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ORIGINAL ARTICLE

Mitochondrial genomes of Bombyliidae (Diptera): Phylogenetic analysis recovers monophyletic Bombyliidae sister to Asilidae

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Abstract. Bombyliidae (bee flies) is a large family in the order Diptera. Their larvae are predators or parasitoids of several insect orders, such as Coleoptera, Lepidoptera, Hymenoptera and Diptera, some species of the genus *Systoechus* are predators of grasshopper eggs. The adults visit flowers for nectar and mating, which makes them important pollinators. Their classification and systematic position are still strongly debated. There were only two complete mitochondrial (mt) genomes of Bombyliidae. Mt genomes of *Villa fasciata*, *Bombylius candidus*, *Heteralonia anemosyris*, *Ligyra guangdongana*, *Systropus excisus* and *Exhyalanthrax afer* were sequenced in order to determine the diversity of mt genomes in this family. A comparative mt genomic analysis of these newly sequenced species revealed that the sizes of the mt genome ranged from 15,036 bp to 17,992 bp. All tRNAs had cloverleaf secondary structures, but the dihydrouridine (DHU) arm of $tRNA^{Ser\ (AGN)}$ is absent. The phylogenetic analyses based on both Bayesian Inference (BI) and Maximum Likelihood (ML) supported Bombyliidae being the sister group of Asilidae. Within Bombyliidae, the analysis recovered subfamilies Toxophorinae, Anthracinae and Bombyliinae, and Anthracinae is the sister group of Bombyliinae.

INTRODUCTION

Bombyliidae (bee flies) is a large family belonging to the Brachycera, a suborder of Diptera, and includes about 250 genera and 5000 species occurring worldwide except in the polar regions (Hull, 1973; Greathead & Evenhuis, 1997; Evenhuis & Greathead, 1999). Their larvae are parasitoids (Greathead, 1958) and adults are important pollinators (Hull, 1973; Deyrup, 1988; Johnson & Midgley, 1997; Johnson & Dafni, 1998; Szucsich & Krenn, 2000; Ellis & Johnson, 2009; Yang et al., 2012).

The systematic position of the family is still debated (El-Hawagry et al., 2019; Evenhuis, 2019; Li & Yeates, 2019, 2020). Hennig (1952, 1954) studied the systematic position of Bombyliidae and suggested grouping it with Nemestrinidae and Acroceridae in the superfamily Nemestrinoidea, as they are all hypermetamorphic and their larvae are predators or parasitoids. However, the same author (Hennig, 1972) considered the acanthophorite spines as evidence of an affinity between Bombyliidae and Asiloidea, rather than Nemestrinoidea. Many researchers agree that Bombyliidae is the sister group of the remaining Asiloidea (Woodley, 1989; Yeates, 1994, 2002), however, there is no morphological or molecular data to support these placements (Trautwein et al., 2010; Wiegmann et al., 2011).

The relationships within Bombyliidae are also controversial. Becker (1913) described 11 subfamilies and increased the subfamilies of Bombyliidae to a total of 15. Bezzi (1924) further divided the Bombyliidae into two groups, which he named "Tomophthalmae" for those subfamilies with an indentation on the hind margin of the eyes as well as a concave post cranium, and "Homeophthalmae" for those subfamilies with normal hind eye margin and post cranium (Bezzi, 1924). Hull's book "Bee flies of the World," published in 1973, is currently the authoritative text for the Bombyliidae. It includes a large proportion of the references to the family, including host records and fossils, describes all genera known at that time, and proposes 14 subfamilies in Bombyliidae. "Catalogue of the Diptera of the Afrotropical Region" by Bowden (1980) treated Systropodinae and Gerontinae as tribes in the Toxophorinae, downgraded Exoprosopinae to a tribe within the Anthracinae, proposed that Platypyginae be included in the Mythicomyiinae and created the monogeneric subfamily Antoniinae. Yeates (1994) established the subfamily Lor-

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dotinae based on 174 morphological characters of adults and larvae of 87 species, belonging to 15 sufamilies. Trautwein et al. (2011) used two molecular markers to construct a phylogeny for this family, which is unlike that based on morphology. The most recent bee fly phylogeny was constructed by Li et al. (2021) using phylogenomic data for 550 loci of 94 species belonging to 14 subfamilies. The subfamily status of Phthiriinae and Ecliminae was recovered, and the previously incertae sedis genus *Sericosoma* was placed in the subfamily Oniromyiinae.

