resolved, but neither multi-locus nor transcriptome data are consistent in its position.

The next subfamily with an ambiguous position was the Protanypodinae. This subfamily previously understood to be a tribe within Diamesinae (Brundin 1966) includes 2 genera, *Protanypus* Kieffer and *Linevitshia* Makarchenko (Makarchenko and Semenchenko 2014). A recent study excluded this tribe from Diamesinae and elevated it to subfamily rank as Protanypodinae based on polyphyly of Diamesinae and morphological implications (Semenchenko et al. 2024). In our phylogenies, the trees with and extended outgroup and the highest branch support 13 PCG12 + rDNA suggested that Protanypodinae are sister to Diamesinae (Fig. 1, Table 1, Supplementary Figs. S31 and S32), while AA trees, on the contrary, showed non-monophyletic relationships of 2 subfamilies (Table 1, Supplementary Figs. S23 and S24). Both topologies raise no doubts about the justification for elevation of Protanypodinae elevation; however, they show variability in the topologies of mitochondrial trees.

The topology of the remaining 3 sampled subfamilies Prodiamesinae + (Orthocladiinae + Chironominae) was highly stable using mitochondrial (Fang et al. 2023, this study) multi-locus (Cranston et

al. 2012, Semenchenko et al. 2024) and morphological data (Sæther 1977, 2000, Brundin and Sæther 1978, Murray and Ashe 1981, 1985, Andersen and Sæther 1994).

Monophyly and Relationships Within Chironomidae Subfamilies

Tanypodinae is a diverse subfamily of Chironomidae with strong development of the prementum and mentum of larva (Roback 1989). We included 4 species of Tanypodinae in the phylogenetic analysis. The highly supported topology of which was unchanged on all trees: *Trissopelopia nemorum* Goetghebuer + (*Clinotanypus yani* Cheng et Wang + (*Procladius villosimanus* Kieffer + *Tanypus punctipennis* Sublette)) (Fig. 1, Supplementary Figs. S3–S32). Despite the limited data, the resulting topology was completely consistent with multi-locus (Krosch et al. 2022), mitochondrial (Xiao et al. 2025), and morphological analysis (Silva, Ekrem, 2016) using similar genera. In an earlier molecular study, Krosch et al. (2017) lacked *Trissopelopia* Kieffer in the analysis, but the topology of tribes Pentaneurini + C linotanypodini + (Procladiini + Tanypodini) coincides with the mitogenomic phylogeny.

Diamesinae are a relatively species-poor subfamily of chironomids, most of whose species live in cold lotic or oligotrophic lentic habitats and are distributed globally, except for Antarctica (Semenchenko et al. 2024). A detailed highly sampled multi-locus phylogeny of austral and Holarctic diamesines has been proposed recently (Semenchenko et al. 2024). Austral and boreal diamesines diverged in the Jurassic and since then have developed an amphitropical ("bipolar") distribution. Monophyly of tribes Boreoheptagyiini and Diamesini was strongly supported by Bayesian inference and Maximum likelihood analyses. Genera *Pagastia* + *Pseudodiamesa* formed the sister clade of tribe Diamesini while *Arctodiamesa* Makarchenko + (*Sympotthastia* + *Potthastia*) was sister clade to *Pseudokiefferiella* Zavrel + (*Diamesa* + *Syndiamesa* Kieffer) (Semenchenko et al. 2024).

On the other hand, a phylogeny for 6 of the 12 Holarctic diamesines had been reconstructed using complete mitogenomes with *Prodiamesa* Kieffer and *Propsilocerus* as outgroups (Lin et al. 2022b). This study suggests *Potthastia* is not a sister to *Sympotthastia*, occupying a sister position within the remaining Diamesinae. The tribe Boreoheptagyiini was non-monophyletic, and 4 out of 6 trees assumed a sister relation of *Boreoheptagyia* Brundin and *Sympotthastia*. And finally, sister of the genus *Diamesa* were *Pagastia* + *Pseudodiamesa* (Lin et al. 2022b) which contradicts established morphological trees (Serra-Tosio 1968, 1973, Rossaro 1995).

