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First mitochondrial genomes of the crane fly tribe Elephantomyiini (Diptera, Tipuloidea, Limoniidae): comparative analysis and phylogenetic implications

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Abstract

Limoniidae, the most speciose family in the superfamily Tipuloidea, consists of four subfamilies and more than 11,000 species. However, mitochondrial (mt) genome sequences, which have been widely used for phylogenetic study, are available for only 11 species across three subfamilies. Thus, a larger variety of mt genome sequences in Limoniidae are required to improve our understanding of tipuloid phylogeny and genomic evolution. Here we present mt genomes of *Elephantomyia* (*Elephantomyia*) *inulta* Alexander, 1938 and *Helius* (*Helius*) *pluto* Alexander, 1932, representing the first mt genomes of the tribe Elephantomyiini (Limoniidae). The two mt genomes are typical circular DNA molecules and show similar gene order, nucleotide composition and codon usage. Standard ATN start and TAR stop codons are present in most protein-coding genes. All transfer RNA (tRNA) genes exhibited the cloverleaf secondary structure typical for metazoans except in $tRNA^{Ser(AGN)}$, which lacks the dihydrouridine arm. Phylogenetic analyses were performed based on four nucleotide matrixes for the currently sequenced species of Tipuloidea using Bayesian inference and maximum likelihood methods. Four-cluster likelihood mapping was used to study incongruent signals between different topologies. Pediciidae is supported as the earliest lineage in Tipuloidea, and the sister-group relationship between Cylindrotomidae and Tipulidae is also supported, but the monophyly of Limoniidae is not supported. Our study also supports the monophyly of Elephantomyiini (*Elephantomyia* + *Helius*), as one of origins of flower-visiting in Limoniidae. Although Elephantomyiini is sister to Limoniinae + *Epiphragma* (Limnophilinae) in our study, a more precise understanding of its phylogenetic position in Tipuloidea will require additional studies that include a broader species sample.

Keywords

Elephantomyiinae, *Elephantomyia*, flower-visiting, *Helius*, mitogenome, phylogeny

1. Introduction

Crane flies are one of the most taxonomically diverse groups of flies with more than 15,000 described species in about 500 genera and subgenera (Oosterbroek 2022). They were first treated as a single family (i.e. Tipulidae), mainly due to the work of Alexander (1919, 1920, 1965) and Edwards (1911, 1912, 1916a, 1916b, 1921, 1923, 1926). However, Savchenko (1966, 1979, 1983) and Lackschewitz (1925, 1964) preferred to treat crane flies as a superfamily (i.e. Tipuloidea), which was supported by Hennig (1973) (Petersen et al. 2010). In the classification of Soós et al. (1992), Tipuloidea was divided into three families: Limoniidae, Cylindrotomidae and Tipulidae. Starý (1992) established a four-family classification system with the elevation of Pediciidae from Limoniidae, which was widely supported (Ribeiro 2008; Petersen et al. 2010; Zhang et al. 2016; Kang et al. 2017).

Limoniidae is the most speciose family in Tipuloidea and consists of about 150 genera and more than 11,000 species around the world (Oosterbroek 2022), accounting for four-fifths of the worlds crane flies. As 'short-palped' crane flies, members of Limoniidae (including Pediciidae) were first recognized based on the length of the terminal segment of palpus, with subsequent qualitative works by Alexander (1919, 1920) and Savchenko (1966, 1979, 1983) further framing the group. Although synapomorphies for Limoniidae appear to be a flattened antepronotum and presence of the subspiracular sclerite (Starý 1992), members of Limoniidae are usually diagnosed based on the absence of characters defining the other tipuloid families (Petersen et al. 2010). Limoniidae is further subdivided into four subfamilies (i.e. Chioneinae, Dactylolabidinae, Limnophilinae and Limoniinae) (Starý 1992), based on the numbers of radial and medial wing veins, the adult tibial spurs and the adult male gonostyli. However, the delineations of these subfamilies are unclear (Petersen et al. 2010). In addition, Limoniidae has long been controversial and has been frequently discussed with respect to both its intragroup relationships and phylogenetic position within Tipuloidea. Early analyses by Alexander (1919, 1920) and Savchenko (1966, 1979, 1983) presented the first evolutionary hypotheses for Limoniidae (Fig. 1A, B), although both were qualitative and proposed relationships based on somewhat unstated criteria. Based on 105 morphological characters from larvae and pupae, Oosterbroek and Theowald (1991) recovered a polytomy between Pediciinae, Chioneinae + Limnophilinae, and a clade containing several unplaced Limoniidae genera, Dactylolabidinae, Limoniinae and Cylindrotomidae + Tipulidae (Fig. 1C). Several more recent studies based on morphological and molecular data have demonstrated that Pediciidae is sister to the remaining Tipuloidea and that Limoniidae is not a natural group (Ribeiro 2008; Petersen et al. 2010) (Fig. 1D, E).

Elephantomyiini is a tribe within Limoniidae and includes three genera: *Elephantomyia* Osten Sacken, 1860, *Helius* Lepeletier and Serville, 1828 and *Protohelius* Alexander, 1928 (Savchenko et al. 1992; Podenas and Gelhaus 2007). In addition, the genus *Toxorhina* Loew, 1850 has also been placed in this tribe due to a close relationship with *Elephantomyia*, which was supported by Alexander (1920) based on adult characters and by Hynes (1997) based on immature characters. With a four-genus system, Elephantomyiini has 528 species/subspecies widely distributed in all biogeographic regions, of which 231 belong to *Helius*, 152 to *Toxorhina*, 137 to *Elephantomyia* and eight to *Protohelius* (Oosterbroek 2022). The origin and evolution of their flower-visiting habits and related morphological characteristics are very interesting topics. Except for *Protohelius* species, members of Elephantomyiini differ from most limoniid crane flies in their elongate mouthparts (Fig. 2A–E), which can visit flowers to ingest nectar (Oosterbroek and Lukashevich 2021). Another widespread genus with elongate mouthparts and flower-visiting habits in Limoniidae is *Geranomyia* Haliday, 1833. However, it should be noted that only the rostrum is elongate in Elephantomyiini, while in *Geranomyia*, the labial lobe is elongate but the rostrum is short (Fig. 2F).

In the past three decades, a large number of taxonomic studies have been carried out on the tribe Elephantomyiini, mainly focusing on the species in Asia (Zhang et al. 2015a, 2015b; Podenas et al. 2020), South America (Welch and Gelhaus 1994; Ribeiro and Amorim 2002) and Australia (Theischinger 1994, 1996, 2000). In addition, there is research on fossil species by Krzemiński and Kania et al. (Krzemiński 1991, 1993, 2002; Krzemiński and Freiwald 1991; Kania et al. 2013, 2016a, 2016b, 2018; Kania 2014, 2015; Krzemiński et al. 2014; Kopeć et al. 2016; Kania-Kłosok and Krzemiński 2021; Kania-Kłosok et al. 2021, 2022), while other researchers have also made some contributions (Podenas 2002; Ribeiro 2003; Wu et al. 2019). Although some larval records exist for the tribe (Hynes 1997; Hancock et al. 2000; Krivosheina 2010, 2012), the biology of the larval stages of most species is unknown.