There are very few mt genomes for Bombyliidae, with only two in the GenBank: *Geron pallipilosus* (accession No. MG732929) and *Hemipenthes neimengguensis* (accession No. MT043309) (Yao et al., 2019; Luo et al., 2020). This paper reports the results of a comparative mt genomic analysis of six newly sequenced species, which together with 15 mt genomes downloaded from GenBank, were used to construct a phylogeny for Asiloidea and investigate the phylogenetic relationships within Bombyliidae.

MATERIALS AND METHODS

Sampling and genomic DNA extraction

Species of six genera of Bombyliidae, with different morphologies, such as sand chamber absent or present, hind margin of the eye complete or with an indentation, post cranium flat/tumid or concave, different types of mouthparts and hairiness. In addition, their modes of oviposition and host specificity are very different. Specimens of *Villa fasciata* (Meigen, 1804) (July 2016, 103°43′52″E, 34°54′60″N), *Bombylius candidus* Loew, 1855 (May 2019, 118°53′21″E, 28°21′41″N), *Heteralonia anemosyris* Yao, Yang & Evenhuis, 2009 (July 2019, 99°58′15″E, 29°07′53″N), *Ligyra guangdongana* Yang, Yao & Cui, 2012 (July 2019, 118°03′52″E, 36°16′34″N), *Systropus excisus* (Enderlein, 1926) (July 2019, 117°36′28″E, 35°01′43″N) and *Exhyalanthrax afer* (Fabricius, 1794) (July 2019, 117°36′28″E, 35°01′43″N) were collected in Gansu, Ningxia, Zhejiang, Sichuan and Shandong, respectively. The species were identified by Gang Yao (the senior author). Specimens were preserved in absolute ethanol at –20°C for storage at China Agricultural University (CAU), Beijing. Total DNA was extracted from thoracic muscle using DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany).

Genome sequencing and analyses

An Illumina TruSeq library was prepared with an insert size of 350 bp and was sequenced on the Illumina NovaSeq 6000 platform with 150 bp pair-end reads. Raw reads were checked using FastQC 0.11.3 (Andrews, 2010), with adapters and low-quality reads filtered out using Trimmomatic (Bolger et al., 2014). A total of 6 Gb of clean data was obtained and used in the de novo assembly using IDBA-UD (Peng et al., 2012) with minimum and maximum k values of 45 bp and 145 bp. To identify the mt genome sequences, the contigs obtained were searched with the *COI* and *rrnS* gene sequences using BLAST with at least 98% similarity. Illumina HiSeq 2500 Platform (Illumina, San Diego, CA, USA) was used to sequence the whole genomes by Majorbio (Shanghai). Assembly, annotation and prediction of secondary structures of tRNAs were initially conducted using MitoS (http://mitos2.bioinf.uni-leipzig.de/index.py) (Meng et al., 2019) and then manually checked by comparing them with other published annotations of closely related species. MEGA 7.0 was used to calculate the nucleotide composition and codon usage (Sudhir et al., 2016). Composition skew values were obtained using AT-skew = [A – T] / [A + T] and GC-skew = [G – C] / [G + C] (Perna & Kocher, 1995).

Phylogenetic analyses

In this study, 21 mt genomes were used in the phylogenetic analysis, including 15 downloaded from GenBank and six newly sequenced. Mt genomes of eight Bombyliidae include species of different lineages and subfamilies with flat/tumid and concave post cranium, with and without a sand chamber and the hind margin of the eye complete or with an indentation. The included taxa belong to 8 families listed in Table 1.

Clustal_X was used to align the DNA based on the amino acid (aa) alignment of protein-coding genes (PCGs) (Thompson, 1997). All sequences (excluding the stop codons) were aligned using MEGA 7.0 (Sudhir et al., 2016). The phylogenetic trees from Mr Bayes (Ronquist & Huelsenbeck, 2003) and RAxML (Liu et al., 2011; Alexandros, 2014) were constructed on CIPRES (http://www.phylo.org/). In the BI analysis, 2 runs of 2,000,000 generations were conducted. The BI tree was sampled every 200

Table 1. List of taxonomic groups used in the phylogenetic analyses.

