

The mitochondrial genome of the *Ornithophila metallica* (Diptera: Hippoboscidae) and its phylogenetic significance

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Abstract: The Hippoboscidae (Diptera: Calyptratae: Hippoboscoidea), or louse flies, are ectoparasites that suck blood of their avian or mammalian hosts with strong host specificity. Therefore, they are of great interest in evolutionary research. Here, we sequenced the mitochondrial genome of *Ornithophila metallica* (Schiner, 1864) for the first time. It is 15,739 bp in length and possesses 37 mitochondrial genes, comprising 13 protein-coding genes (PCGs), 22 tRNA and two rRNA genes, and a non-coding control region. The gene arrangement is consistent with that of most Dipteran mitochondrial genomes. The mitogenome is subsequently used for phylogeny reconstruction using all available mitogenomes of Hippoboscoidea. The Hippoboscidae is recovered as a monophyletic group, sister to the clade of (streblid + Nycteribiidae), which is the sister group to the monophyletic Glossinidae. Within the Hippoboscidae, the *Ornithophila* clusters together with *Ornithomya*, which is a sister group to (*Melophagus* + *Lipoptena*). This study broadens our knowledge of the Hippoboscidae and contributes to research in entomology, veterinary science, and ecology.

Key words: Hippoboscoidea; molecular data; phylogeny; taxonomy

金光喜鸟虱蝇线粒体基因组及系统发育分析（双翅目：虱蝇科）

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摘要: 虱蝇科 Hippoboscidae 隶属于双翅目 Diptera 有瓣蝇类 Calyptratae 虱蝇总科 Hippoboscoidea, 为专性吸食鸟类或哺乳动物宿主血液的脊椎动物外寄生虫, 具有很强的宿主特异性, 在进化生物学研究领域具有重要的研究价值。本研究首次测序了金光喜鸟虱蝇 *Ornithophila metallica* (Schiner, 1864) 的线粒体基因组, 其全长 15739 bp, 有 37 个基因, 其中蛋白编码基因 13 个, tRNA 基因 22 个, rRNA 基因 2 个。该线粒体基因组排列与大多数双翅目昆虫一致。系统发育研究支持虱蝇科为单系, 并与 (蝠蝇科 Streblidae + 蛛蝇科 Nycteribiidae) 形成姐妹群, 单系的舌蝇科 Glossinidae 与其他虱蝇总科形成姐妹群。在虱蝇科中, 喜鸟虱蝇属 *Ornithophila* 与鸟虱蝇属 *Ornithomya* 聚为一支并构成了 (虱蝇属 *Melophagus* + 利虱蝇属 *Lipoptena*) 的姐妹群。本研究有助于拓宽对虱蝇科的认识, 并可为昆虫学、兽医学和生态学等研究提供基础。

关键词: 虱蝇总科; 分子数据; 系统发育; 分类

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Introduction

The Hippoboscidae (Calypttratae: Hippoboscoidea), commonly known as louse flies, are widely distributed throughout the world, with most species found in the tropics and the subtropics (Pape *et al.* 2011; Reeves & Lloyd 2019). The Hippoboscidae are divided into three subfamilies, Lipopteninae, Ornithomyinae, and Hippoboscinae (Reeves & Lloyd 2019). The Ornithomyinae are the largest group and are ectoparasites unique to birds, some of which are known to be vectors of avian zoonotic pathogens (Suh *et al.* 2012). *Ornithophila metallica* (Schiner, 1864) has an exceptionally wide distribution across all biogeographic regions (except Antarctica) and is known from a wide host range of up to 134 genera (Maa 1969a, b, c; Suh *et al.* 2012). Therefore, it is a valuable group for studying Dipteran parasitic species adapted to widespread host distribution, using the approach of phylogenetics (Trowbridge *et al.* 2006; Petersen *et al.* 2007).

Mitochondrial genomes of insects have been widely used in phylogenetic inference (Cameron 2014, 2025; Zhang *et al.* 2016; Tang *et al.* 2018; Pei *et al.* 2019; Yan *et al.* 2019; Lin *et al.* 2022a, b) and molecular evolution of various groups (Ding *et al.* 2015; Li *et al.* 2017, 2020; Wang *et al.* 2017; Yan *et al.* 2021a). With the popularity of High-Throughput Sequencing technology, it is possible to rapidly obtain the mitochondrial genome of insects in an affordable and efficient way (Ramakodi *et al.* 2015; Pei *et al.* 2024;). However, the sequenced molecular data for Hippoboscidae are very limited, due to the difficulty collecting specimens. A representative mitogenome for the Ornithomyinae lineage is currently lacking, with only one species, *Ornithom. biloba*, having been sequenced (Li *et al.* 2022).

