Zootaxa 5120 (1): 128–142
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5120.1.8
http://zoobank.org/urn:lsid:zoobank.org:pub:79C3A3C1-2D21-411B-8A5E-40C9AAB09130

Four new stink bug mitogenomes corroborate the internal inconsistencies in the classification of Pentatomidae (Hemiptera)

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Abstract

Stink bugs (Pentatomidae) are a speciose group of insects that feed mostly on plants. Many species are considered agricultural pests of economically important crops around the globe. Mitochondrial genomes are valuable for evolutionary and phylogenetic studies, but have been little explored for Pentatomidae. Here, we characterized the mitochondrial genomes of four pentatomid species (*Diceraeus melacanthus*, *Euschistus heros*, *Piezodorus guildinii*, and *Stiretrus anchorago*) and performed a comparative analysis for this family and its subfamilies. Stink bug mitogenomes disclosed a conserved gene order and content, although we detected two uncommon armless tRNAs in *E. heros* and *D. melacanthus*. Phylogenetic results indicate that Pentatominae is polyphyletic, showing that internal relationships of Pentatomidae should be further investigated. Stink bug mitochondrial genes are under strong purifying selection, except for ATP8 which showed signs of positive selection.

Key words: Asopinae, crop pest, evolution, mitogenome, phylogeny

Introduction

Stink bugs (Hemiptera: Pentatomidae) are a group of conspicuous and morphologically diverse insects that present a broad range of coloration, from bright and aposematic hues to dull and cryptic shades. They occur in all zoogeographical regions, with the exception of the Antarctic continent, and have had a greater diversification in the tropical and subtropical zones (Grazia *et al.* 2015). Stink bugs are organized in ten subfamilies (*sensu* Rider *et al.* 2018) and comprise about 5,000 species, representing the third largest family of Heteroptera (Schuh & Weirauch 2020). Most species are phytophagous, usually oligophagous, and some species are considered agricultural pests of economically important crops around the globe. The only exceptions concerning feeding habits are the Asopinae, the predatory stink bugs, which sometimes are used as biological control agents against pests (Rider *et al.* 2018).

The descriptions of Pentatomidae are based on morphology, being the genitalic features one of the main sources of characters for taxa description (e.g. Barros *et al.* 2020; Mendonça *et al.* 2021; Salini & Roca-Cusachs 2021). For taxonomic purposes, any inherited physical structure (macroscopic, microscopic or molecular) or behavior (e.g. mating, communication, diapause) with more than one form (character state) has potential to provide phylogenetic information (Quicke 1993). The combination of different taxonomic characters provides more robust and precise species delimitations (Padial *et al.* 2010). Several complementary taxonomic characters, such as cuticular hydrocarbons (e.g. Sessa *et al.* 2021), vibratory signals (e.g. Caorsi *et al.* 2021), and mitochondrial DNA (e.g. Hickmann *et al.* 2019) have allowed integrative taxonomic approaches of Pentatomidae, mainly for those economically important species.

Mitogenomes are an attractive resource for taxonomic, phylogenetic, and evolutionary studies due to their small

 Accepted by D. Rider: 25 Feb. 2022; published: 24 Mar. 2022

size, compact structure, conserved gene content, rare recombination, and high mutation rate (Cameron 2014; Smith 2015). Mitochondrial DNA sequences of Pentatomidae, mostly COX1, have been accumulated in public databases due to different kinds of studies, mainly focusing on economically relevant species (Bianchi & Gonçalves 2021). Until 2010, a couple of complete mitochondrial genomes of Pentatomidae species were published (e.g., Hua *et al.* 2008; Lee *et al.* 2009). Currently, this number is increasing exponentially, and dozens of pentatomid mitochondrial genomes are available. High-throughput sequencing technologies usually yield sufficient data to assemble complete organellar genomes, but many species with genomic data available in public databases still lack an assembled mitogenome (Vieira & Prosdocimi 2019).

Here, we characterized the mitochondrial genomes of three pentatomid species: *Diceraeus melacanthus* (Dallas) (green belly stink bug), *Piezodorus guildinii* (Westwood) (redbanded stink bug), and *Stiretrus anchorago* (Fabricius) (anchor stink bug). In addition, we reassembled the *Euschistus heros* (Fabricius) (neotropical brown stink bug) mitogenome after finding inconsistencies in the available sequence for this species. We used the generated data to compare mitogenomic structure among the Pentatomidae, investigate phylogenetic relationships inside this family, and search for signs of selection in stink bug mitogenomes. The characterized mitogenomes of this study has increased the available information for Pentatomidae and may be used to address important evolutionary and taxonomic questions within this family.

Materials and methods

Sequence obtention and curation. We downloaded Illumina paired-end data from the NCBI Sequence Read Archive (SRA) database (Table 1). The datasets containing both mitochondrial and nuclear data were converted to FASTQ using fastq-dump of the SRA Toolkit 2.11.0 (https://trace.ncbi.nlm.nih.gov/Traces/sra/). The datasets were quality-checked using FastQC (Andrews 2010), and sequence adapters were trimmed with Trimmomatic (Bolger *et al.* 2014). During preliminary screening for the mitochondrial genomes of Pentatomidae species, we detected through the Basic Local Alignment Search Tool—Nucleotide (BLASTn) an unexpected high similarity between the COX1 gene of *E. heros* mitochondrial genome (accession number MG253270) and *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) (accession number MN123622). Thus, we decided to make a new assembly for *Euschistus heros* to double-check its accuracy.