Family	Species	Genbank	Size(bp)	Sequencing methods	Year of sequencing
Asilidae	*Dasypogon diadema*	NC_045239	16941 bp		
Asilidae	*Leptogaster longicauda*	KT225296	14407 bp		
Asilidae	*Satanas* sp.	KT225300	14415 bp		
Bombyliidae	*Bombylius candidus*	MW548253	15036bp	Illumina NGS	2020
Bombyliidae	*Exhyalanthrax afer*	MW125545	16062bp	Illumina NGS	2020
Bombyliidae	*Geron pallipilosus*	MG732929	15588 bp	Illumina NGS	2018
Bombyliidae	*Hemipenthes neimengguensis*	MT043309	15405 bp	Illumina NGS	2018
Bombyliidae	*Heteralonia anemosyris*	MW548252	17451bp	Illumina NGS	2020
Bombyliidae	*Ligyra guangdongana*	MW077532	16670bp	Illumina NGS	2019
Bombyliidae	*Systropus excisus*	MW561429	14647bp	Illumina NGS	2020
Bombyliidae	*Villa fasciata*	MW548254	17992bp	Illumina NGS	2020
Empididae	*Chelipoda* sp.	MT396991	14976 bp		
Empididae	*Oreogeton* sp.	MK639348	15718 bp		
Hybotidae	*Leptopeza flavipes*	MT610901	15267 bp		
Hybotidae	*Ocydromia* sp.	MK514083	16078 bp		
Stratiomyidae	*Hermetia illucens*	NC_035232	15698 bp		
Xylomyidae	*Xylomya moiwana*	KT225302	14681 bp		
Xylophagidae	*Dialysis* sp.	KT225293	14465 bp		
Xylophagidae	*Heterostomus curvipalpis*	MH817480	15897 bp		
Tabanidae	*Atylotus miser*	NC_030000	15858 bp		
Tabanidae	*Cydistomyia duplonotata*	NC_008756	16247 bp		