In addition, the monophyly, taxonomic status and position of Elephantomyiini have been subject to debate (Fig. 1). The genera *Elephantomyia* and *Helius* were classified into two different tribes (Alexander 1965; Alexander and Alexander 1973). In the opinion of Savchenko, the genera *Elephantomyia* and *Helius* are related, but their placement into one tribe would be an arbitrary decision, as only similarity of adult characters (e.g. an elongate rostrum) supported it (Krivosheina 2012). Based on combined analysis of morphological characters and two nuclear genes, Petersen et al. (2010) supported the sistergroup *Elephantomyia* + *Helius* and suggested that this clade should be treated as a subfamily. However, based on larval characters, Krivosheina (2012) questioned the monophyly of *Elephantomyia* + *Helius* as well as the position of these two genera in Limoniinae, and considered that *Elephantomyia* should be placed in the subfamily Limnophilinae, while *Helius* cannot be assigned to any known subfamily thus should be elevated into a separate subfamily.

The mitochondrial (mt) genome is a double strand molecule of 15–16 kb in size that typically contains 13

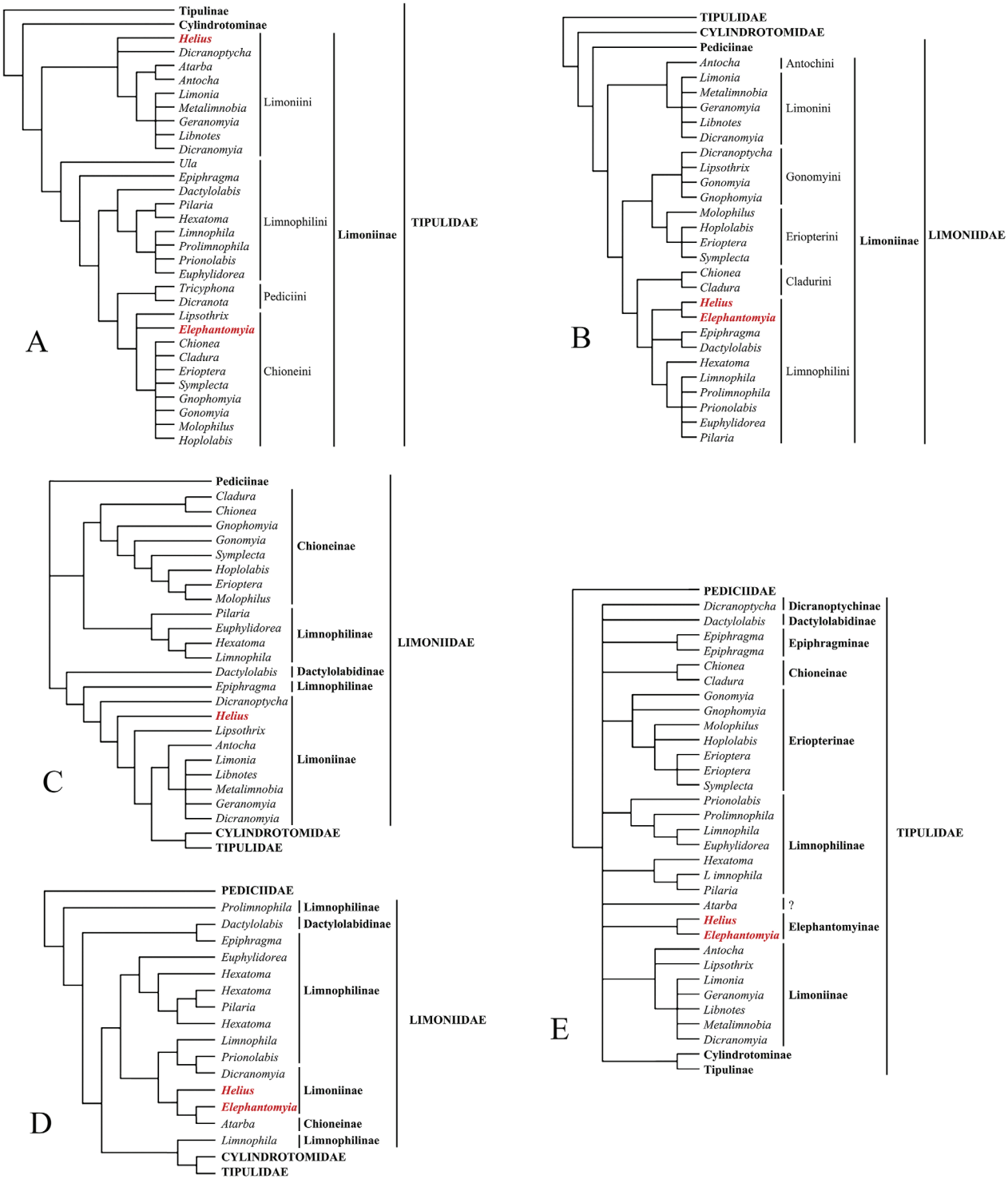

Figure 1. Previous hypotheses for the relationships among major Tipuloidea groups proposed by **A** Alexander (1919, 1920), **B** Savchenko (1966, 1979, 1983), **C** Oosterbroek and Theowald (1991), **D** Ribeiro (2008) and **E** Petersen et al. (2010). After Petersen et al. 2010. The genera of Elephantomyiini are in red color.

protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes and a noncoding A + T-rich region (control region). It has become the most extensively studied genomic system in insects. It is now widely used in the study of insect phylogenetics and molecular evolution due to its maternal inheritance, fast evolutionary rate, and highly conserved gene content (Song et al. 2016; Chen et al. 2017; Liu et al. 2017; Wang et al. 2017; Feng et al. 2018; Zhang et al. 2018, 2022; Zhu et al. 2018; Ren et al. 2019; Su and Liang 2019; Wang and Huang 2019; Zhao et al. 2019; Tang et al. 2020; Li et al. 2021; Wang et al. 2021; Shi et al. 2021; Sun et al. 2021; Zheng et al. 2021; Lin et al. 2022; Mo et al. 2022; Song et al. 2022). Beckenbach (2012) reported the first mt genome of Tipuloidea. In the following decade, many mt genome sequences for the superfamily Tipuloidea have been produced, including the first mt genomes for the families Cylindrotomidae, Pediciidae and Limoniidae, for