TABLE 1. Taxonomic information and GenBank accession numbers of data used in mitochondrial genome assembly of four Pentatomidae (Hemiptera: Heteroptera) species.

Subfamily	Species	SRA	NOVOPlasty Seed
Asopinae	*Stiretrus anchorago*	SRX2485272	HQ985170
Pentatominae	*Diceraeus melacanthus*	SRX2888372	JQ218458
	Euschistus heros	SRX4782131	KU892543
	Piezodorus guildinii	SRX729935	HQ985132

Assembly, annotation and genome feature analysis. We used NOVOPlasty v4.2 (Dierckxsens *et al.* 2017) to assemble *de novo* the mitochondrial genomes, setting as a seed the COX1 gene from the same species (Table 1). Automatic annotation was carried out with MITOS2 (Bernt *et al.* 2013) implemented in Galaxy (Jalili *et al.* 2020), followed by manual revision of predicted annotations. The taxonomic identity of the assembled mitogenomes was confirmed using each recovered COX1 as a query in the Barcode of Life Data System identification workbench (www.boldsystems.org). The transfer RNA (tRNA) secondary structure was predicted using ARWEN v1.2 (Laslett & Canbäck 2008). We generated the genome maps with Unipro UGENE (Okonechnikov *et al.* 2012) and the tRNA figures with the forna webapp (http://rna.tbi.univie.ac.at/forna/) (Kerpedjiev *et al.* 2015). We assessed the base composition and relative synonymous codon usage (RSCU) of protein coding genes (PCGs) with MEGA X (Kumar *et al.* 2018). Strand asymmetry was calculated following the formulas AT skew = (A−T)/(A+T) and GC skew = (G−C)/(G + C) (Perna & Kocher 1995).

Phylogenomic analysis. To put our results in an evolutionary context, we selected 40 additional mitogenomes from Pentatomidae (Table 2) for a phylogenetic analysis using the 13 mitochondrial PCGs and the two ribosomal

RNAs (rRNAs). Four mitogenomes from other families of Pentatomoidea were used as outgroups, whereas *Lygaeus* sp. (Lygaeoidea: Lygaeidae), a pentatomomorphan outgroup of Pentatomoidea, rooted the tree (Table 2).

TABLE 2. Taxonomic information and GenBank accession numbers of the mitochondrial genomes used in phylogenomic analysis.

Family	Subfamily	Species	Accession number	Reference
Pentatomidae	Asopinae	*Arma custos*	MT535604	Wu *et al.* (2020)
		Cazira horvathi	MF497718	Liu *et al.* (2019)
		Dinorhynchus dybowskyi	MG450552	Zhao *et al.* (2018)
		Eocanthecona thomsoni	MF497715	Liu *et al.* (2019)
		Picromerus griseus	MF805778	Zhao *et al.* (2017a)
		Stiretrus anchorago	BK059217	This study
		Zicrona caerulea	MK759656	Zhao *et al.* (2020)
	Pentatominae	*Antestiopsis thunbergii*	MW679031	Liu *et al.* (2019)
		Brachymna tenuis	MF497711	Liu *et al.* (2019)
		Carbula sinica	KY069964	Liu *et al.* (2019)
		Catacanthus incarnatus	MF497716	Liu *et al.* (2019)
		Caystrus obscurus	MF497717	Liu *et al.* (2019)
		Chinavia impicticornis	MG253262	Unpublished
		Chinavia ubica	MG253263	Unpublished
		Diceraeus melacanthus	BK059216	This study
		Dolycoris baccarum	KJ507135	Zhang *et al.* (2019)
		Erthesina fullo	MK374364	Gao *et al.* (2016)
		Eurydema dominulus	MG584833	Zhao *et al.* (2019b)
		Eurydema gebleri	KP207595	Yuan *et al.* (2015)
		Eurydema liturifera	MG584834	Zhao *et al.* (2019b)
		Eurydema maracandica	MF135553	Zhao *et al.* (2017b)
		Eurydema oleracea	MG584835	Zhao *et al.* (2019b)
		Eurydema qinlingensis	MG584836	Zhao *et al.* (2019b)
		Euschistus heros	BK059218	This study
		Euschistus variolarius	KJ778885	Unpublished
		Eysarcoris aeneus	MK841489	Zhao *et al.* (2019a)
		Eysarcoris guttigerus	MN831205	Chen *et al.* (2020)
		Halyomorpha halys	FJ685650	Lee *et al.* (2009)
		Hoplistodera incisa	MF620037	Liu *et al.* (2019)
		Menida violacea	MK617948	Liu *et al.* (2019)
		Nezara viridula	EF208087	Hua *et al.* (2008)
		Palomena viridissima	MT242599	Chen *et al.* (2021)
		Pentatoma semiannulata	MT985377	Wang *et al.* (2021)
		Piezodorus guildinii	BK059215	This study
		Placosternum urus	MF497730	Liu *et al.* (2019)
		Plautia crossota	MK757497	Wang *et al.* (2019)
		Plautia fimbriata	MF497731	Liu *et al.* (2019)
		Rubiconia intermedia	KP207596	Yuan *et al.* (2015)
	Phyllocephalinae	*Dalsira scabrata*	KX505855	Unpublished
		Gonopsis affinis	MG182695	Chen *et al.* (2017)