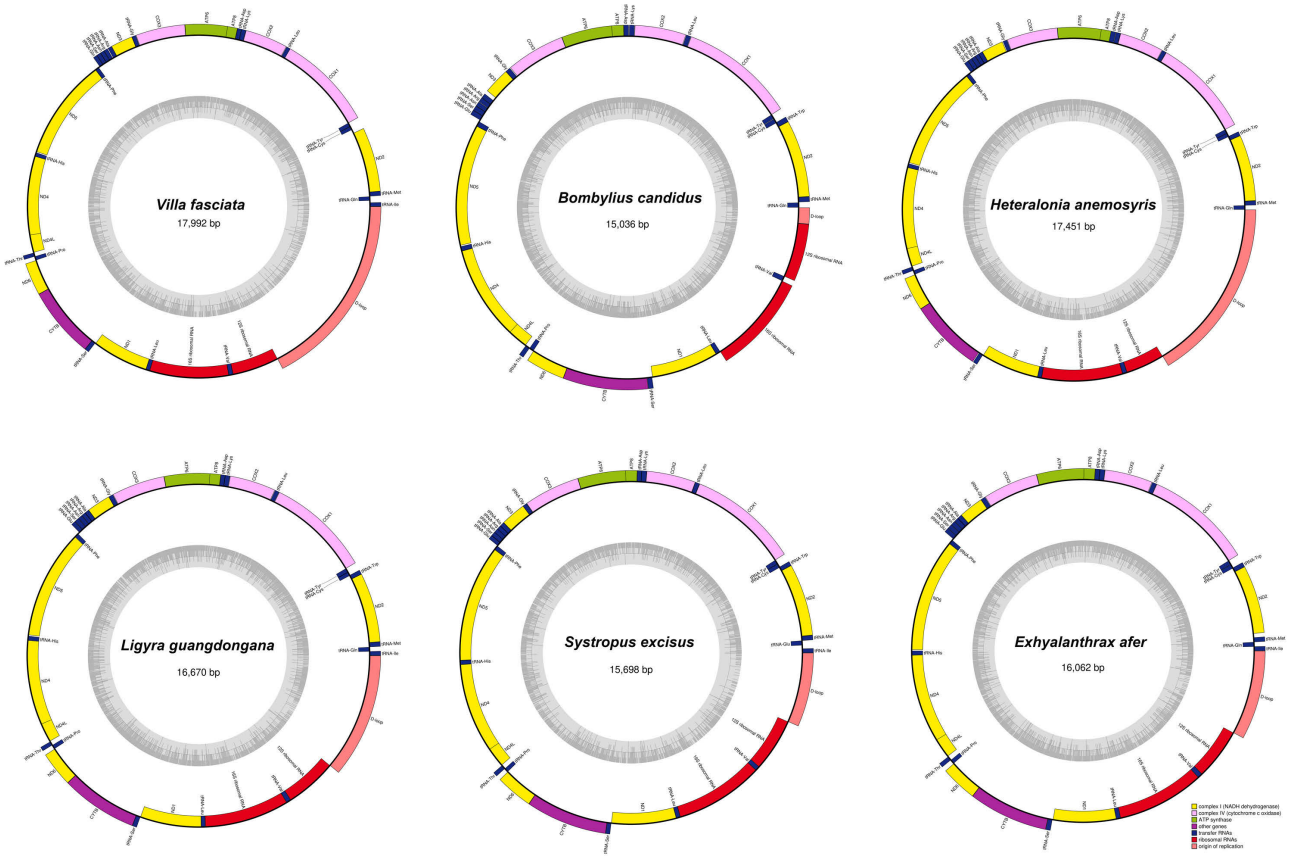

Fig. 1. Mitochondrial maps for *V. fasciata*, *B. candidus*, *H. anemosyris*, *L. guangdongana*, *S. excisus* and *E. afer*. The blocks of tRNAs, rRNA and PCGs are denoted in different colours. Genes outside the map are transcribed clockwise and those inside counter-clockwise.

generations with a burn-in of 25%. The analysis was continued until the average standard deviation of split frequencies was below 0.01. The model of GTR + I + G was optimal for ML analysis. The ML tree was calculated with branch support based on an estimated 500 bootstrap replicates. Finally, the phylogenetic trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Genome organization and base composition

Of the six mt genomes sequenced that of *V. fasciata* (MW548254), *L. guangdongana* (MW077532), *S. excisus* (MW561429) and *E. afer* (MW125545) contained 37 genes with 13 PCGs, 22 tRNAs, 2 rRNAs and a control region, which is typical for metazoan mt genomes. Those of *B. candidus* (MW548253) and *H. anemosyris* (MW548252) were nearly complete, with $tRNA^{Ile}$ missing and the control region incomplete (Tables S1–S6). The mt genomes of the species sequenced ranged from 15,036 bp to 17,992 bp in length (Table S7). The structures of the mitochondrial genomes of *V. fasciata*, *B. candidus*, *H. anemosyris*, *L. guangdongana*, *S. excisus* and *E. afer* are shown in Fig. 1.

Almost all species have 23 genes located on the J strand, while the remaining 14 are located on the N strand, except that *B. candidus* and *H. anemosyris* lacked a gene located on the J strand. The longest overlap (8 bp) for all species was between $tRNA^{Trp}$ and $tRNA^{Cys}$ (Tables S1–S6).

AT-skews and GC-skews are usually measured in terms of the strand bias in nucleotide composition of metazoans mt genomes (Hassanin, 2006). The nucleotide compositions of all the species sequenced are obviously biased in terms of the A + T content, which ranges from 71.73% to 78.29% (Table 2). The comparative analysis of A + T% vs AT-skew and G + C% vs GC-skew for all available mt genomes is shown in Fig. 2. Bombyliidae have an obvious AT bias, with the AT-skew ranging from –0.09 (*L. guangdongana*) to 0.10 (*E. afer*) and the GC-skew ranging from –0.27 (*E. afer*) to 0.27 (*L. guangdongana*) (Fig. 2).

Protein-coding genes (PCGs) and codon usage

In the sequenced species, the PCGs of *B. candidus* are the longest with a length of 11136–11203 bp (Table S7). In the mt genomes of many insects, seven nucleotides (ATGNTAA) overlap between *ATP8* and *ATP6* as well as *ND4* and *ND4L*, which are believed to be bicistronic (Stewart & Beckenbach, 2009). In the mt genome of the species sequenced, there are overlaps between *ATP8*/*ATP6* and between *ND4*/*ND4L*.