In the present study, we document the mitogenome of *Ornithop. metallica* sequenced by High-Throughput Sequencing, and infer the phylogenetic relationship of the Hippoboscidae, which will improve our understanding of the diversity and evolution of Hippoboscoidea and other widespread host species from the perspective of mitogenomics.

Material and methods

Specimen collecting and identification

The specimens in this study were collected in Dongzhai, Henan, China, in December 2022. The specimens were identified based on morphological characters (Oboňa *et al.* 2022) and stored in 95% alcohol until DNA extraction. The vouchers are currently stored in Beijing Forestry University.

DNA extraction, sequencing, assembly, and genome annotation

The genomic DNA was extracted from a single intact adult specimen of *Ornithop. metallica* (Diptera: Hippoboscidae) (sample ID: BFU-I-d1) using a TIANamp Genomic DNA Kit (TIANGEN Biotech, China) according to the manufacturer's protocols. The *COI* fragment was amplified and sequenced with the primer pair of LCO1490 and HCO2198 (Folmer *et al.* 1994). Paired-end sequencing (PE150) using the platform NovaSeq was then conducted to sequence the genomic DNA. The paired-end sequencing was performed on the Illumina NovaSeq 6000 platform (Gene+, Beijing, China), generating 6.0 gigabase pairs (Gbp) of raw data, with a Q30 of 97.5%. The raw data was checked by FastQC 0.11.3 (Andrews 2010) before

filtering low-quality reads and sites by Trimmomatic 3.2.57 (Bolger *et al.* 2014). We subsequently used IDBA_UD (Peng *et al.* 2012) to assemble the genomic data with similarity set to be 98%. The mitochondrial genome was identified by BLAST 2.11.0 searches of *COI* against the whole-genome assembly, followed by extraction of matching scaffolds.

The protein-coding genes (PCGs) and ribosomal RNA (rRNA) genes of the mitochondrial genome of *Ornithop. metallica* were annotated using Geneious Prime (Kearse *et al.* 2012) by aligning with the mitochondrial genes of calyptrates. The transfer RNA genes (tRNA) were identified using tRNAscan-SE Search Server (<http://lowelab.ucsc.edu/tRNAscan-SE/>). A circular map of the mitochondrial genome was generated using the online server proksee (<https://proksee.ca/>) (accessed March 1, 2024).

Genome composition and codon usage

The base composition and codon usage of PCGs were estimated with MEGA 7 (Kumar *et al.* 2016). AT-skew and GC-skew were calculated to evaluate strand asymmetry of the *Ornithop. metallica* mitochondrial genomes, following the formula: AT-skew = $(A - T) / (A + T)$ and GC-skew = $(G - C) / (G + C)$.

Phylogenetic analysis

Mitochondrial genomes of Hippoboscoidea were harvested from GenBank, and used for phylogenetic analyses with data of *Ornithop. metallica*, with four species of muscoid grade and three species of Oestroidea selected as outgroups, and *Drosophila melanogaster* was used to root to the tree (Table 1).