.....continued on the next page

TABLE 2. (Continued)

Family	Subfamily	Species	Accession number	Reference
	Podopinae	*Graphosoma rubrolineatum*	KX267740	Unpublished
		Scotinophara horvathi	MK251145	Song *et al.* (2019)
		Scotinophara lurida	MF497733	Liu *et al.* (2019)
Achantosomatidae		*Acanthosoma labiduroides*	JQ743670	Li *et al.* (2017)
Cydnidae		*Adrisa magna*	KU053339	Liu *et al.* (2019)
Dinidoridae		*Cyclopelta parva*	KY069962	Liu *et al.* (2019)
Lygaeidae		*Lygaeus* sp.	MF497725	Liu *et al.* (2019)
Urostylididae		*Urostylis flavoannulata*	KY069970	Liu *et al.* (2019)

We removed the stop codons of each PCG and aligned the sequences using MAFFT v7 (Katoh *et al.* 2019). After sequence concatenation, we assessed sequence divergence heterogeneity using AliGROOVE 1.7 (Kück *et al.* 2014) for the whole genetic matrix. Substitution saturation was estimated for each PCG using Xia's method (Xia *et al.* 2003) implemented in DAMBE 7.3.2 (Xia 2018). The optimal model for the partitioned alignment was selected in PartitionFinder v.2.1.1 (Lanfear *et al.* 2012) using a greedy search algorithm and Bayesian Information Criterion (BIC) embedded in Phylosuite (Zhang *et al.* 2020). A phylogenetic tree was constructed using MrBayes 3.2.7a (Ronquist *et al.* 2012) through the CIPRES Science Gateway (Miller *et al.* 2010), with Markov Chain Monte Carlo (MCMC) analysis run for 25,000,000 generations. Tracer v.1.7 (Rambaut *et al.* 2018) was used to inspect the convergence to the stationary distribution of the chains. The first 25% of the generations were discarded as "burn-in", and then the chains were combined. The combined ESS values for each parameter were higher than 200. We estimated phylogenetic informativeness profiles of each PCG with HyPhy (Pond *et al.* 2005) as implemented in PhyDesign (López-Giráldez & Townsend 2011), using as input the MrBayes tree, which was converted into an ultrametric tree using the chronos() function of the R package ape 5.0 (Paradis & Schliep 2019).

Substitution rate and selection tests. We assessed the number of synonymous (dS) and non-synonymous (dN) substitutions for each PCG, and the pairwise dN/dS ratio (ω) was calculated according to Yang & Nielsen (2000), using the YN00 program in the PAML v 4.9 package (Yang 2007). We searched for positive selection for each PCG and the whole set of genes using nested site models through EasyCodeML (Gao *et al.* 2019), a wrapper tool of CodeML from PAML. A comparison of the model M0 (one-ratio) vs. model M3 (discrete) was used to test whether ω was variable among codons. The sets of pairs M1a (nearly neutral) vs. M2a (positive selection) and M7 (beta) vs. M8 (beta and $\omega > 1$) were used to identify positive selection. An unrooted tree constructed from the previous analysis was used as the input tree. We conducted a pairwise likelihood ratio test (LRT) to find the best fit model for our dataset by calculating twice the log-likelihood difference ($2\Delta Ln$) and comparing it with a Chi-square ($\chi 2$) distribution to determine the statistical significance. For genes that showed evidence of positive selection, codon positions with $\omega > 1$ were detected based on the Bayes empirical Bayes (BEB) method (Yang *et al.* 2005) with a posterior probability threshold of 0.95. All significant p-values were corrected by a false discovery rate analysis (FDR) (Benjamini & Yekutieli 2001), and q-values represent the correct p-values.

Results and discussion

Genome features and nucleotide composition

We successfully assembled the complete mitochondrial genomes of *E. heros* (15,499 bp), *P. guildinii* (15,887 bp), and *S. anchorago* (16,600 bp). These molecules disclose the canonical double-stranded circular structure of animal mitochondrial genomes, encoding 13 PCGs, 22 tRNAs, two rRNAs, and an A+T rich control region (Fig. 1). For *D. melacanthus*, we were able to recover the near-complete mitochondrial genome (15,367 bp), lacking only tRNA-Ile and part of the control region. Gene order and PCG open reading frame direction were the same as in the available pentatomid mitogenomes. The majority of genes are encoded in the J strand (nine PCGs and 14 tRNAs), with the remaining genes being encoded in the N strand (four PCGs, eight tRNAs, and two rRNAs). GenBank accession numbers of the assembled mitogenomes are available in Table 2.

The assembled mitochondrial genomes showed average nucleotide composition of A (42.21%), T (32.39%), C (14.58%), and G (10.52%); these values are close to the nucleotide composition of the pentatomid mitogenomes (Table S1). The high AT content reflects in the codon usage, as discussed below. All analyzed species have positive AT skews and negative GC skews (Fig. S1), consistent with previous surveys of strand asymmetry in insect mitochondrial genomes (Wei *et al.* 2010).

