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Complete Mitochondrial Genome of *Eurostus validus* (Hemiptera: Tessaratomidae): Genomic Features and Comparative Analysis

Dongkai LIU[1] Liyingzi HE[2] Chao XUE[3] Yingying GAO[4] Ran LI[5]*

School of Life Sciences, Qufu Normal University, Qufu 273165, CHINA

e-mails: [1]liu224@qfnu.edu.cn, [2]Heli0123haha@163.com, [3]xuebhc1001@163.com, [4]flyying-01@163.com, [5]li471329014@163.com

ORCID IDs: [1]0009-0003-0683-9472, [2]0009-0007-7852-1577, [3]0009-0007-0271-1411, [4]0009-0001-6492-6545, [5]0000-0001-8567-3527

*Corresponding author

ABSTRACT

The genus *Eurostus* is distributed across East and Southeast Asia, inhabiting forest and agricultural ecosystems and participating in plant-insect interactions. However, the absence of a complete mitochondrial genome has limited understanding of its evolution and phylogeny. Here, we report the first complete mitogenome of *Eurostus validus*. The circular genome is 15,948 bp and contains the typical 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNAs, two ribosomal RNAs, and a control region. Comparative analyses with other tessaratomid mitogenomes revealed strong AT bias, consistent strand asymmetry, and conserved patterns of amino acid and codon usage. Among Pentatomoidea, atp8 showed the highest nucleotide diversity, whereas cox1 was the most conserved. ENC-plot and neutrality analyses indicated that both mutation pressure and natural selection shape codon usage bias, with natural selection playing a dominant role. Phylogenetic analyses based on the PCG12RNA dataset using Bayesian inference and maximum likelihood strongly supported family monophyly. The topology recovered Dinidoridae and Tessaratomidae as sister groups, with well-resolved relationships within Tessaratomidae. These results provide a valuable genomic resource and improve understanding of Pentatomoidea evolution.

Keywords: Comparative analysis, *Eurostus validus*, Mitochondrial genome, Pentatomoidea, Tessaratomidae.

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INTRODUCTION

The mitochondrial genome is a compact circular double-stranded DNA molecule, typically ranging from 14 to 20 kilobases (kb) in insects, and encodes 37 conserved genes including 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), and two ribosomal RNA genes (rRNAs), along with a non-coding control region that regulates replication and transcription (Wolstenholme, 1992; Simon & Hadrys, 2013). Owing to its small genome size, maternal inheritance, low recombination frequency, high mutation rate, and relatively conserved gene content, the insect mitogenome has become a powerful molecular marker in studies of species identification, population genetics, evolutionary biology, and phylogenetic reconstruction (Luo, Liang, Jin, & Yi, 2025; Xue, Zhang, Li, Li, & Yi, 2026; Duangphakdee, Poolprasert, & Rattanawannee, 2025; Chen et al., 2025). Compared with analyses based on individual mitochondrial genes, complete mitogenomes offer greater resolution and stronger statistical support for deciphering evolutionary relationships across taxa (Gong, Yang, & Chen, 2021).

The rapid development of next-generation sequencing (NGS) technology has dramatically accelerated the acquisition of mitogenomic data across diverse insect orders. These data have provided novel insights into gene rearrangements, nucleotide composition, codon usage bias, and adaptive evolution, thereby improving our understanding of insect phylogeny and evolutionary diversification (Naro et al., 2025; Yao et al., 2024; Yang, Xiao, Zhang, Shang, & Guo, 2024; Wang, Sun, Hu, Zhang, & Li, 2024a). Recent mitogenomic studies on Lepidoptera, Coleoptera, and Hemiptera have revealed lineage-specific patterns of gene organization and compositional bias, highlighting both conserved and derived features among insect lineages (Xue et al., 2024; Wang et al., 2024b; Tian, Yang, Si, Guo, & Zhang, 2022). The increasing number of complete mitogenomes available in public databases has thus provided an essential foundation for comparative genomic and evolutionary analyses within and between insect families.

The order Hemiptera, one of the largest and most diverse groups within Insecta, comprises more than 100,000 described species (Li, Zhang, Zhao, Chen, & Tian, 2024). Members of the order Hemiptera exhibit remarkable ecological diversity, including phytophagous, predatory, and hematophagous lifestyles, with many species such as aphids and planthoppers being major agricultural pests that damage crops and transmit plant pathogens (Xu et al., 2021). Within Hemiptera, the superfamily Pentatomoidea (true stink bugs) represents an ecologically and economically important lineage, but available mitogenomic data remain limited (Jia, Wei, Niu, Zhang, & Zhao, 2023; Yan & Chen, 2023). Among these families, Tessaratomidae is particularly underrepresented. Enhancing taxon sampling within this family is critical for reconstructing deeper-level phylogenetic relationships. The genus *Eurostus*, a member of Tessaratomidae, includes species distributed mainly in East and Southeast Asia (Ho & Chen, 2010). Among them, *Eurostus validus* is a distinctive species inhabiting forest and agricultural ecosystems, where it plays a role in plant-insect interactions. However, molecular studies on this genus are scarce, and its phylogenetic placement within Tessaratomidae has not been well resolved (Xu et al., 2021; Jia et al., 2023;

Yan & Chen, 2023). Comparative mitogenomic analyses among related genera can provide valuable insights into the evolutionary history, adaptive diversification, and taxonomic classification of *Eurostus* and its relatives.