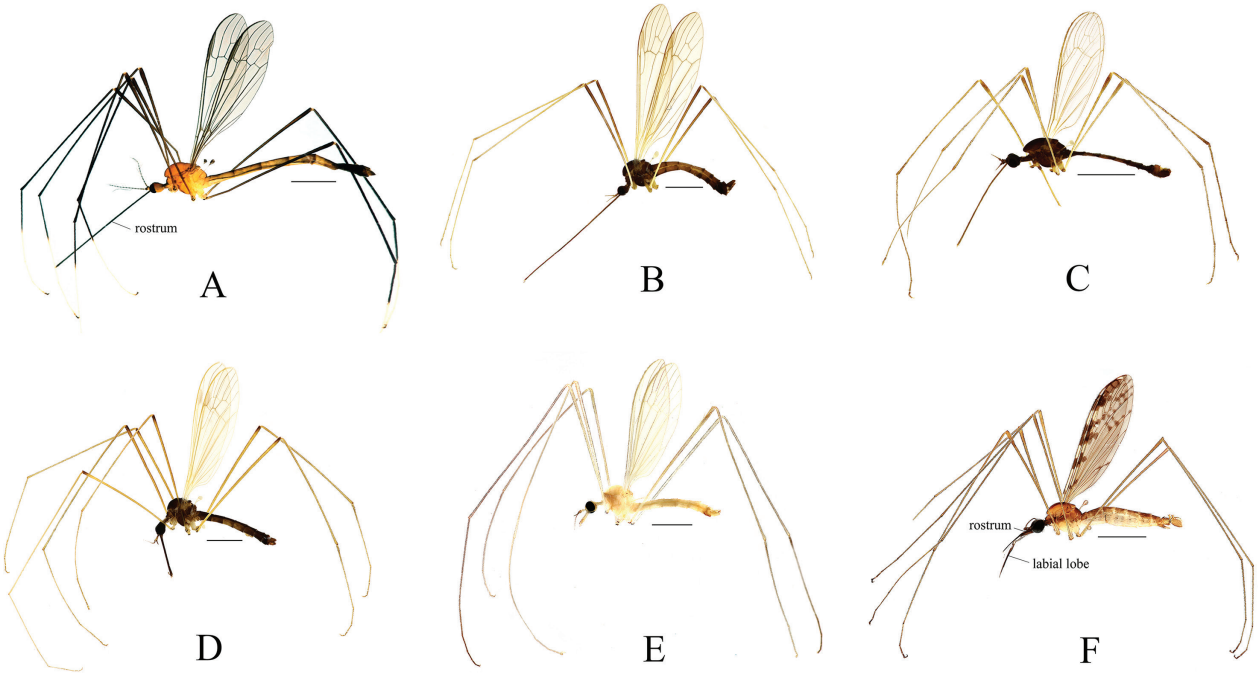

Figure 2. General morphology of limoniid crane flies with elongate mouthparts, represented by **A** *Elephantomyia* (*Elephantomyodes*) *tianmushana* Zhang, Li and Yang, 2015, **B** *Elephantomyia* (*E.*) *laohegouensis* Zhang, Li and Yang, 2015, **C** *Toxorhina* (*Ceratocheilus*) *omnifusca* Zhang, Li and Yang, 2015, **D** *Helius* (*H.*) *pluto* Alexander, 1932, **E** *Helius* (*H.*) *pallidissimus* Alexander, 1930 and **F** *Geranomyia subablusa* Qian and Zhang, 2020. Scale bars = 2.0 mm.

the limoniid subfamilies Chioneinae, Limnophilinae and Limoniinae, and for the tipulid subfamily Ctenophorinae (Zhang et al. 2016; Kang et al. 2019; Zhao et al. 2019; Zhao et al. 2021). Mitochondrial genomes for Tipuloidea, however, are far from sufficient. Before the present study (November 2022), 27 complete or nearly complete mt genomes of Tipuloidea were available in GenBank of NCBI (https://www.ncbi.nlm.nih.gov), of which 14 represent Tipulidae, 11 Limoniidae, one Cylindrotomidae and one Pediciidae. In this study, we analyzed the first two mt genome sequences from the tribe Elephantomyiini (Limoniidae) and reconstructed phylogenetic relationships in Tipuloidea, using maximum likelihood and Bayesian inference methods, aiming to provide new genomic data for the phylogeny of Tipuloidea. The monophyly and taxonomic status of Elephantomyiini will be explored, to test whether the elongate rostrum of Elephantomyiini is a synapomorphy or the result of parallel evolution, as well as examine the origin and evolution of flower-visiting in Limoniidae.

2. Materials and methods

2.1. Specimen sample and DNA extraction

Adult specimens of *Elephantomyia* (*Elephantomyia*) *inulta* Alexander, 1938 were collected from Motuo, Linzhi, Tibet, China, and adult specimens of *Helius* (*Helius*) *pluto* Alexander, 1932 were collected from Mount Daming, Nanning, Guangxi, China. Specimens were identified based on Zhang et al. (2015b) and Alexander (1932). All specimens were preserved in 96% ethanol at –20 °C for long-term storage at the China Agricultural University, Beijing, China. For each species, genomic DNA was extracted from thoracic muscle tissue using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturers protocol.

2.2. Mitochondrial genome sequencing and assembly

An Illumina TruSeq library was prepared with 450 bp average insert size and sequenced on the Illumina Hiseq 2500 platform with 250 bp paired-end reads. The genomes of the two species were sequenced on one lane. About 4 Gb of clean data was obtained from the library after trimming using Trimmomatic (Bolger et al. 2014). De novo assemblies of high-quality reads were conducted using IDBA-UD 1.1.1 (Peng et al. 2012), with minimum and maximum k values of 80 bp and 240 bp, respectively. Fragments of *COI* near the 5'-terminus (~610 bp) were amplified for each species by polymerase chain reaction (PCR) with primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3' forward) and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3' reverse) (Folmer et al. 1994). The PCR was performed in a 25 μL volume containing 12.5 μL Taq PCR Master Mix, 1.0 μL of DNA extract, 1.0 μL primer LCO1490, 1.0 μL Primer HCO2198 and 9.5 μL ddH2O. The cycling profile was 94 °C for 4 min, 30 cycles of 94 °C for 30 sec, 45 °C for 30 sec, 72 °C for 1 min, and a final extension period of 72 °C for 10 min. Successful PCR products were purified

Table 1. Information of species used in molecular analysis with GenBank accession numbers of mitochondrial genome sequences.

Family	Subfamily	Species	Accession Number
Pediciidae	Pediciinae	*Pedicia* sp.	KT970062
Limoniidae	Chioneinae	*Chionea crassipes gracilistyla* Alexander, 1936	MK941181
		Symplecta (*Symplecta*) *hybrida* (Meigen, 1804)	NC_030519
	Limnophilinae	*Conosia irrorata* (Wiedemann, 1828)	NC_057072
		Epiphragma (*Epiphragma*) *mediale* Mao and Yang, 2009	NC_057085
		Euphylidorea (*Euphylidorea*) *dispar* (Meigen, 1818)	MT410841
		Paradelphomyia sp.	KT970061
		Pseudolimnophila (*Pseudolimnophila*) *brunneinota* Alexander, 1933	MN398932
	Limoniinae	*Dicranomyia* (*Dicranomyia*) *modesta* (Meigen, 1818)	MT628560
		Elephantomyia (*Elephantomyia*) *inulta* Alexander, 1938	This study
		Helius (*Helius*) *pluto* Alexander, 1932	This study
		Limonia phragmitidis (Schrank, 1781)	NC_044484
		Metalimnobia (*Metalimnobia*) *quadrinotata* (Meigen, 1818)	MT584154
		Rhipidia (*Rhipidia*) *chenwenyoungi* Zhang, Li and Yang, 2012	KT970063
Cylindrotomidae	Cylindrotominae	*Cylindrotoma* sp.	KT970060
Tipulidae	Ctenophorinae	*Tanyptera* (*Tanyptera*) *hebeiensis* Yang and Yang, 1988	NC_053795
	Tipulinae	*Nephrotoma flavescens* (Linnaeus, 1758)	MT628586
		Nephrotoma quadrifaria quadrifaria (Meigen, 1804)	MT872674
		Nephrotoma tenuipes (Riedel, 1910)	MN053900
		Nigrotipula nigra nigra (Linnaeus, 1758)	MT483653
		Tipula (*Acutipula*) *cockerelliana* Alexander, 1925	NC_030520
		Tipula (*Dendrotipula*) *flavolineata* Meigen, 1804	MT410828
		Tipula (*Formotipula*) *melanomera gracilispina* Savchenko, 1960	MK864102
		Tipula (*Lunatipula*) *fascipennis* Meigen, 1818	NC_050319
		Tipula (*Nippotipula*) *abdominalis* (Say, 1823)	JN861743
		Tipula (*Tipula*) *paludosa* Meigen, 1830	MT483696
		Tipula (*Vestiplex*) *aestiva* Savchenko, 1960	NC_063751
		Tipula (*Yamatotipula*) *nova* Walker, 1848	NC_057055
Trichoceridae	Paracladurinae	*Paracladura trichoptera* (Osten Sacken, 1877)	JN861751
	Trichocerinae	*Trichocera bimacula* Walker, 1848	JN861750
		Trichocera sp.	MW263048

and sequenced by Sangon Biotech (Shanghai, China). The *COI* fragments served as bait references to identify best-fit mt contigs under BLAST searches (Altschul et al. 1990) with minimum similarity 98%. For checking assembly accuracy, clean reads were mapped onto the obtained mt contigs using Geneious 9.0.2 (Kearse et al. 2012).