Almost all of the PCGs have the typical ATN start codon, with a few having the start codons TTG and TCG. Most of the PCGs have typical termination codons (TAA/TAG) except those of *V. fasciata* and *S. excisus* with AT-tRNA for *ND1* and *L. guangdongana* with A-tRNA for *ND1* (Tables S1–S6).

Table 2. Base composition and strand bias of the species included in this study.

Species	T(U)	C	A	G	Total (bp)	A+T%	AT-Skew	G+C%	GC-Skew
Dasypogon diadema	33.50	16.34	39.89	10.27	16941	73.39	0.09	26.61	–0.23
Leptogaster longicauda	34.93	16.14	38.93	10.00	14407	73.85	0.05	26.14	–0.23
Satanas sp.	26.13	22.22	39.57	12.08	14415	65.70	0.20	34.30	–0.30
Bombylius candidus	32.73	17.96	38.40	10.91	15036	71.13	0.08	28.87	–0.24
Exhyalanthrax afer	32.46	17.65	39.83	10.07	16062	72.28	0.10	27.72	–0.27
Geron pallipilosus	35.41	14.80	40.11	9.69	15588	75.51	0.06	24.49	–0.21
Hemipenthes neimengguensis	33.86	16.75	38.84	10.55	15405	72.70	0.07	27.30	–0.23
Heteralonia anemosyris	33.44	16.21	40.34	10.01	17451	73.78	0.09	26.22	–0.24
Ligyra guangdongana	39.60	9.88	33.20	17.32	16670	72.80	–0.09	27.20	0.27
Systropus excisus	37.50	12.71	40.79	9.01	15698	78.29	0.04	21.72	–0.17
Villa fasciata	36.89	13.98	40.88	8.24	17992	77.78	0.05	22.22	–0.26
Chelipoda sp.	38.26	13.55	38.94	9.25	14976	77.20	0.01	22.80	–0.19
Oreogeton sp.	37.36	13.58	39.77	9.28	15718	77.13	0.03	22.86	–0.19
Leptopeza flavipes	38.52	12.83	39.59	9.07	15267	78.11	0.01	21.90	–0.17
Ocydromia sp.	39.21	12.27	39.92	8.61	16078	79.13	0.01	20.88	–0.18
Hermetia illucens	35.44	17.59	36.44	10.53	15698	71.88	0.01	28.12	–0.25
Xylomya moiwana	38.31	13.53	38.96	9.20	14681	77.27	0.01	22.73	–0.19
Dialysis sp.	38.21	12.90	39.87	9.02	14465	78.08	0.02	21.92	–0.18
Heterostomus sp.	39.18	12.23	39.52	9.06	15897	78.71	0.00	21.29	–0.15
Atylotus miser	38.98	13.02	38.67	9.33	15858	77.66	0.00	22.35	–0.17
Cydistomyia duplonotata	38.84	12.99	39.09	9.08	16247	77.93	0.00	22.07	–0.18

Transfer RNAs

In the species sequenced, the tRNAs of *E. afer* are the longest (Table S7). Twenty-two typical tRNAs range in length from 60 to 73 bp in *V. fasciata*, *L. guangdongana*, *S. excisus* and *E. afer*, for which 14 tRNAs are encoded on the J strand, and the remaining tRNAs on the N strand. The $tRNA^{Ile}$ encoded on the J strand is missing in *B. candidus* and *H. anemosyris*. The supplementary Fig. S1 shows the predicted secondary structures. Almost all tRNAs are folded into cloverleaf structures, except the dihydrouridine (DHU) arm of $tRNA^{Ser\ (AGN)}$ that is in the form of a simple loop.