Table 1. Taxon sampling for phylogenetic analyses in the present study

Superfamily	Family	Subfamily	Species	Accession	References
Ephydroidea	Drosophilidae	Drosophilinae	<i>Drosophila melanogaster</i>	NC_024511	Direct Submission
Hippoboscoidea	Glossinidae	Glossininae	<i>Glossina austeni</i>	MZ826152	Porter <i>et al.</i> 2022
			<i>Glossina brevivalpis</i>	MZ826153	Porter <i>et al.</i> 2022
	Hippoboscoidea	Lipopteninae	<i>Lipoptena</i> sp.	MT679542	Direct Submission
			<i>Melophagus ovinus</i>	NC_037368	Liu <i>et al.</i> 2017
			<i>Ornithomya biloba</i>	NC_061211	Li <i>et al.</i> 2022
			<i>Ornithophila metallica</i>	OR936035	This study
	Nycteribiidae	Cyclopediinae	<i>Dipseliopoda setosa</i>	MZ826151	Porter <i>et al.</i> 2022
			Nycteribiinae	<i>Basilina ansifera</i>	MZ826150
		<i>Nycteribia allotopa</i>		NC_080267	Direct Submission
		<i>Nycteribia formosana</i>		NC_079664	Direct Submission
		<i>Nycteribia parvula</i>		NC_068095	Direct Submission
		<i>Penicillidia dufourii</i>		NC_079651	Direct Submission
		<i>Phthiridium</i> sp.		OQ301748	Direct Submission
		<i>Phthiridium szechuanum</i>	NC_068222	Direct Submission	
Streblidae	Brachytarsinae	<i>Brachytarsina</i>	NC_071917	Direct Submission	
		<i>amboinensis</i>			
	<i>Raymondia</i> sp.	OQ301747	Poon <i>et al.</i> 2023		
	Trichobiinae	<i>Paratrachobius longicrus</i>	NC_044652	Direct Submission	
<i>Paradyschiria parvula</i>		NC_044702	Direct Submission		

Table 1 (continued)

Superfamily	Family	Subfamily	Species	Accession	References
muscoïd grade	Anthomyiidae	Hylemyiinae	<i>Delia platura</i>	KP901268	Ding <i>et al.</i> 2015
	Faniidae	Fanniinae	<i>Fannia scalaris</i>	NC_053661	Ren <i>et al.</i> 2020
	Muscidae	Muscinae	<i>Musca domestica</i>	NC_024855	Li <i>et al.</i> 2016
	Scathophagidae	Scathophaginae	<i>Scathophaga stercoraria</i>	NC_024856	Li <i>et al.</i> 2016
Oestroïdea	Calliphoridae	Calliphorinae	<i>Calliphora vicina</i>	NC_019639	Nelson <i>et al.</i> 2012b
	Gasterophilidae	Gasterophilinae	<i>Gasterophilus pecorum</i>	NC_029812	Zhang <i>et al.</i> 2016
	Sarcophagidae	Sarcophaginae	<i>Sarcophaga impatiens</i>	NC_017605	Nelson <i>et al.</i> 2012a

The PCGs and rRNA genes were aligned separately using MAFFT 7 (Katoh & Standley 2013). The 13 PCGs were concatenated using SequenceMatrix 1.8 (Vaidya *et al.* 2011) for phylogenetic analyses. The matrices were analyzed using maximum likelihood (ML) on IQ-TREE web server (Trifinopoulos *et al.* 2016). The ML analysis was inferred with best model for each partition determined by the self-implemented modelfinder (Kalyaanamoorthy *et al.* 2017), and branch support estimated using 1000 replicates of ultrafast bootstraps. Bayesian inference (BI) was conducted using MrBayes-3.2.7 (Ronquist *et al.* 2012) with 1,000,000 generations, and the results were sampled for each 1,000 generations. A consensus tree was calculated from the remaining samples after discarding the first 25% of the trees. The phylogenetic trees were visualized using ITOL 5 (Letunic & Bork 2021).

Results and discussion

The General Feature of the Mitochondrial Genome

The mitochondrial genome of *Ornithop. metallica* was 15,739 bp in length (Fig. 1) (GenBank accession: OR936035). The gene composition is consistent with that of the typical insect mitochondrial genome, including 13 PCGs, two rRNA genes, and 22 tRNA genes. The minority strand (L-strand) encoded four PCGs (*ND1*, *ND4*, *ND4L* and *ND5*), two rRNAs (*12S* and *16S*) and eight tRNAs (*trnV*, *trnL2*, *trnP*, *trnH*, *trnF*, *trnY*, *trnC*, and *trnQ*), and the remaining genes, including nine PCGs and 14 tRNAs, were encoded on the majority strand (H-strand).

The number of codons in the mitochondrial genome is 5328. The five most frequently used codons were Leu-UUA, Ile-AUU, Lys-AAA, Phe-UUU, and Asn-AAU respectively, in order of quantity from high to low (Fig. 2).

Eleven PCGs start with the codon ATT or ATG (*COII*, *COIII*, *CYTB*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6*, *ATP6* and *ATP8*), and the start codon of the remaining PCGs are identified as ATA (*COI*, *ND1*). The stop codon TAA or TAG was used for all 13 PCGs.