2.3. Mitochondrial genome annotation and sequence analysis

The protein-coding, rRNA and tRNA genes were identified using the MITOS2 Webserver (http://mitos2.bioinf.unileipzig.de/index.py). Protein-coding genes that could not be predicted by that program were annotated by alignment with the homologous genes reported in other crane flies. Nucleotide composition of mt genomes and PCG codon usage was analyzed in MEGA 7.0 (Kumar et al. 2016). AT-skew [(A–T)/(A + T)] and GC-skew [(G–C)/(G + C)] were used to measure nucleotide compositional differences between genes (Perna and Kocher 1995). DnaSP 5.0 (Librado and Rozas 2009) was used to calculate synonymous (Ks) and non-synonymous (Ka) substitution rates, and Ka/Ks ratio (Hurst 2002) was calculated manually.

2.4. Molecular analyses

A total of 31 mt genomes were used for molecular analyses, including the two newly sequenced mt genomes of Elephantomyiini, 26 complete or nearly complete mt genomes of Tipuloidea available in GenBank, and three mt genomes of Trichoceridae used as outgroup (Table 1). The mt genome of *Tipula* (*Pterelachisus*) *varipennis* Meigen, 1818 in Tipuloidea was not included because it lacks both rRNA genes.

The protein-coding and RNA genes were aligned individually with the MAFFT 7.0 online server with the algorithm G-INS-i strategy (Katoh and Standley 2013). Individual gene alignments were checked manually in MEGA 7.0 after removing poorly aligned sites using GBlocks 0.91b (Talavera and Castresana 2007). Some studies suggest that RNA genes evolve too quickly or are too difficult to align to provide phylogenetic signal at deep nodes, but others suggest that the addition of these genes in mt genome phylogenies can improve the resolution and support (Cameron et al. 2007). Alternatively, the third codon positions of PCG have been shown to contain high AT content and accelerated rates of evolutionary change, and so, third codon positions are removed in

most phylogenetic studies (Cameron et al. 2009; Mao et al. 2012). However, third position removal also drastically lowers the phylogenetic information content provided by these sites, and so comparison among trees inferred both with and without these 3rd sites included is required to fully evaluate their impact on relationships (Cameron et al. 2007; Fenn et al. 2008; Cameron 2014). Therefore, alignments of individual genes were concatenated using SequenceMatrix 1.8 (Vaidya et al. 2011) to generate the following four datasets: (1) PCGRNA matrix, including all three codon positions of 13 PCGs, large rRNA (lrRNA) and 18 tRNA genes (only these RNA genes are available for all species) (11,353 bp); (2) PCG matrix, including all three codon positions of 13 PCGs (9,444 bp); (3) PCG12RNA matrix, including the first and second codon positions of 13 PCGs, lrRNA and 18 tRNA genes (8,205 bp); and (4) PCG12 matrix, including the first and second codon positions of 13 PCGs (6,296 bp).

Heterogeneous sequence divergence can lead to strong biases in tree reconstructions, such as long branch effects or the misplacement of rogue taxa (Kück et al. 2014), in particular when the taxon sampling is poor, or the outgroup is distant (Felsenstein 1978; Philippe and Laurent 1998). AliGROOVE 1.07, a tool that can detect strongly derived sequences, was used to offer the possibility to exclude taxa or gene partitions (Kück et al. 2014). Phylogenetic trees were inferred for each dataset using Bayesian inference (BI) and maximum likelihood (ML) methods. Partitioning schemes and the best-fit substitution models were determined in PartitionFinder 2.1.1 with the BIC criterion and greedy-search algorithm (Lanfear et al. 2017). The BI analysis was performed in MrBayes 3.2.7 (Huelsenbeck and Ronquist 2001) with the default settings and four independent runs of 5–10 million generations with sampling every 1000 generations; after the average standard deviation of split frequencies fell below 0.01, the initial 25% of samples were discarded as burn-in. The ML analysis was conducted with RAxML 8.2.4 (Stamatakis 2014) using the GTRGAMMAI model; the best ML tree was calculated with branch support estimated from 1000 bootstrap replicates. The program TreePuzzle 5.3 (Strimmer and von Haeseler 1997; Schmidt et al. 2002) was used for four-cluster likelihood mapping (FcLM) analysis to evaluate single topological splits.

3. Results and discussion

3.1. General characters of mt genomes

The mt genomes of two crane fly species in the tribe Elephantomyiini, *E.* (*E.*) *inulta* and *H.* (*H.*) *pluto*, are sequenced and analyzed for the first time. The nearly complete mt genomes of *E.* (*E.*) *inulta* (GenBank accession no. OP556661) and *H.* (*H.*) *pluto* (GenBank accession no. OP556662) are 14,551 bp and 14,358 bp in length, respectively. The control regions and short stretches on either side of the control regions are not obtained for either species. In the mt genome of *E.* (*E.*) *inulta*, 35 genes are detected ($tRNA^{Ile}$, $tRNA^{Gln}$ and partial small rRNA (srRNA) are not detected), while in the mt genome of *H.* (*H.*) *pluto*, 34 genes are detected ($tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, partial srRNA and partial *ND2* are not detected) (Fig. 3; Table 2). The composition and arrangement in both two mt genomes are identical to the presumed ancestral insect mt genome (Boore 1999). The organization of both mt genomes are generally compact. Intergenic spacers in *E.* (*E.*) *inulta* are 14 in number and generally less than 18 bp, with the largest between $tRNA^{Ser(UCN)}$ and *ND1*, while the intergenic spacers in *H.* (*H.*) *pluto* are 19 in number and generally less than 22 bp, with the largest between $tRNA^{Cys}$ and $tRNA^{Tyr}$. Both mt genomes also have overlapping genes but no genes overlapped by more than 8 bp (Table 2).

3.2. Nucleotide composition

The mt genomes of both Elephantomyiini species are biased to high A+T% across their four major genome partitions (i.e. PCGs, tRNA genes, lrRNA gene and srRNA gene). The AT contents of whole mt genome, PCGs, tRNA genes and lrRNA gene in *E.* (*E.*) *inulta* (76.4%, 75.2%, 78.8% and 81.5%) are lower than those in *H.* (*H.*) *pluto* (76.8%, 75.8%, 79.4% and 82.1%), but the AT content of the srRNA gene in *E.* (*E.*) *inulta* (78.9%) is higher than that in *H.* (*H.*) *pluto* (76.3%). Both species show slightly positive AT-skew (0.01, 0.02) and negative GC-skew (–0.18, –0.21) for the whole mt genome, but show negative AT-skew (–0.16, –0.16; –0.04, –0.05) and positive GC-skew (0.04, 0.03; 0.33, 0.33) for PCGs and the lrRNA gene. For tRNAs, both species show insignificant or no AT-skew (0.01, 0.00) and positive GC-skew (0.11, 0.14). For the srRNA gene, *E.* (*E.*) *inulta* shows positive AT (0.02) and GC-skews (0.32), while *H.* (*H.*) *pluto* shows negative AT-skew (–0.03) and positive GC-skew (0.27) (Table 3). For both Elephantomyiini species, the whole mt genome and the four major partitions all have the same trends in AT content, AT and GC-skews consistent with the common nucleotide composition of mt genomes of Tipuloidea (Zhang et al. 2016).