In almost all the tRNAs of the species sequenced the amino acid acceptor (AA) arm is of the same size (7bp), but the AA stem of $tRNA^{Ala}$ and $tRNA^{Arg}$ is 6 bp long except in *S. excisus*. The $tRNA^{Ser\ (AGN)}$ in *S. excisus* is 6 bp long and the $tRNA^{Leu\ (CUN)}$ in *S. excisus* 5 bp long. In all tRNAs the anticodon (AC) loop is of the same length (7 nucleotides). The length of the TψC (T) arm varied between 2 to 5 bp and that of the DHU stem from 2 to 4 bp, except $tRNA^{Ser\ (AGN)}$. The AC arms are mostly 5 bp long except for $tRNA^{Ser\ (AGN)}$, whose AC stems are 6 bp long.

Based on the secondary structures, 13–20 pairs of G-U are located on 4 arms. 2–4 pairs of U-U are located on the T arm, AA arm and AC arm. One pair of A-G and 1 pair of U-C constitute bonds located on the AA arm.

Ribosomal RNAs

The *rrnL* of all the species sequenced are located between $tRNA^{Leu\ (CUN)}$ and $tRNA^{Val}$ and the *rrnS* between $tRNA^{Val}$ and the control region. The *rrnL* of *H. anemosyris* and the *rrnS* of *L. guangdongana* are the longest (Table S7). The length of *rrnL* ranges in length from 1312 to 1333 bp, and that of *rrnS* from 635 to 813 bp (Table S1–S6).

The control region

Of all the species sequenced, the control regions in *V. fasciata*, *L. guangdongana*, *S. excisus* and *E. afer* are 1273–1845 bp long and encoded between *rrnS* and $tRNA^{Ile}$. The control regions of *B. candidus* and *H. anemosyris* are incomplete and located between *rrnS* and $tRNA^{Gln}$ (Table S7).

Phylogeny

A total of 21 species were used in the phylogenetic analysis and are listed in Table 1. Two datasets, consisting of different types of data and inclusion/exclusion of particular sites, were used in the analysis.

There were 7370 sites in the PCG12 matrix (containing the first and the second codon positions of PCGs) and in the PCG12r matrix 9373 sites (containing the first and the second codon positions of PCGs, plus the two rRNA genes). The phylogenetic trees based on the PCG12 and PCG12r matrices inferred by both Bayesian methods (BI) and Maximum Likelihood (ML) have similar topologies. Four phylogenetic trees were obtained, of which two have the same topology (PCG12 by BI and ML); differences occurred only within the bombyliid subfamily Anthracinae (Fig. 3). The phylogeny of this group of Diptera is still in part uncertain and our sample of families is limited, but in view of the results of Shin et al. (2018), we rooted our tree by their group labelled "SXT" (Stratiomyomorpha + Xylophagidae + Tabanomorpha) represented in our tree by the monophyletic group containing the families Stratiomyidae, Xylomyidae, Xylophagidae and Tabanidae.

In all analyses, the Bombyliidae and Asilidae are sister groups, supporting placement of Bombyliidae in the superfamily Asiloidea. This clade is sister to the monophyletic Empidoidea (Empididae + Hybotidae). Within Bombyliidae, the phylogenetic relationship of *V. fasciata*, *H.*