Nucleotide composition

This mitogenome is strongly biased toward A + T (80.9%), ranging from 71.8% of *COI* to 88.3% of *ATP8* (Fig. 3), which was found for other species of Hippoboscidae (Liu *et al.* 2017) and similar to the A+T bias (79.6%) found in *Melophagus ovinus* (Tang *et al.* 2018).

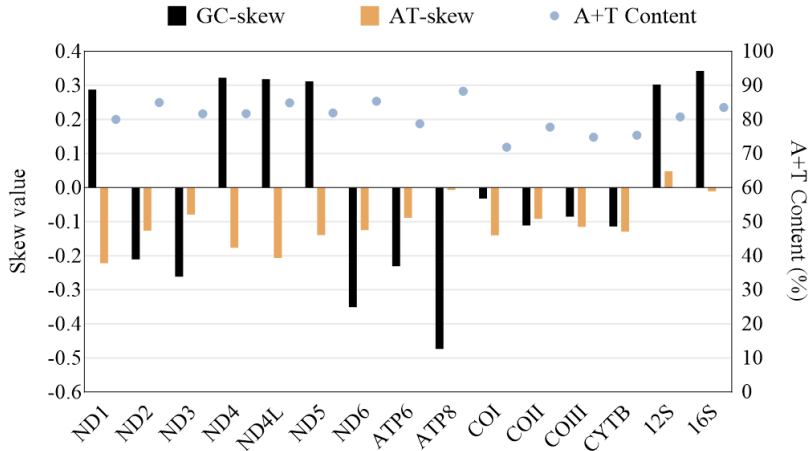


Figure 3. Nucleotide composition of mitochondrial genes of *Ornithophila metallica*.

The GC skew values for most genes were negative except for six genes (0.29 in *ND1*, 0.32 in *ND4*, 0.32 in *ND4L*, 0.31 in *ND5*, 0.30 in *12S*, and 0.34 in *16S*). The AT skew values were all negative except for 0.05 in *12S*, which is positive.

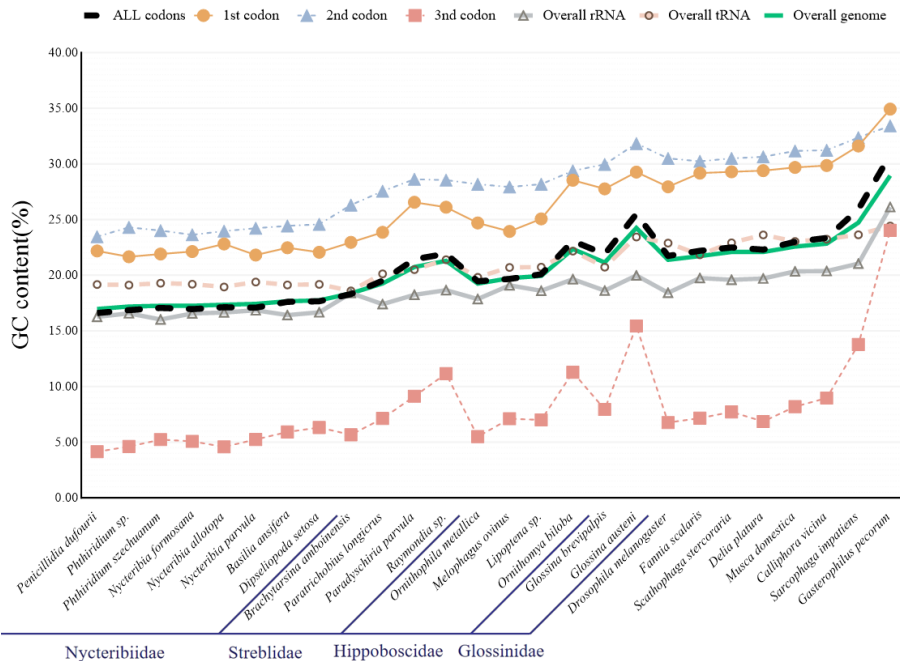


Figure 4. GC content of calyptrate mitochondrial genome.