3.3. Protein-coding genes and codon usage

Each of the two newly sequenced mt genomes has 13 PCGs, of which *COI*, *COII*, *COIII*, *CytB*, *ATP6*, *ATP8*, *ND2*, *ND3* and *ND6* are coded on the majority strand, and *ND4*, *ND4L*, *ND5* and *ND1* are coded on the minority strand (Fig. 3; Table 2). A majority of PCGs in both mt genomes show the typical ATN start codons (ATT/ATG); TCG is the start codon for *COI* in both species and TTG is the start codon for *ND1* in *H.* (*H.*) *pluto*. The majority of PCGs also show the typical TAR (TAA/TAG) stop codons, while the partial stop codon T for *COII* is found in both species and for *ND5* in *E.* (*E.*) *inulta* (Table 2).

Table 2. Organization of the mitochondrial genomes of *Elephantomyia* (*Elephantomyia*) *inulta* and *Helius* (*Helius*) *pluto*.

Gene	Direction	Location	Size (bp)	Anticodon	Codon		Intergenic nucleotide*
					Start	Stop	
$tRNA^{Met}$	J	1-70/-	70/-	CAT/-			
ND2	J	71-1096/1-912	1026/912		ATT/-	TAA	0/–
$tRNA^{Trp}$	J	1106-1174/911-980	69/70	TCA			9/–2
$tRNA^{Cys}$	N	1167-1236/973-1042	70/70	GCA			–8/18
$tRNA^{Tyr}$	N	1251-1315/1065-1134	65/70	GTA			14/22
COI	J	1314-2849/1150-2685	1536/1536		TCG	TAA	–2/15
$tRNA^{Leu(UUR)}$	J	2852-2918/2689-2756	67/68	TAA			2/3
COII	J	2920-3601/2766-3450	682/685		ATG	T-tRNA	1/9
$tRNA^{Lys}$	J	3602-3672/3451-3521	71/71	CTT			0/0
$tRNA^{Asp}$	J	3672-3736/3525-3591	65/67	GTC			–1/3
ATP8	J	3737-3898/3592-3756	162/165		ATT	TAA/TAG	0/0
ATP6	J	3892-4566/3750-4427	675/678		ATG	TAA	–7/–7
COIII	J	4572-5360/4427-5215	789/789		ATG	TAA	5/–1
$tRNA^{Gly}$	J	5361-5424/5219-5283	64/65	TCC			0/3
ND3	J	5425-5778/5284-5637	354/354		ATG/ATT	TAA	0/0
$tRNA^{Ala}$	J	5780-5843/5642-5709	64/68	TGC			1/4
$tRNA^{Arg}$	J	5844-5910/5712-5777	67/66	TCG			0/2
$tRNA^{Asn}$	J	5911-5976/5779-5844	66/66	GTT			0/1
$tRNA^{Ser(AGN)}$	J	5977-6043/5845-5911	67/67	GCT			0/0
$tRNA^{Glu}$	J	6049-6117/5914-5979	69/66	TTC			5/2
$tRNA^{Phe}$	N	6138-6204/5999-6064	67/66	GAA			10/19
ND5	N	6205-7939/6072-7808	1735/1737		ATG	T-tRNA/TAA	0/7
$tRNA^{His}$	N	7940-8005/7809-7875	66/67	GTG			0/0
ND4	N	8013-9353/7877-9217	1341/1341		ATG	TAA	7/1
ND4L	N	9347-9643/9211-9507	297/297		ATG	TAA	–7/–7
$tRNA^{Thr}$	J	9646-9712/9510-9575	67/66	TGT			2/2
$tRNA^{Pro}$	N	9713-9776/9576-9640	64/65	TGG			0/0
ND6	J	9779-10303/9642-10166	525/525		ATT	TAA	2/1
CytB	J	10303-11439/10170-11306	1137/1137		ATG	TAA	–1/3
$tRNA^{Ser(UCN)}$	J	11445-11512/11313-11380	68/68	TGA			5/6
ND1	N	11531-12472/11397-12341	942/945		ATG/TTG	TAG	18/16
$tRNA^{Leu(CUN)}$	N	12474-12538/12343-12407	65/65	TAG			1/1
lrRNA	N	12539-13864/12408-13728	1326/1321				0/0
$tRNA^{Val}$	N	13865-13936/13729-13800	72/72	TAC			0/0
srRNA	N	13937-14551/13801-14358	615/558				0/0

* Intergenic nucleotide: minus indicates overlapping between genes.

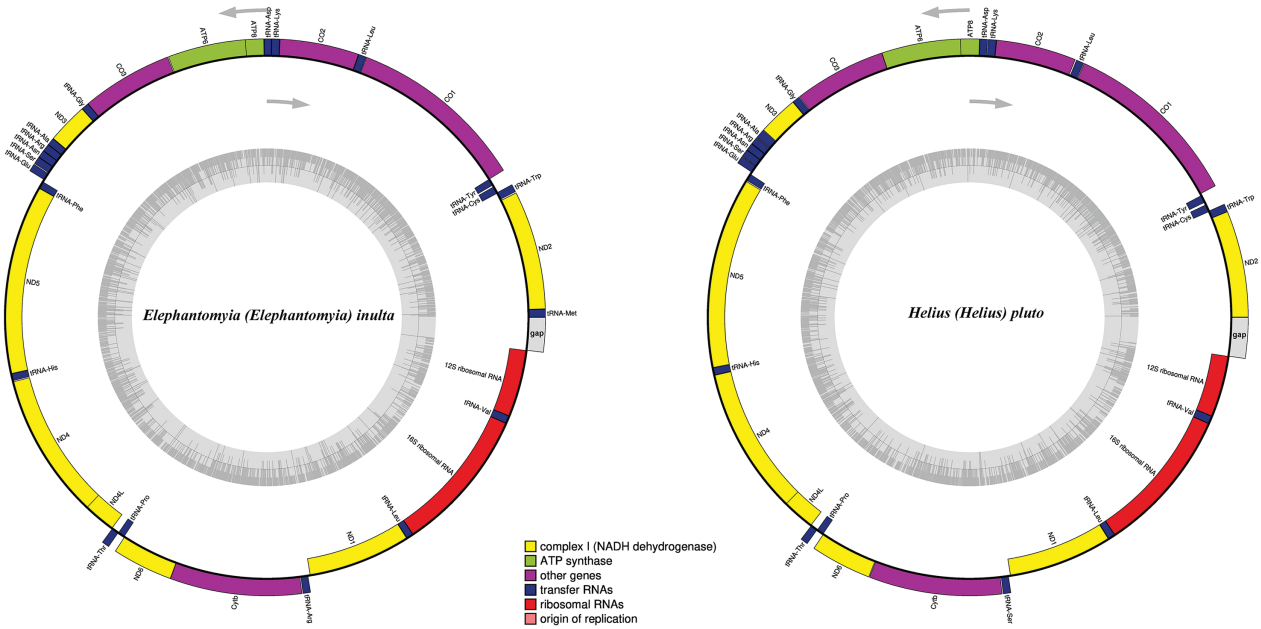

Figure 3. Gene maps of the mitochondrial genomes of two Elephantomyiini species sequenced in this study. The circular maps were drawn with OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html). The transcriptional direction is indicated by arrows.