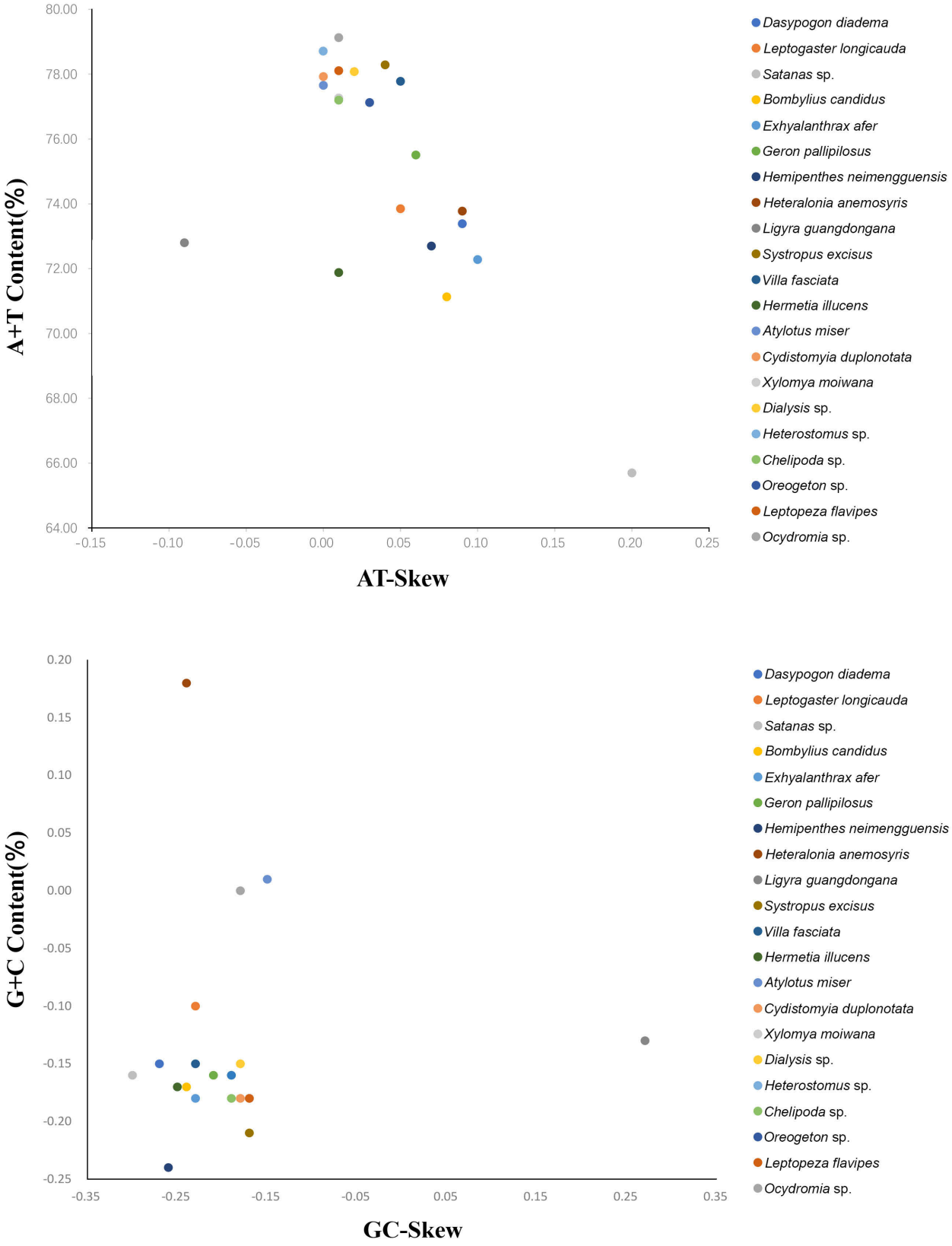

Fig. 2. AT% vs AT-Skew and GC% vs GC-Skew in the mitochondrial genomes included in this study. Measured nucleotide contents (Y-axis) and level of nucleotide skew (X-axis). Values are calculated for the 21 mt genomes included in this study.

neimengguensis, *E. afer*, *H. anemosyris* and *L. guangdongana* was equivocal – three different results were obtained (Fig. 3).

DISCUSSION AND CONCLUSIONS

Although we could include only a limited number of families, the BI and ML based phylogenetic reconstruc-