The GC content of *Ornithop. metallica* is generally lower than other louse flies and Calypterae as well, particularly at the 3rd codon positions of PCGs. The overall GC content of the mitochondrial genome is 19.24% for *Ornithop. metallica*, and that of PCG, rRNA, and tRNA genes are 19.45%, 17.85%, and 19.81%, respectively (Fig. 4). For PCGs, the GC content of the 3rd codon positions of *Ornithop. metallica* is 5.49%, while the 1st codon and the 2nd codon positions are 24.69% and 28.18%.

Phylogenetic reconstruction

The Hippoboscoidea are fully supported to be monophyletic (BS = 100, PP = 1.00; Fig. 5), with the families Glossinidae, Nycteribiidae, and Hippoboscidae also recovered as monophyletic groups with full support (BS = 100, PP = 1.00). However, Streblidae is recovered as paraphyletic in terms of Nycteribiidae with modest support (BS = 88). The family-level relationship of the Hippoboscoidea is reconstructed as (Glossinidae, (Hippoboscidae, (streblids, Nycteribiidae))). Relationships at the genus level are inferred to be identical between ML and Bayesian analyses, with supported values of only several branches different in Hippoboscoidea (Fig. 5).

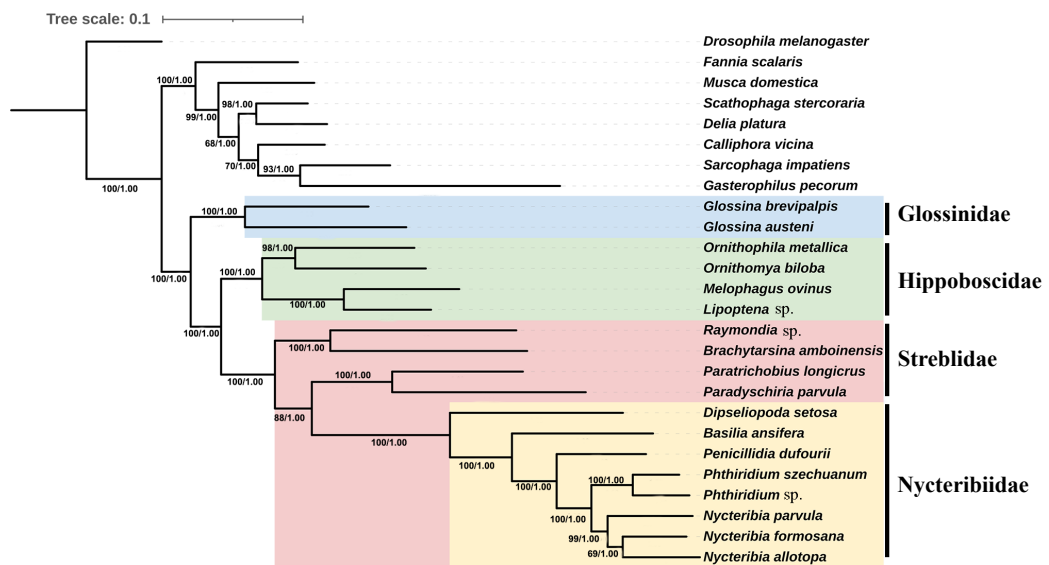


Figure 5. Phylogenetic tree of the Hippoboscoidea using mitochondrial genomes. Numbers at the nodes are bootstraps of ML construction (BS) / posterior probability of BI (PP).

The hippoboscooid flies, traditionally divided into four families, Glossinidae, Hippoboscidae, Streblidae, and Nycteribiidae (Petersen *et al.* 2007; Rahola *et al.* 2011; Reeves & Lloyd 2019), are ectoparasites of birds and mammals (Liu *et al.* 2017; Zhao *et al.* 2020; Sun *et al.* 2021, 2022), some of which transmit severe diseases such as sleeping sickness (Huang *et al.* 2024), and therefore are of great medical and ecological importance. The unique reproductive strategy of adenotrophic viviparity (pupiparous) (Meier *et al.* 1999) and the ectoparasitism of the adults are strong evidence in favor of Hippoboscoidea being monophyletic. The monophyly of Hippoboscoidea, as well as the families Hippoboscidae, Glossinidae, and