In this study, we obtained mitogenomes of *Syndiamesa*, supplemented by the genera *Pagastia*, *Pseudodiamesa*, *Potthastia*, *Sympotthastia*, and *Boreoheptagyia*. The monophyly of Diamesinae was strongly supported (Fig. 1, Supplementary Figs. S3–S32). High similarity of diamesine topology was observed both in the resulting mitochondrial trees and in comparison, with the multi-locus phylogeny (Semenchenko et al. 2024). Austral *Paraheptagyia tonnoiri* (Freeman) was a sister for boreal diamesines. Tribe Boreoheptagyiini is restored high supported monophyly. On the most trees *Potthastia* were sister to *Sympotthastia*, which is consistent with both morphological (Serra-Tosio 1968, 1973) and multi-locus data (Semenchenko et al. 2024). At the same time, the sister relation of clades *Diamesa* + *Syndiamesa* and *Pagastia* + *Pseudodiamesa* on most trees (Table 1, Supplementary Figs. S3–S32) confirm the topology of Lin et al. (2022b). However, this discrepancy may be due to missing genera *Pseudokiefferiella*, *Arctodiamesa*, and *Lappodiamesa* Serra-Tosio and not to the inconsistency of mitogenomes for the phylogeny of chironomids.

The subfamily Prodiamesinae included 4 genera, *Compteromesa* Sæther, *Monodiamesa* Kieffer, *Odontomesa* Pagast, and *Prodiamesa* Kieffer (Ache and Connor 2009). Multi-locus analysis confirmed the monophyly of Prodiamesinae, also if including *Propsilocerus* that previously belonged to Orchocladiinae (Cranston et al. 2012). The transfer of this genus was supported subsequently with morphological data (Baranov 2021, Makarchenko and Semenchenko 2023), although some studies still consider *Propsilocerus* to be part of Orchocladiinae (Lin et al. 2022a, Fang et al. 2023). Relationships within the Prodiamesinae are consistent across all molecular studies using both mitogenomes (Lin et al. 2022a, Fang et al. 2023, this study) and multi-locus approaches (Cranston et al. 2012).

Orthocladiinae are the most species-rich and poorly studied subfamily. A relatively highly sampled phylogeny was obtained by Cranston et al. (2012), in which monophyly of Orthocladiinae were confirmed, but this node was supported neither by bootstrap analysis of Maximum likelihood trees or Bayesian posterior probability. Based on mitogenomic data, Lin et al. (2022a) reconstructed a phylogeny and showed paraphyly of Orthocladiinae since Prodiamesinae was found to be deeply nested within Orthocladiinae taxa. The authors conclude that the monophyly of Orthocladiinae is possible when transferred Prodiamesinae to the Orthocladiinae.

Our study also does not confirm the monophyly of Orthocladiinae. Most of the orthoclads samples formed a clade (hereinafter Orchocladiinae *sensu stricto*) on the tree with the basal position of *Corynoneura* group is represented by the genus *Thienemanniella*. The *Corynoneura* group consists of the genera *Corynoneura* Winnertz, *Corynoneurella* Brundin, *Onconeura* Andersen et Sæther, *Tempisquitoneura* Epler, *Thienemanniella* Kieffer, *Ubatubaneura* Wiedenbrug et Trivinho-Strixino and perhaps *Notocladius* Harrison. The main distinguishing feature of the group is the wing structure. Veins R_1 and R_{4+5} are short, thick, and fused with the costa in a thickened clavus, ending before midpoint of wing. The internal relationships of this clade and the position of *Thienemanniella* remain stable in most of the constructed trees (Table 1, Supplementary Figs. S3–S32).

However, 2 genera, *Brillia* Kieffer and *Abiskomyia* Edwards do not belong to the clade Orthocladiinae *sensu stricto* on any of the trees. The position of *Abiskomyia* was stable as sister to Chironominae. *Brillia* was sister to orthoclads (*Abiskomyia* + Chironominae) in most trees, but some trees suggested that it was sister to *Abiskomyia* and Chironominae (Table 1, Supplementary Figs. S3–S32). In this study, we refer to Orthocladiinae + *Brillia* + *Abiskomyia* as Orchocladiinae *sensu lato*.