Table 3. Nucleotide composition of the mitochondrial genomes of two Elephantomyiini species.

Region		*E.* (*E.*) *inulta*	*H.* (*H.*) *pluto*
Whole mt genome	A+T%	76.4	76.8
	G+C %	23.7	23.2
	AT-skew	0.01	0.02
	GC-skew	–0.18	–0.21
PCGs	A+T%	75.2	75.8
	G+C %	24.7	24.3
	AT-skew	–0.16	–0.16
	GC-skew	0.04	0.03
PCGs(J)	A+T%	74.3	74.5
	G+C %	25.7	25.5
	AT-skew	–0.13	–0.12
	GC-skew	–0.11	–0.14
PCGs(N)	A+T%	76.8	77.7
	G+C %	23.1	22.3
	AT-skew	–0.22	–0.22
	GC-skew	0.28	0.32
tRNAs	A+T%	78.8	79.4
	G+C %	21.2	20.7
	AT-skew	0.01	0.00
	GC-skew	0.11	0.14
lrRNA	A+T%	81.5	82.1
	G+C %	18.5	17.9
	AT-skew	–0.04	–0.05
	GC-skew	0.33	0.33
srRNA	A+T%	78.9	76.3
	G+C %	21.2	23.7
	AT-skew	0.02	–0.03
	GC-skew	0.32	0.27
AT-skew = (A-T)/(A+T); GC-skew = (G-C)/(G +C)			

The total number of codons of mt genomes are 3,733 in *E.* (*E.*) *inulta*, and 3,700 in *H.* (*H.*) *pluto* but with incomplete *ND2* (Tables S1, S2). Codon usage values are described by relative synonymous codon usage (RSCU), which reflects how often each codon is used relative to the expected number in the absence of usage bias. All RSCU values for each amino acid are similar between the two mt genomes, with Leu (UUR) and Ser (UCN) being the two most frequently used amino acids, and Leu (CUN), Met and Trp being the least. The most frequently used codon in each amino acids solely comprises A or T, reflecting the high AT content of PCGs (Fig. S1). These phenomena were also recorded from other mt genomes of lower Diptera (Zhang et al. 2022).

To further investigate evolutionary patterns across the PCGs, the ratio of Ka (rates of nonsynonymous mutations)/Ks (rates of synonymous mutations) are calculated for each (Fig. S2). The Ka/Ks values for all 13 PCGs are lower than 1 (<0.70), implying purifying selection on all these genes. The Ka/Ks ratio of *ND2* is obviously higher than other PCGs, which indicates that *ND2* has a relatively higher evolutionary rate. In contrast, *COI* has the lowest Ka/Ks ratio, indicating that this gene has been subjected to the highest purifying selection.

3.4. Transfer and ribosomal RNA genes

Twenty tRNA genes are detected in *E.* (*E.*) *inulta* and 19 tRNAs in *H.* (*H.*) *pluto*. The tRNA genes lengths range from 64 bp to 72 bp (Table 2). Most tRNAs can be folded into the typical cloverleaf structure, except for $tRNA^{Ser(AGN)}$ whose dihydrouridine (DHU) arm is replaced by a simple loop (Fig. S3). It is common for $tRNA^{Ser(AGN)}$ to lack the DHU arm in insect mt genomes (Zhang et al. 2016; Zhang et al. 2018; Zhu et al. 2018; Ren et al. 2019; Su and Liang 2019; Wang and Huang 2019; Li et al. 2021; Mo et al. 2022; Zhang et al. 2022). Nucleotide substitutions of tRNAs between *E.* (*E.*) *inulta* and *H.* (*H.*) *pluto* range from three to 23: $tRNA^{Asp}$ has the least variation with three substitutions, while $tRNA^{Ala}$ has the most variation with 23 substitutions and indels.

As in the ancestral insect (Zhang et al. 2016, 2022; Chen et al. 2017; Liu et al. 2017; Feng et al. 2018; Zhang et al. 2018; Zhu et al. 2018; Ren et al. 2019; Su and Liang 2019; Wang and Huang 2019; Zhao et al. 2019; Tang et al. 2020; Li et al. 2021; Wang et al. 2021; Shi et al. 2021; Sun et al. 2021; Zheng et al. 2021; Mo et al. 2022), the lrRNA gene in each Elephantomyiini species is located between $tRNA^{Leu(CUN)}$ gene and $tRNA^{Val}$ gene, while the srRNA gene is located between $tRNA^{Val}$ gene and the control region (not obtained in this study) (Fig. 3). The lengths of lrRNAs are 1,326 bp in *E.* (*E.*) *inulta* and 1,321 bp in *H.* (*H.*) *pluto*. The assembled srRNA gene in both species are incomplete, and the obtained sequence lengths are 615 bp and 558 bp respectively (Table 2).

3.5. Phylogenetic Analyses

AliGROOVE analysis indicates that *Chionea crassipes gracilistyla* Alexander, 1936 has the strongest heterogeneity relative to other Tipuloidea species in all four datasets (Fig. S4), which may cause bias tree reconstructions and node support in phylogenetic analysis (Kück et al. 2014). An effective solution is to avoid using this species (Soltis et al. 2004). Therefore, *C. crassipes gracilistyla* was excluded when constructing phylogenetic trees. Twenty-eight representatives from all four families of Tipuloidea and three representatives from Trichoceridae were included in the phylogenetic analysis. Eight phylogenetic trees inferred from the four datasets under BI and ML methods were finally constructed (Figs 4, S5–S10), resulting in four hypotheses of relationships among major groups of Tipuloidea (Fig. 5).

In all BI and ML trees, Pediciidae is sister to all other Tipuloidea, and a sister relationship between Cylindrotomidae and Tipulidae is strongly supported. These arrangements are consistent with the phylogeny by Ribeiro (2008) based on 88 morphological characters, Petersen et al. (2010) based on combined morphological characters and two nuclear genes, Zhang et al. (2016) based on mt genomes and Kang et al. (2017) based on transcriptomes.

Limoniidae is not supported as monophyletic clade in any phylogenetic trees. *Symplecta* (*Symplecta*) *hybrida*