The mitogenome of *E. heros* available on GenBank (accession number MG253270) presented anomalous discrepancies in previous analyses. We used BLASTn to check its COX1 similarity to congeneric sequences and found mismatches between this entry and Hemiptera sequences on GenBank. Moreover, a simple pairwise comparison using this sequence, our *E. heros* assembly, and the mitogenome of *E. variolarius*, demonstrated that our assembly is closer to *E. variolarius* (p-distance 0.176) than to the available entry of *E. heros*-MG253270 (p-distance 0.210). Last, we recovered our assembly as sister species of *E. variolarius*, as shown below.

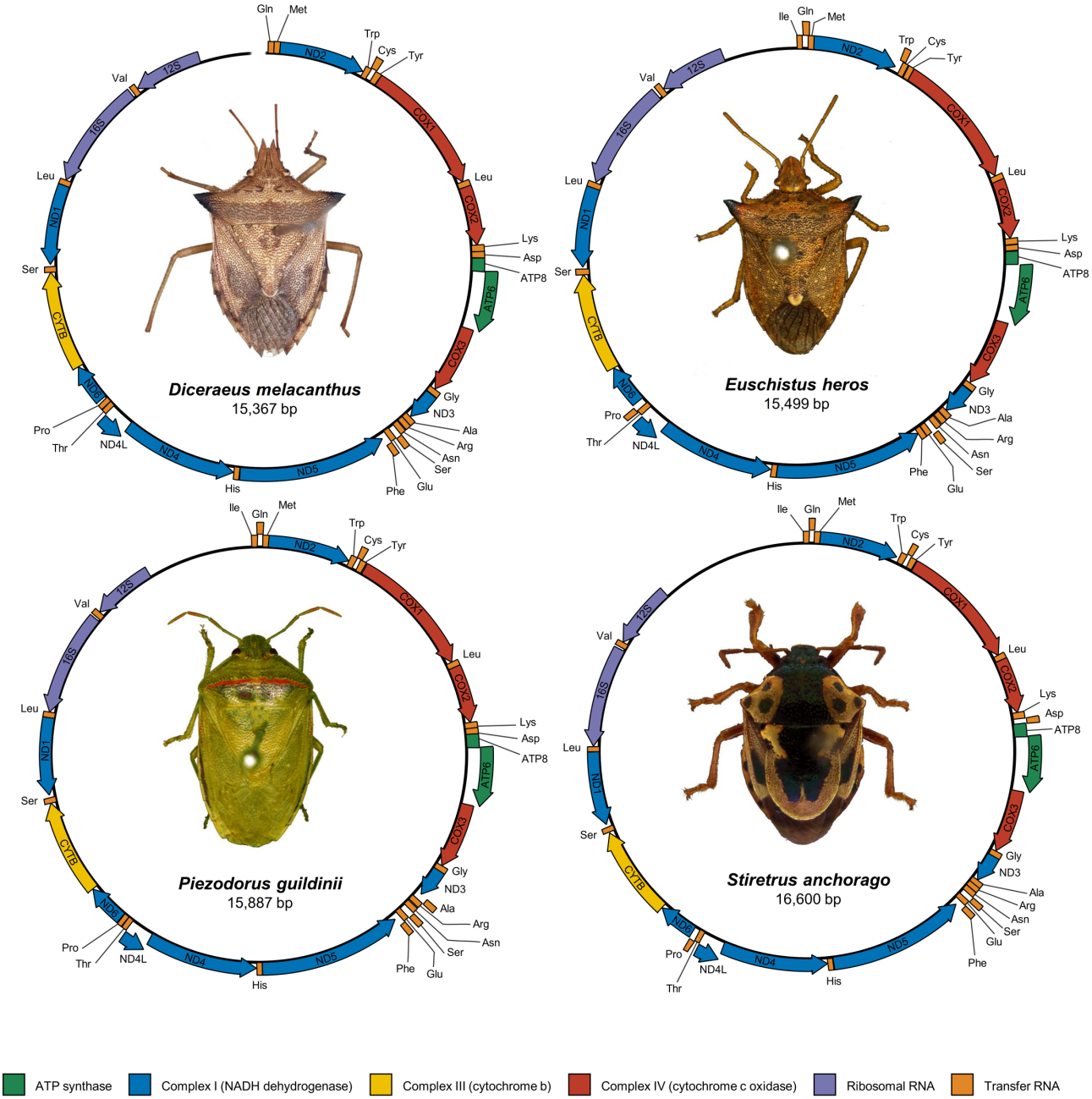

FIGURE 1. Mitochondrial genomes of *Diceraeus melacanthus*, *Euschistus heros*, *Piezodorus guildinii*, and *Stiretrus anchorago*. Direction of gene transcription is as indicated by the strand arrows. Transfer RNAs (tRNAs) are represented by the three-letter IUPAC-IUB amino acid code.

Protein coding genes (PCGs)

Overall, PCGs of the assembled mitogenomes feature the standard ATN start codon except for ATP8 of *P. guildinii* and *S. anchorago*, which start with GTG; also, COX1 and ND1 of these same species start with TTG. The PCG stop codons are TAA or TAG; in some instances, however, we detected truncated T as termination codons. Incomplete stop codons are common in insect mitogenomes (e.g. Wang *et al.* 2021; Wang & Tang 2018; Zhao *et al.* 2019b), and they are completed with A residues through post-transcriptional polyadenylation (Ojala *et al.* 1981).