Our topology is completely consistent with Lin et al. (2021); however, it includes many subfamilies as outgroups. We cannot confirm Lin's et al. (2021) conclusion about the transfer of Prodiamesinae to Orthocladiinae, as this would lead to non-monophyletic Chironominae as well. On the contrary, our results suggest transferring *Brillia*-group (genera *Brillia* Kieffer, and probably *Eurycnemus* van der Wulp, *Euryhapsis* Oliver, *Neobrillia* Kawai, *Tokyobrillia* Kobayashi et Sasa, and *Xylotopus* Oliver), to a new higher-ranked grouping (Sæther 2000). The *Brillia*-group, characterized by the following features of the male imago, distinguishing it from other Orthocladiinae: gonostylus bifurcate, wing membrane with setae and dorsal anteprotals setae present. Paraphyly of Orthocladiinae due to *Brillia*-group assumed that the listed set of features are a symplesiomorphies.

Abiskomyia is located at stem of Chironominae on all reconstructed trees (Fig. 1, Table 1, Supplementary Figs. S3–S32). Sister position for all subfamily occupied by genera *Xiaomyia* Sæther et Wang and *Shangomyia* Sæther et Wang from tribe Xiaomyiini

(Cranston et al. 2012, Tang and Cranston 2019, Krosch et al. 2020) for which mitogenomes are missing in the GenBank.

Some morphological features of the genus *Abiskomyia* are related to the Chironominae. The gonostylus of some *Abiskomyia* may be without a terminal spine (megaseta), as in the Chironominae. The pupae of the genus have tergites IV–VI and sometimes tergite VII with anteromedian oval group of brown spines, as in some Tanytarsini, such as the genus *Paratanytarsus*, and some Orthocladiinae, to which *Abiskomyia* is proposed to be related. The thoracic horn tapering to pointed apex, with spinules and spines, as in many Orthocladiinae and Tanytarsini. The larval antenna is located on pedestal with pointed projection, as in Tanytarsini. All the above-mentioned features, coupled with new molecular data, allow us to take a new look at the systematic position of the genus. It is necessary to reconstruct multi-locus phylogeny including both Xylomyini and *Abiskomyia* on a single analysis in future studies.

The intergeneric relationships of the subfamily Chironominae are not well studied. The phylogeny of some genera or tribes, such as *Polypedilum* (Kawai et al. 2012, Song et al. 2016, 2018, Tang et al. 2022a, Zhang et al. 2023), *Riethia* (Krosch et al. 2020) *Stenochironomus* (Zheng et al. 2022), Chironomini (Martin et al. 2007, Li et al. 2022), Tanytarsini (Ekrem, Willassen 2004, Ekrem et al. 2010, Huang et al. 2014, Lin et al. 2018, Kodama et al. 2022) has been studied in detail.

We have found a relatively stable topology of the subfamily Chironominae, which included 2 clusters of the tribe Chironomini, and *Tanytarsus* van der Wulp with *Microtendipes* Kieffer as sister to both clusters (Fig. 1, Supplementary Figs. S3–S32). The main variability in the topology between the various trees obtained is associated with the rearrangement of genera within the 2 clusters of Chironomini. The resulting topology was completely consistent with the phylogeny revealed by Fang et al. (2023) using mitogenomes but from fewer samples. The genera included in the phylogenetic analysis differed significantly from each other in the mitochondrial (Fang et al. 2023, this study) and multi-locus datasets (Cranston et al. 2012, Qi et al. 2019, Tang et al. 2022b), which prevents comparison of the obtained topologies. Nevertheless, the identified clusters within the tribe Chironomini are consistent with multi-locus results (Cranston et al. 2012, Qi et al. 2019, Tang et al. 2022b). In contrast to Cranston et al. (2012) results, the sister position of the genera *Microtendipes* and *Tanytarsus* is not supported by the mitogenomic phylogeny, which is probably due to inadequate sampling.