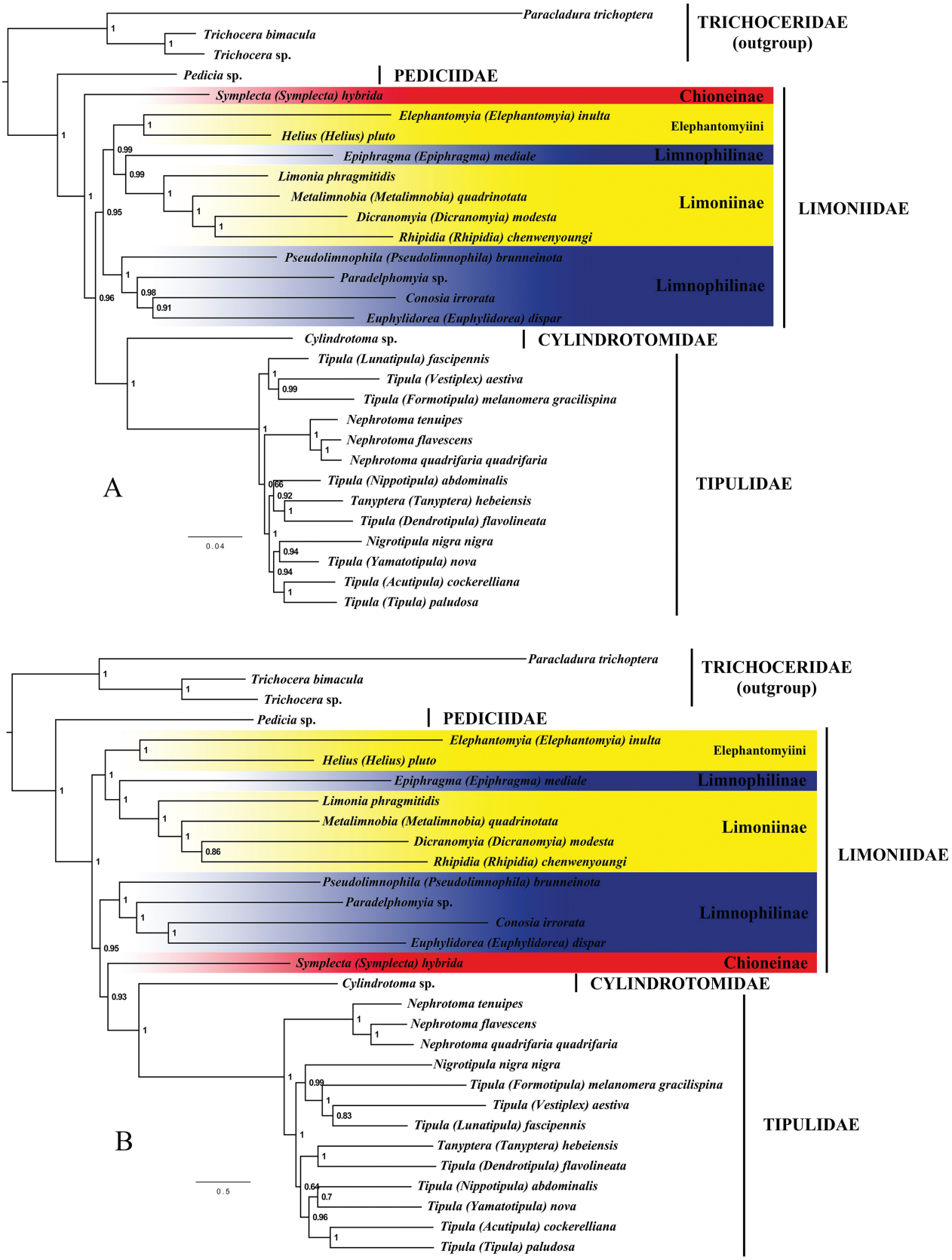

Figure 4. Phylogenetic trees of Tipuloidea inferred from the datasets **A** PCG12RNA and **B** PCG under BI method. Numbers at the nodes are posterior probabilities. The family Trichoceridae was set as the outgroup.

(Meigen, 1804) (Chioneinae) is sister to all non-pedicid crane flies in trees inferred from the PCG12RNA and PCG12 datasets under BI and ML methods (96% PP, 45% BV; 64% PP, 42% BV) (Figs 4A, S5, S7, S8), while in the BI tree inferred from the PCG dataset (Fig. 4B), *Symplecta* is sister to a clade of Cylindrotomidae + Tipulidae (93% PP). In the both BI and ML trees inferred from the PCGRNA dataset (Figs S9, S10), *Symplecta* + the Lim-

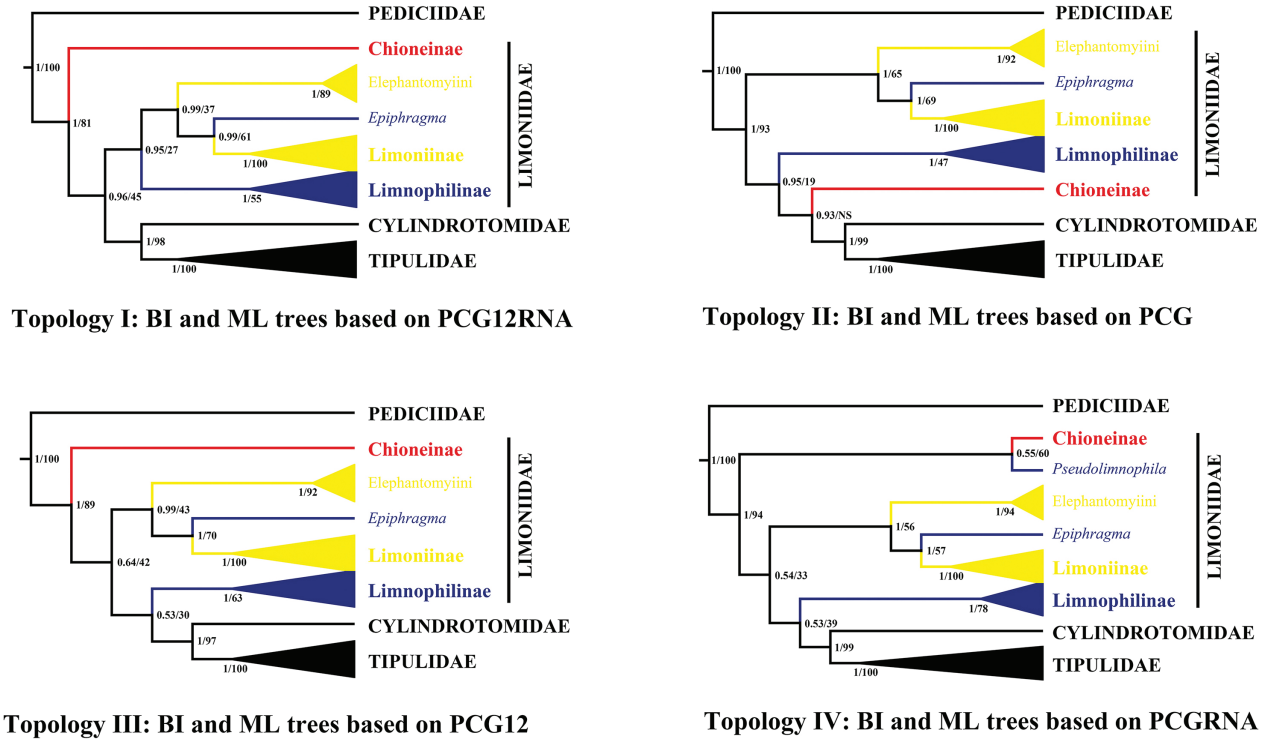

Figure 5. Four hypotheses for the relationships among major groups of Tipuloidea in this study. Multiple sampling of different species from a single family/subfamily/tribe are collapsed into triangles. Numbers at the nodes are posterior probabilities/bootstrap values, NS = not support.

nophilinae species *Pseudolimnophila* (*Pseudolimnophila*) *brunneinota* Alexander, 1933 is weakly supported (55% PP, 60% BV), as sister to all non-pediciid crane flies (54% PP, 33% BV). In the ML tree inferred from the PCG dataset (Fig. S6), *Symplecta* is sister to a clade of Limnophilinae + (Cylindrotomidae + Tipulidae) with a very low bootstrap value (19% BV). However, our current taxon sampling for Chioneinae is far from extensive, and further detailed studies with more taxa are needed before the monophyly of Chioneinae can be confidently defined.

Limoniinae (including Elephantomyiini) + *Epiphragma* (*Epiphragma*) *mediale* Mao and Yang, 2009 (Limnophilinae) forms a clade in all phylogenetic trees (100% or 99% PPs for all BI trees; 65%, 56%, 43% and 37% BVs for ML trees). Elephantomyiini (*Elephantomyia* + *Helius*) (100% PP for all BI trees; 94%, 92%, 92% and 89% BV for ML trees) and Limoniinae (100% PP/BV for all trees) are two well-supported clades, which to some extent supports the suggestion of Petersen et al. (2010) to treat the monophyletic Elephantomyiini as a subfamily on the basis of morphological and molecular data. Although *Epiphragma* consistly shows a distant relationship to other Limnophilinae in our study and was also treated as a subfamily in Petersen et al. 2010, the taxonomic status of the group it represents needs further study.