Relative synonymous codon usage (RSCU) of assembled sequences and available stink bug mitogenomes is as depicted in Fig. S2. All possible codon combinations of the 20 amino acids are present, although there is a bias towards A, T, and C-rich codons, reflecting the high AT content and negative GC skews. Ser2 showed the highest RSCU, while Leu1 was the least represented amino acid. These results support the consistent RSCU within Pentatomidae (Yuan *et al.* 2015; Zhao *et al.* 2019b).

Transfer RNA (tRNA) and ribosomal RNA (rRNA) genes

Predicted tRNAs from the assembled mitogenomes ranged between 62 and 72 bp, showing the typical cloverleaf secondary structure (Figs. S3–S6), with a few exceptions. The tRNA-Ser(gct) from *E. heros*, *P. guildinii*, and *S. anchorago* lack the dihydrouridine arm (D-arm), a common feature of metazoan mitogenomes (Jühling *et al.* 2012; Wolstenholme 1992). Moreover, tRNA-Val from *D. melacanthus* also lacks the D-arm, whereas tRNA-His from *E. heros* lacks the TΨC arm (T-arm) (Fig. 2). Previous studies showed D-armless tRNA-Val in *Eurydema* (Pentatominae) mitogenomes (Zhao *et al.* 2019b). However, to our knowledge, this is the first time a T-armless tRNA-His is described for insects, although truncation of this tRNA is reasonably common in mites (Xue *et al.* 2016) and nematodes (Palomares-Rius *et al.* 2017).

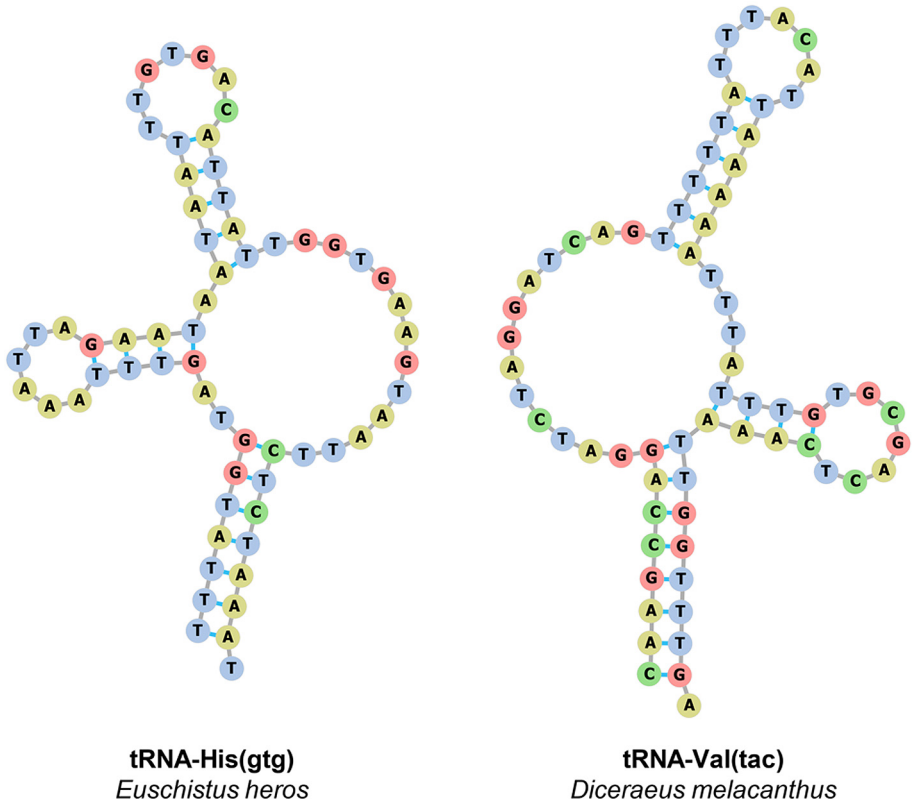

FIGURE 2. Uncommon transfer RNA (tRNA) structures predicted for the assembled mitochondrial genomes. The tRNA-His of *Euschistus heros* lacks the TΨC arm (T-arm), while tRNA-Val of *Diceraeus melacanthus* lacks the dihydrouridine arm (D-arm).

The large subunit rRNA (16S) of the assembled mitochondrial genomes is located between tRNA-Leu and tRNA-Val, ranging from 1,242 to 1,261 bp. The small subunit rRNA (12S), on the other hand, is situated between tRNA-Val and the control region, ranging from 742 to 803 bp. Thus, the rRNAs position and length are similar among all available stink bug mitogenomes, exhibiting a solid pattern for Pentatomidae.

Phylogenomic analyses

We used a broad sample of available pentatomid mitogenomes to construct the phylogeny (Fig. 3). Out of the 43 Pentatomidae terminal taxa, 31 belong to Pentatominae, seven to Asopinae, three to Podopinae, and two to Phyllocephalinae. Pentatominae is the largest subfamily of Pentatomidae, comprising several of its economically relevant species (Rider *et al.* 2018). Our sample represented four of the ten recognized subfamilies of Pentatomidae (*sensu* Rider *et al.* 2018), reflecting the paucity of mitogenomes for this family.

Most of the pairwise comparisons performed in AliGROOVE resulted in positive scores, indicating low sequence heterogeneity (Fig. S7), thus being adequate to use in phylogenetic analysis. Base substitution saturation analysis showed the I_{ss} values were significantly smaller than the $I_{ss.c}$ for all tested datasets, except for ATP8 and ND6 (Table S2), indicating low substitution saturation for our sample. High levels of substitution saturation decrease the sequences' phylogenetic informativity, possibly adding noise to phylogenetic inferences, such as long-branch attraction (Xia & Lemey 2009). Although ATP8 and ND6 showed substantial saturation, they only represented a small portion of our dataset (4.92%), and were maintained for the subsequent analyses.