Nycteribiidae, has been consistently well supported by both morphological characters (Griffiths 1972; Hennig 1973) and molecular evidence (Nirmala *et al.* 2001; Dittmar *et al.* 2006, 2015; Petersen *et al.* 2007; Yatsuk *et al.* 2024). However, the streblids have been mostly supported to be paraphyletic in terms of Nycteribiidae by both morphological (Schlein 1970; Pollock 1971; Hennig 1973; McAlpine 1989) and molecular (Dittmar *et al.* 2006; Petersen *et al.* 2007) evidence. Therefore, some research proposed that streblids should be merged into nycteribiids, making it a sister group with Hippoboscidae (Trowbridge *et al.* 2006; Petersen *et al.* 2007), or that the bat flies should be sunk into Hippoboscidae as the subfamily Nycteribiinae (Griffiths 1972; Dittmar *et al.* 2015).

The phylogeny of Hippoboscidae remains controversial because of insufficient taxon sampling and inadequate phylogenetic signals for phylogenetic construction (Hennig 1973; Petersen *et al.* 2007; Yatsuk *et al.* 2024). Hippoboscidae is generally divided into three subfamilies, Hippoboscinae, Lipopteninae, and Ornithomyinae (Reeves & Lloyd 2019), with only Lipopteninae and Ornithomyinae sampled in the present study. The Hippoboscinae has been consistently recovered as monophyletic previously (Petersen *et al.* 2007; Yatsuk *et al.* 2024). The Lipopteninae (represented by *Lipoptena* sp. and *Melophagus ovinus*) and Ornithomyinae (represented by *Ornithom. biloba* and *Ornithop. metallica*) are supported to be monophyletic in the present study. However, the monophyly of these two subfamilies is still uncertain because of limited taxa sampling. The Lipopteninae was generated to be paraphyletic (Yatsuk *et al.* 2024), which is likely the result of uncertain placement of *Melophagus ovinus* (Petersen *et al.* 2007; Yatsuk *et al.* 2024). The Ornithomyinae was recovered as paraphyletic in previous studies, based on morphological characters (Maa 1966; Yatsuk *et al.* 2024) and molecular evidence (Petersen *et al.* 2007; de Moya 2019; Keve *et al.* 2024; Yatsuk *et al.* 2024). The majority of molecular phylogenetic studies recovered Lipopteninae and Hippoboscinae as nested within a paraphyletic Ornithomyinae (Petersen *et al.* 2007; de Moya 2019; Keve *et al.* 2024; Yatsuk *et al.* 2024). Relationships at the genus and subfamily level are generally the same among the present study and previous research using mitochondrial genes (Keve *et al.* 2024; Yatsuk *et al.* 2024) and research combining mitochondrial and nuclear genes (Nirmala *et al.* 2001; Dittmar *et al.* 2006; Petersen *et al.* 2007; de Moya 2019), except for the contentious placement of *Melophagus ovinus*. Therefore, the reliability of mitochondrial genes resolving relationships at both familial and intra-familial levels of calyptrates is validated again (Cameron 2014; Zhang *et al.* 2016). However, various phylogenetic information, including characteristics of mitochondrial and nuclear genes, as well as morphology, often result in a different phylogeny for groups (Petersen *et al.* 2007; Whitworth *et al.* 2007; Misof *et al.* 2014; Yan *et al.* 2021b). The placement of the controversial *Melophagus ovinus* might depend on integrative study with more and different types of phylogenetic information. Taxon sampling continues to play a critical role in phylogenetic reconstruction (Linder *et al.* 2005; Cameron 2014, 2025; Zhang *et al.* 2016). Considering that no other mitogenome of Hippoboscinae has been sequenced, the relationship among the three subfamilies of Hippoboscidae from the perspective of mitogenomics depends on the documentation of mitogenomic data of Hippoboscinae.

Conclusion

The Hippoboscidae are ectoparasitic flies that suck the blood of birds and mammals with significant host specificity, therefore, this study is of great medical, ecological, and evolutionary importance. This study reports the newly sequenced mitochondrial genome of *Ornithop. metallica*. The mitogenome is highly conserved in terms of gene content, gene order, nucleotide composition, and codon usage of protein-coding genes, which is consistent with that of other calyptrates. Phylogenetic reconstruction supports the monophyly of Hippoboscidae, as well as the subfamilies Lipopteninae and Ornithomyinae. However, with the absence of mitogenomes for Hippoboscinae, the relationship of the subfamilies is still open for further investigation.

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