Divergence Time Estimations for Chironomids

Origination of the stem Chironomidae (node A, Fig. 2, Table 2) is reconstructed as of Upper Triassic age (211.3, HPD 185.8 to 236.3 Mya) These data are consistent with previous studies (Cranston et al. 2010, 2012, Krosch et al. 2020, Tang et al. 2022b, Semenchenko et al. 2024), which is not surprising given that these studies use the same fossil calibration *Aenne triassica* Krzeminski and Jarzembowski 1999. Crown-group divergence of subfamilies occurred in the Lower Jurassic—Upper Cretaceous (nodes B, C, E, F, G, H, L, and potential J and K). Node C (197.1, HPD 173.1 to 223.6 Mya) containing Chironomoinae + Telmatogetoninae have shown the same date with Cranston et al. (2012) although more ancient than 125 Mya according to Tang et al. (2022). Nodes D and E cannot be compared due to the topological differences with multi-locus analysis. It is noteworthy that the origin of diamesines (node F) occurred in Upper Cretaceous (89.8, HPD 57.2 to 130.4 Mya) which is significantly more recent than previous studies (Cranston et al. 2010, 2012, Semenchenko et al. 2024). In our opinion, this discrepancy is a consequence of the sister relationship of Diamesinae and Protanypodinae. The ancient diamesines are known from the Lower Cretaceous (*Eugenodiamesa makarchenkoi*, Lukashevich, Przhiboro, 2015) and Upper Cretaceous (*Craetodiamesa taimyrica*, Kalugina, 1976), but additional morphological studies are needed to use these fossils as calibration points. We obtained a slight overestimation of the divergence time for node G uniting Prodiamesinae + Orthocladiinae s.l. + Chironominae and node H uniting Orthocladiinae s.l. + Chironominae in comparison with multi-locus data (Table 2). The divergence of the genera *Brillia* and *Abiskomyia* occurred in the Upper Jurassic and Lower Cretaceous, respectively. The timing of the onset of chironominae diversification coincides with the data of Cranston et al. (2012) and Krosch et al. (2020), although in their case the first branching tribe was Xiaomyiini (Tang and Cranston 2019) for which mitogenomes are missing.

The Utility of Mitochondrial DNA for Chironomid Phylogeny

Mitochondrial DNA contains strong phylogenetic signals at subfamily and genus level within Chironomidae. The best results are determined by analyzing only the first and second codon positions of protein-coding genes, with and without ribosomal genetic data. The amino acid datasets also showed acceptable results, but the topology differed somewhat from the first set of genes. Topological accuracy, node support, log-likelihood scores, and effective sample number of Bayesian trees significantly worsened when using all 3 positions of codons of protein-coding genes. The choice of outgroup plays an important role. The best results were shown by using all possible Culicomorpha, while reduction to ceratopogonids and culicids leads to a false topology within the Chironomidae. In contrast to multi-locus trees, mitochondrial-derived phylogenies consistently suggest a sister position of Tanypodinae and Podonominae. Other mismatches, such as sister *Diamesa* + *Syndiamesa* and *Pagastia* + *Pseudodiamesa*, polyphyletic *Microtendipes* and *Tanytarsus* may be due to the lowly sampled datasets rather than the lack of mitogenomes. The divergence time estimation of Chironomidae coincides with multi-locus data with some differences related to the discrepancy in topology of phylogenies. A possible future improvement in the accuracy of the phylogeny is associated with the exclusion of genes ND1, ND2, and ND3 from the analysis due to experimental natural selection across most chironomids genera (Lei et al. 2024).

Supplementary material

Supplementary material is available at *Journal of Insect Science* online.

Author contributions

Alexander Semenchenko (Conceptualization [lead], Writing – original draft [lead]), Andrei Krasheninnikov (Conceptualization [equal]), Elena Khamenkova (Data curation [equal], Resources [equal]), Kirill Vinnikov (Funding acquisition [lead], Supervision [lead]), and Nikita Seliverstov (Methodology [lead])

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The authors report no conflict of interest.

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