Pentatomidae was recovered as a monophyletic group with high posterior probability (PP=1), agreeing with phylogenies based on morphological, molecular, and combined data (Bianchi *et al.* 2021; Gapud 1991; Grazia *et al.* 2008; Lis *et al.* 2017; Xu *et al.* 2021; but see Roca-Cusachs *et al.* 2022). Conversely, the phylogenetic relationships among pentatomid lineages have been almost completely ignored (Rider *et al.* 2018). Our results question the monophyly of Pentatominae and Podopinae. Phyllocephalinae and Asopinae were recovered as independent clades (PP=1), both as lineages within the polyphyletic Pentatominae.

The current internal classification of Pentatomidae has been considered stable, except for Pentatominae (Rider *et al.* 2018). Groupings are mostly based on exclusive features or set of characteristics, but not necessarily under a systematic perspective (Schuh & Weirauch 2020). Some phylogenetic analyses have diverged to the current classification of Pentatomidae subfamilies, placing Asopinae within Pentatominae (Genevcius *et al.* 2021; Liu *et al.* 2019; Roca-Cusachs *et al.* 2022; Xu *et al.* 2021) and recovering Podopinae as non-monophyletic (Roca-Cusachs *et al.* 2022; Xu *et al.* 2021). Besides the limited sampling of Pentatomidae, we are aware of the caveats of using only mitochondrial data to infer evolutionary relationships—phylogenetic hypotheses based only on nuclear, mitochondrial, or morphological markers may conflict (Lee *et al.* 2019; Massardo *et al.* 2020). However, our findings emphasize how the current classification requires substantial reformulation to reflect the evolutionary hypotheses generated by recent phylogenetic studies (Genevcius *et al.* 2021; Roca-Cusachs *et al.* 2022).

The phylogenetic informativeness profiles of most PCG presented quite similar patterns across time, showing double peaks; a smooth curve with an optimal rate at time 0.2, and a narrow peak near time 0 (Fig. S8). In general, optimal rates for mitochondrial PCGs were clustered on the same temporal slice. Informativeness varied drastically among PCGs, with ND5, ND4, 16S, ND2, and COX1 bearing higher relative phylogenetic informativeness. However, our dataset was unable to resolve the tree completely (Fig. 3). Low support values and polytomies on deep nodes of Pentatomidae have been recovered in other studies (e.g. Bianchi *et al.* 2021; Grazia *et al.* 2008; Roca-Cusachs *et al.* 2022). The rate of change for a character that maximizes informativeness is fundamental to phylogenetics, as is the heterogeneous phylogenetic signals provided by distinct characters (Townsend 2007). Thus, future analyses intending to propose resolution to the deep nodes of Pentatomidae (e.g. relationships among subfamilies) should look for additional characters evolving slower than mitochondrial genes and incorporate a broader taxon sampling.