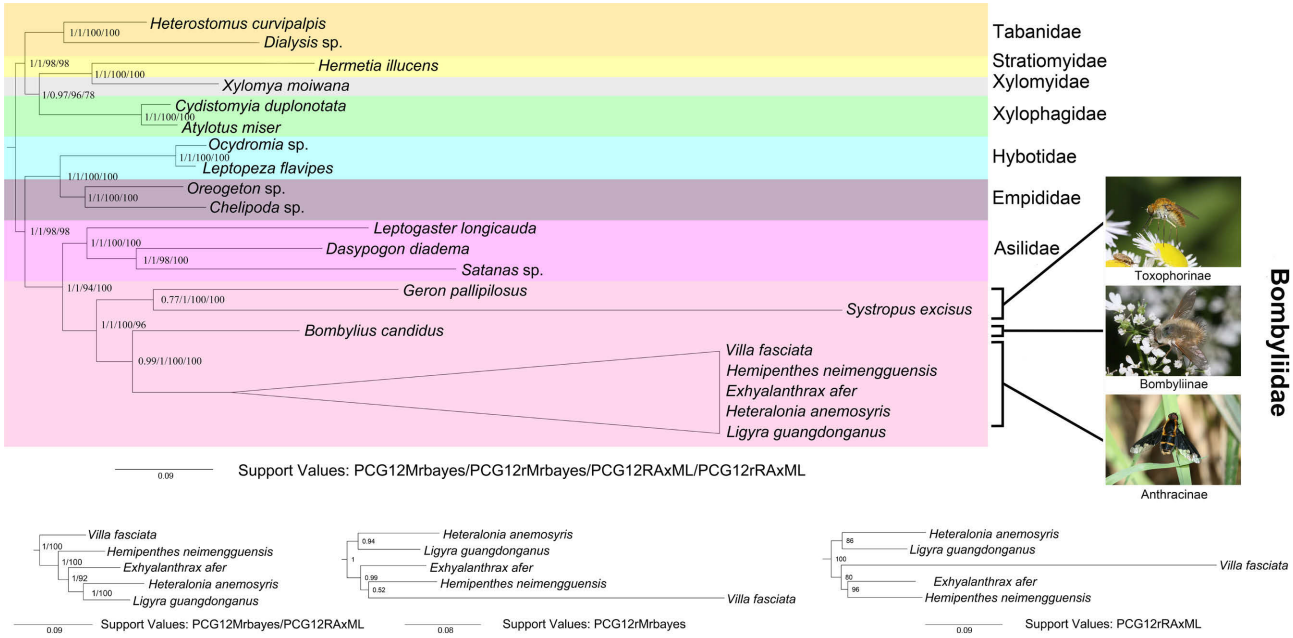

Fig. 3. Phylogenetic relationships of Bombyliidae inferred using Bayesian Inference (BI) and Maximum Likelihood (ML). Numbers at the nodes are posterior probabilities (left) and bootstrap values (right). The tree is rooted by the clade containing the families Tabanidae, Stratiomyidae, Xylomyidae and Xylophagidae.

tions place Bombyliidae and Asilidae as sister groups, thus supporting the view of most researchers that Bombyliidae should be placed in Asiloidea (Woodley, 1989; Yeates, 1994; Yeates et al., 2003). Molecular phylogenies have so far remained inconclusive (Trautwein et al., 2010; Shin et al., 2018). The relationships within the family Bombyliidae revealed monophyletic Toxophorinae and Anthracinae, and Anthracinae is a sister group of the single species of Bombyliinae sampled. This result is consistent with Bowden (1974) who considers the female genitalia of Bombyliinae and Anthracinae to be advanced. In addition, the females in the subfamilies Bombyliinae and Anthracinae have a sand chamber, which serves as a receptacle and is where the eggs are coated with sand, whereas the subfamily Toxophorinae lacks a sand chamber (Merle, 1971; Mühlenberg, 1971; Yeates, 1994). *Systropus excisus* is on a very long branch in the phylogenetic tree, which might correspond with its considerable differences such as very long and slender antennae, a long petiolate abdomen and being host specific on Eucleidae (Lepidoptera).

In this study, six mt genomes of members of the family Bombyliidae (*V. fasciata*, *L. guangdongana*, *S. excisus*, *E. afer*, *B. candidus* and *H. anemosyris*) were sequenced. This revealed that codon usage, protein-coding genes, transfer RNAs structure, ribosomal RNAs and structural elements in the control region are highly conserved in Bombyliidae. Phylogenetic relationships support the monophyly of Bombyliidae and the relationships within the family Bombyliidae separated the subfamilies Toxophorinae, Anthracinae, and Bombyliinae. To better understand the phylogenetic position of Bombyliidae, as well as the relationships within that family, more mt genomes of Asiloidea and Bombyliidae are needed. A genome-wide sequencing (including nuclear genes) needs to be used in the future to resolve the higher-level relationships of Bombyliidae.

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DATA AVAILABILITY. The data that support the findings of this study are available in National Centre for Biotechnology Information at https://www.ncbi.nlm.nih.gov/nuccore, with reference numbers MW548254, MW548253, MW548252, MW077532, MW561429, MW125545.

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CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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S1 (http://www.eje.cz/2023/038/S01.pdf). Tables S1–S7.
S2 (http://www.eje.cz/2023/038/S02.pdf). Fig. S1.