Limnophilinae is a controversial group with respect to both its monophyly and relationships with other Limoniidae. The main clade of Limnophilinae (including four species) is sister to the clade containing Elephantomyiini, *Epiphragma* and Limoniinae in the trees inferred from the PCG12RNA dataset under BI and ML methods (95% PP, 27% BV) (Figs 4A, S5), while in the remaining trees, a clade of Limnophilinae (including three or four species) shows a closer relationship to Cylindrotomidae + Tipulidae.

Topologies I and II (Fig. 5) based on the PCG12RNA and PCG datasets under BI method have high PPs (Fig. 4), but are contradictory. FcLM analysis is often used to evaluate single topological splits (Trautwein et al. 2010; Misof et al. 2014; Peters et al 2014; Winterton and Ware 2015; Kang et al. 2017; Narayanan et al. 2018, 2019; Vasilikopoulos et al. 2019; Zhang et al. 2019; Karmeinski et al. 2021; Wang et al. 2022). Two questions that reflect the main differences between topologies I and II were used for FcLM testing to further evaluate these two topologies: 1) Is *Symplecta* sister to all non-pediciid crane flies, or to Cylindrotomidae + Tipulidae? 2) Is Limnophilinae part of Limoniinae, or does Limnophilinae have a closer relationship with Cylindrotomidae + Tipulidae? (Table 4). For these tests, species in four datasets were grouped into four clusters representing alternative resolutions of these topologies.

Our FcLM analysis shows a support for the sister-group relationship between *Symplecta* and all non-pediciid crane flies (51.0%/43.9%/81.4%/81.3%) (Fig. S11). FcLM results for the placement of *Symplecta* are concordant with topology I (Fig. 5). However, the status of Limoniinae (except Chioneinae) is not well resolved by FcLM analysis (Fig. S12): Limnophilinae is supported as part of Limoniinae (as shown in topology I) with the support rate of 40.8%/31.4%/36%/18.1% whereas Limnophilinae is supported as sister to Cylindrotomidae + Tipulidae (as shown in topology II) with the support rate of 30.5%/31.8%/40.4%/49.7%.

Table 4. Two splits designed to evaluate two questions.

Questions	Groups	Number of Species
Is *Symplecta* sister to all non-pediciid crane flies, or to Cylindrotomidae + Tipulidae?	G1: Pediciidae	1
	G2: *Symplecta*	1
	G3: remaining Limoniidae	11
	G4: Cylindrotomidae + Tipulidae	14
Is Limnophilinae part of Limoniinae, or does Limnophilinae have a closer relationship with Cylindrotomidae + Tipulidae?	G1: Pediciidae	1
	G2: Limnophilinae (except *E.* (*E.*) *mediale*)	4
	G3: Limoniidae (except Chioneinae and Limnophilinae)	7
	G4: Cylindrotomidae + Tipulidae	14

4. Conclusions

Here, we present the first two mt genomes for the tribe Elephantomyiini, which are typical circular DNA molecules with lengths of 14,551 bp and 14,358 bp. Like the mt genomes of other crane flies, these two mt genomes show similar gene order, nucleotide composition and codon usage. Phylogenetic results support both new and traditional arrangements. The traditional views, that Pediciidae is sister to all remaining Tipuloidea, while Cylindrotomidae and Tipulidae are sister groups, are reconfirmed in this study. The four-family system of Tipuloidea and four-subfamily system of Limoniidae are found to be unstable classification systems. The monophyly of Limoniidae is not supported in our study, which indicates that Limoniidae may not be a natural group. In addition, two limoniid subfamilies (i.e. Limoniinae and Limnophilinae) may be para- or polyphyletic, as *Epiphragma* (Limnophilinae) has a closer relationship with Limoniinae. Our study supports the monophyly of Elephantomyiini, as *Elephantomyia* and *Helius* form a strongly supported clade, which represents a significant origin of flower-visiting in Limoniidae. However, the more precise phylogenetic position of Elephantomyiini in Tipuloidea, as well as other phylogenetic arrangements within Limoniidae, needs to be further revealed through additional studies with more species.

5. Competing Interests

The authors have declared that no competing interests exist.

6. Acknowledgments

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Supplementary Material 1

Tables S1, S2

Authors: Kang Z, Xu Y, Wang G, Yang D, Zhang X (2023)
Data type: .docx
Explanation note: Table S1. Codon usage of the mitochondrial genome of *Elephantomyia* (*Elephantomyia*) *inulta*. — **Table S2.** Codon usage of the mitochondrial genome of *Helius* (*Helius*) *pluto*.

Link: https://doi.org/10.3897/asp.81.e97946.suppl1

Supplementary Material 2

Figures S1–S12

Authors: Kang Z, Xu Y, Wang G, Yang D, Zhang X (2023)
Data type: .pdf
Explanation note: Figure S1. Relative synonymous codon usage (RSCU) of the protein-coding genes in two newly sequenced mitochondrial genomes of Elephantomyiini. Leu1 = Leu (CUN); Leu2 = Leu (UUR); Ser1 = Ser (AGN); Ser2 = Ser (UCN). — **Figure S2.** The ratio of Ka/Ks of 13 PCGs in two newly sequenced mitochondrial genomes of Elephantomyiini. — **Figure S3.** Secondary structures of tRNAs of two Elephantomyiini species. All tRNAs are labeled with the abbreviations of their corresponding amino acids. Dashes indicate Watson-Crick base pairing and dots indicate G-U base pairing. — **Figure S4.** AliGROOVE analysis for four datasets. The mean similarity score between sequences is represented by a colored square, based on AliGROOVE scores ranging from minus one, indicating a large difference in sequence composition from the remainder of the dataset (red coloration), to plus one, indicating similarity to all other comparisons (blue coloration). — **Figure S5.** Phylogenetic tree of Tipuloidea inferred from the dataset PCG12RNA under ML method. Numbers at the nodes are bootstrap values. The family Trichoceridae was set as the outgroup. — **Figure S6.** Phylogenetic tree of Tipuloidea inferred from the dataset PCG under ML method. Numbers at the nodes are bootstrap values. The family Trichoceridae was set as the outgroup. — **Figure S7.** Phylogenetic tree of Tipuloidea inferred from the dataset PCG12 under BI method. Numbers at the nodes are posterior probabilities. The family Trichoceridae was set as the outgroup. — **Figure S8.** Phylogenetic tree of Tipuloidea inferred from the dataset PCG12 under ML method. Numbers at the nodes are bootstrap values. The family Trichoceridae was set as the outgroup. — **Figure S9.** Phylogenetic tree of Tipuloidea inferred from the dataset PCGRNA under BI method. Numbers at the nodes are posterior probabilities. The family Trichoceridae was set as the outgroup. — **Figure S10.** Phylogenetic tree of Tipuloidea inferred from the dataset PCGRNA under ML method. Numbers at the nodes are bootstrap values. The family Trichoceridae was set as the outgroup. — **Figure S11.** Results of four-cluster likelihood mapping of the first question as 2D simplex graphs based on four datasets. — **Figure S12.** Results of four-cluster likelihood mapping of the second question as 2D simplex graphs based on four datasets.

Link: https://doi.org/10.3897/asp.81.e97946.suppl2