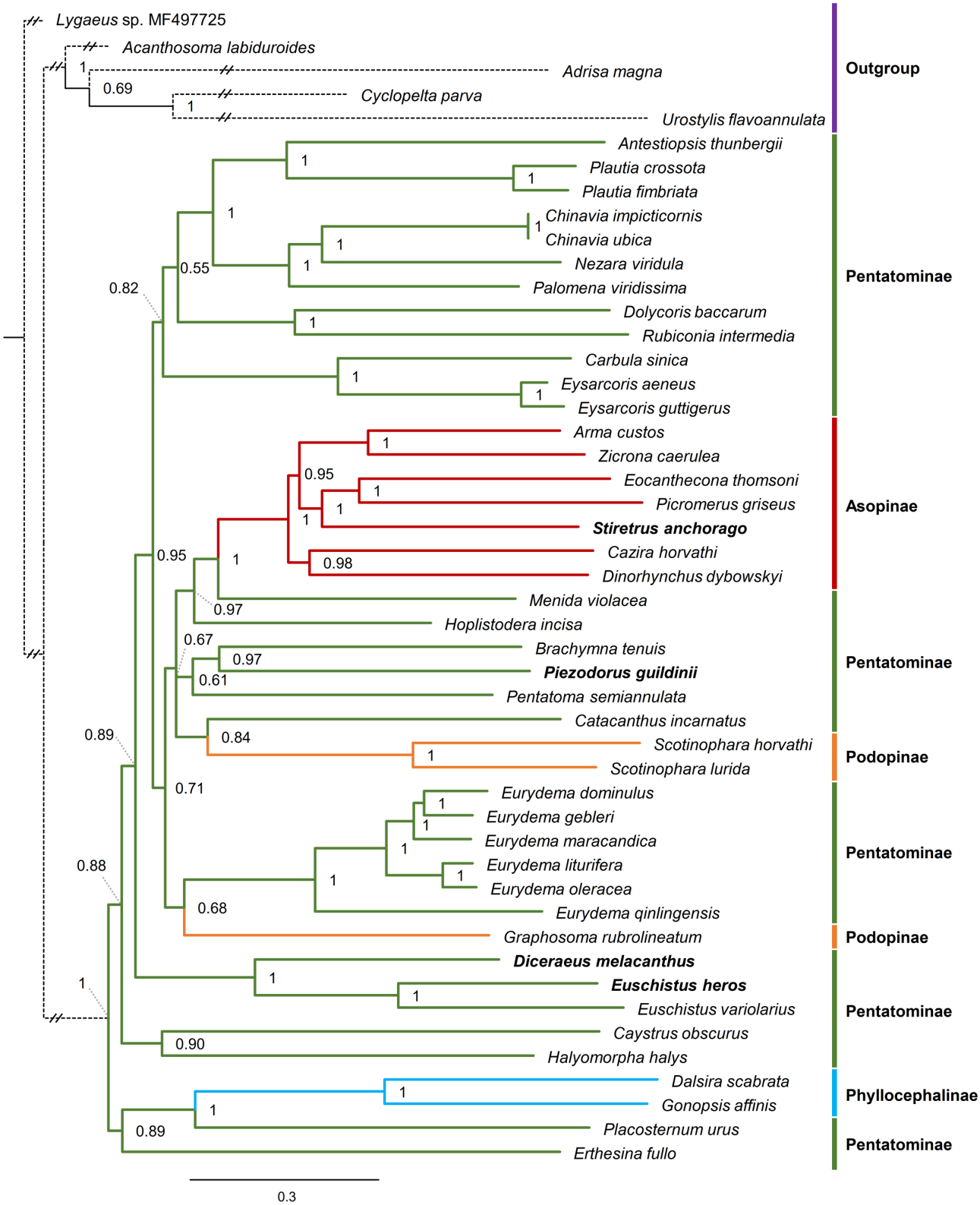

FIGURE 3. Phylogenetic tree of Pentatomidae based on mitochondrial genomes. Tree built based on 13 protein coding genes and 2 ribosomal RNA genes using Bayesian Inference. Species whose mitogenomes were assembled in this work are labeled in bold. Branch labels are the Bayesian posterior probabilities. The length of the branches is proportional to the genetic distance. Outgroup branches are not to scale (dashed lines). The subfamilies of Pentatomidae are shown by vertical bars.

Selection tests

All pairwise comparisons among genes showed a mean ω < 1, suggesting they are under strong purifying selection (Fig. 4). ATP8 has the highest ω ratio, and some of its pairwise comparisons showed ω ≥ 1, indicating this gene has high levels of non-synonymous substitutions and could be under different selection pressures than the other PCGs. LRT results for the site-based models showed that M3 is significantly better than M0 for all tested datasets, i.e., ω is variable among sites (Table S3). The comparison between M3 vs. M0 does not test for positive selection, and thus a comparison should be made between the models that allow positive selection (M2a and M8) against neutral nested models (M1a and M7, respectively). The LRT results showed M1a was significantly better than M2a for all datasets tested, except for ATP8, which has evidence for positive selection for codons 31, 34, and 38 (Table S3). The comparison between models M7 vs. M8 indicated that M8 was significantly better for ATP8, ND1, ND2, ND4, and the concatenated 13 PCG matrix (Table S3). However, no positive selection sites were detected in the BEB test for these instances.

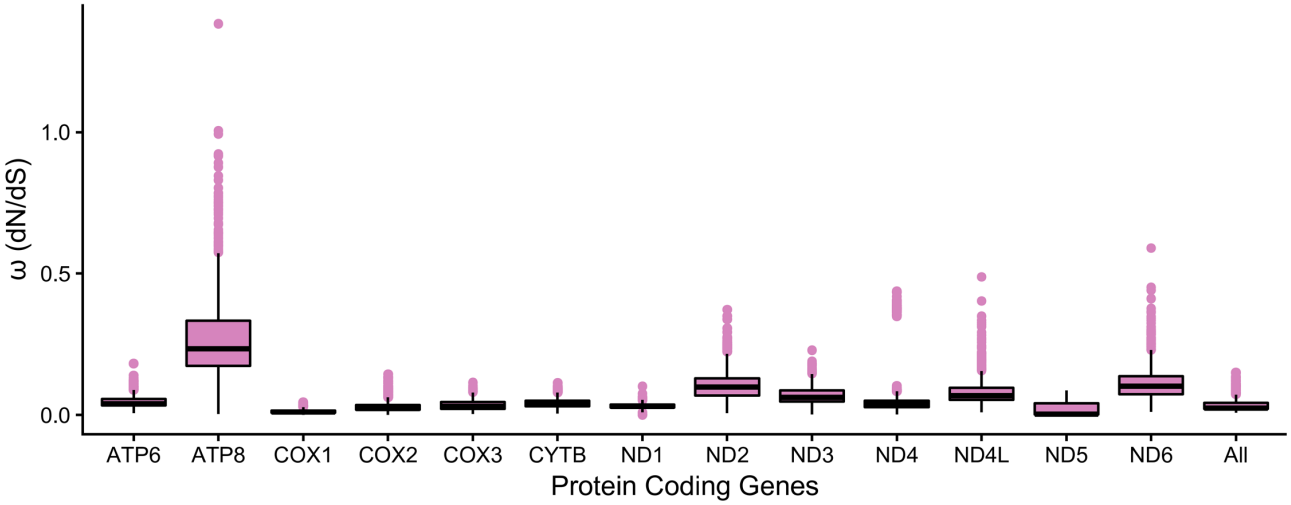

FIGURE 4. Pairwise dN/dS (ω) ratio of the 13 protein coding genes and the concatenated matrix in 48 taxa. The boxplot shows the median (central line), first and third quartiles (box limits), and outlier values (dots).

The lack of evidence of positive selection for most PCGs shown in LRT in association with the low ω ratios indicates the mitogenome is under strong purifying selection to eliminate deleterious mutations. This is an expected result as purifying selection has been described as the major force acting on mitogenome evolution in many insect lineages (e.g. Chang *et al.* 2020; Singh *et al.* 2017; Zhang *et al.* 2019), since it is composed by genes encoding for protein subunits involved in the oxidative phosphorylation process, an indispensable metabolic pathway for cell survival. Furthermore, our results showed that COX1, the canonical DNA barcode of metazoans (Hebert *et al.* 2003), experienced strong evolutionary constraints that yielded low non-synonymous substitutions, ratifying its suitability for barcoding studies of Pentatomidae (Bianchi & Gonçalves 2021).

Mitochondrial DNA was often considered to be under neutral evolution, but growing evidence shows that evolutionary forces act upon mitogenomes (Dowling *et al.* 2008). Previous studies linked positive selection in mitochondrial genes to the increase of energy demand in flying insects (Chang *et al.* 2020; Mitterboeck *et al.* 2017; Yang *et al.* 2014) and to adaptation to high altitudes (Li *et al.* 2018; Zhang *et al.* 2019). ATP8 showed the highest mean ω, indicating this gene may have experienced a different evolutionary pressure than other pentatomid mitochondrial PCGs. The accumulation of non-synonymous mutations in ATP8 could be a result of two scenarios: 1) positive selection, as suggested by LRT and the fairly strong rates of adaptive evolution in animal mitochondrial DNA (James *et al.* 2016); or 2) relaxation of purifying selection which led to accumulation of slightly deleterious mutations (Oliveira *et al.* 2008; Yang *et al.* 2018). Whether the higher mean ω of ATP8 compared to other mitochondrial PCGs is a result of positive or relaxed selection still needs further investigation, but our results identify a candidate gene under selection that might be involved in broad-scale adaptations of Pentatomidae.

Conclusions

Describing a taxon under multiple sources of data promotes a more holistic, stable, and integrative taxonomy (Roe *et al.* 2017). In this study, we assembled and annotated the mitochondrial genomes of four pentatomid species (i.e. *Diceraeus melacanthus*, *Euschistus heros*, *Piezodorus guildinii*, and *Stiretrus anchorago*), expanding their taxonomic characterization. These genomes have a conserved structure and share many features with the available Pentatomidae mitochondrial genomes, although two uncommon tRNA secondary structures were detected. We showed that mitochondrial genomes are useful to solve shallow phylogenetic relationships within this family, whereas the resolution of deep nodes will require slow-evolving markers. Despite the limited sampling, our results indicate that the current subfamily classification of Pentatomidae needs revision to reflect the internal relationships of the family. Moreover, the low substitution rates and the selection tests highlight the strong purifying selection acting on pentatomid mitochondrial genomes. Further investigations are still needed to determine the evolutionary forces acting upon ATP8.

Data availability

Research data associated with this study is available at FigShare (https://doi.org/10.6084/m9.figshare.c.5893874.v1), including the inferred phylogenetic tree and the concatenated nucleotide matrix.

Conflicts of interest

The authors declare none.

Acknowledgements

We thank Dr. Kim Barão and Dr. Talita Roell for providing the pictures of *Diceraeus melacanthus* and *Stiretrus anchorago*, respectively. LTG and PHP are supported by doctoral fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). FMB is supported by a postdoctoral fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (PNPD/CAPES) – Finance code 001.

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Supplementary tables & figures

TABLE S1. Base composition, AT skew, and GC skew of the mitochondrial genomes of Pentatomidae.

TABLE S2. Results of substitution saturation analysis performed in DAMBE using Xia's method for 13 protein coding genes and the concatenated matrix.

TABLE S3. Results of the Likelihood Ratio Tests (LRT) performed in EasyCodeML to evaluate selection pressure for 13 protein coding genes and the concatenated matrix of mitogenomes.

FIGURE S1. Scatterplot showing AT and GC skew values of the mitochondrial genomes of Pentatomidae.

FIGURE S2. Relative synonymous codon usage (RSCU) of mitochondrial protein coding genes of Pentatomidae and the four newly assembled mitochondrial genomes.

FIGURE S3. Predicted secondary structure of transfer RNAs of *Diceraeus melacanthus*.

FIGURE S4. Predicted secondary structure of transfer RNAs of *Euschistus heros*.

FIGURE S5. Predicted secondary structure of transfer RNAs of *Piezodorus guildinii*.

FIGURE S6. Predicted secondary structure of transfer RNAs of *Stiretrus anchorago*.

FIGURE S7. Sequence divergent heterogeneity of the concatenated matrix of 15 mitochondrial genes assessed by AliGROOVE. Heterogeneity scores range from -1 (red), indicating full random accordancy, to +1 (blue), indicating non-random accordancy between pairwise comparisons.

FIGURE S8. Phylogenetic informativeness profiles of 13 protein coding genes and 2 ribosomal RNA mitochondrial genes used to build the phylogenetic tree of Pentatomidae (see Fig. 3).