In this study, we first determined and annotated the complete mitogenome of *E. validus* using NGS technology. We analyzed its gene content, organization, nucleotide composition, codon usage bias, and structural features, and compared them with those of other species in Tessaratomidae. In addition, the evolutionary rate and selection pressure of mitochondrial protein-coding genes were evaluated to explore their molecular evolutionary patterns within Pentatomoidea. Furthermore, phylogenetic relationships were reconstructed based on 13 PCGs and two rRNAs using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Our findings enrich the available mitogenomic data for Tessaratomidae, contribute to the refinement of its phylogenetic relationships, and provide a valuable genetic resource for future studies on the molecular evolution and systematics of true bugs.

MATERIALS AND METHODS

Sample collection and DNA extraction

Specimens of *E. validus* were collected from Jiuxian Mountain, Qufu, Shandong Province, China (35°46′28.40″N, 117°2′4.89″E). Immediately after collection, specimens were preserved in 100% ethanol and stored at -20 °C until DNA extraction. Species identification was conducted based on morphological characteristics according to existing taxonomic keys and descriptions. Voucher specimens were deposited at the School of Life Sciences, Qufu Normal University, Shandong, China. Total genomic DNA was extracted from approximately 50 mg of thoracic and leg muscle tissue using the DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's instructions. DNA integrity was assessed by 1% agarose gel electrophoresis, and DNA purity was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was stored at -20 °C for subsequent analyses.

Mitochondrial genome sequencing, assembly, and annotation

High-quality DNA samples were sent to Biozeron Biotechnology Co., Ltd. (Shanghai, China) for library preparation and sequencing. Libraries with an average insert size of 400 bp were constructed using the Illumina TruSeq® DNA PCR-Free HT Kit and sequenced on the Illumina NovaSeq 6000 platform (Illumina, USA) with a paired-end strategy (2 × 150 bp). Each library generated more than 4 Gb of raw reads. Raw sequencing reads were pre-processed with fastp v0.23.2 (Chen, Zhou, Chen & Gu, 2018) to remove adapter and polyG sequences, and their quality was subsequently assessed using FastQC v0.11.9 (Andrews, 2010). The remaining clean reads were retained for downstream assembly. *De novo* assembly of the mitochondrial genome was carried out using NOVOPlasty v4.0 with a k-mer size of 33 (Dierckxsens, Mardulyn & Smits, 2017). The cox2 gene fragment of *E. validus* was used as the initial seed, and the complete mitogenome of *Eusthenes cupreus* (GenBank accession: JQ910983)

was used as the reference to guide assembly. The assembled mitogenome was first annotated with the MITOS web server (invertebrate mitochondrial code) (Bernt et al., 2013). PCGs were manually verified and corrected by comparison with reference mitogenomes from Tessaratomidae to confirm start and stop codons. tRNAs and rRNAs were identified by alignment with homologous sequences and predicted using tRNAscan-SE v1.21 and ARWEN v1.2 (Chan & Lowe, 2019; Laslett & Canbäck, 2008). Intergenic and overlapping regions were manually inspected and corrected when necessary. A circular map of the mitogenome was generated using the MitoZ visualization module (Meng, Li, Yang & Liu, 2019).

Bioinformatic analysis

For comparative analyses, mitogenome sequences of five additional Tessaratomidae species were downloaded from GenBank. Nucleotide composition, AT- and GC-skews, codon usage of PCGs, and relative synonymous codon usage (RSCU) were calculated using MEGA X and PhyloSuite v1.2.1 (Kumar, Stecher, Li, Knyaz & Tamura, 2018; Zhang et al., 2020). Strand asymmetry was measured with the formulas: AT-skew = (A - T) / (A + T) and GC-skew = (G - C) / (G + C) (Perna & Kocher, 1995). To ensure reliable comparative and evolutionary inferences, and given the currently limited availability of mitogenomic data in Tessaratomidae, subsequent analyses in this study were performed using 74 Pentatomoidea species. Nucleotide diversity (Pi) was calculated using DnaSP v5.0 with a sliding window of 100 bp and a step size of 25 bp (Librado & Rozas, 2009). Synonymous (Ks) and non-synonymous (Ka) substitution rates, as well as Ka/Ks ratios for each PCG, were also estimated with DnaSP5. Codon usage bias was assessed by calculating the effective number of codons (ENC) with CodonW v1.4.4 (Peden, 1999). Neutrality plot analysis was performed to evaluate the effects of mutation and selection (Sueoka, 1988). ENC-plot analysis was conducted in OriginPro 2025b, and the expected ENC values were calculated as follows: ENC = 2 + (GC3 + 29 / ($GC3^2$ + $(1-GC3)^2$)) (Novembre, 2002).

Phylogenetic analysis

Phylogenetic analyses were reconstructed using mitogenome sequences of 74 Pentatomoidea species, with *Aneurus similis* (Aradoidea), *Aradus compar* (Aradoidea), *Geocoris pallidipennis* (Lygaeoidea), *Idioscopus nitidulus* (Membracoidea) and *Nephotettix cincticeps* (Membracoidea) serving as outgroups (Table S1). The concatenated nucleotide sequences of 13 PCGs and two rRNAs were used for the analyses. PCGs were aligned using codon-based multiple sequence alignment in MAFFT v7.205 (L-INS-i algorithm) (Katoh & Standley, 2013), while rRNAs were aligned using MAFFT with the G-INS-i algorithm. Nucleotide saturation was assessed in DAMBE v7 (Xia, 2018). Ambiguously aligned regions were identified and excluded with Gblocks v0.91b (Castresana, 2000). The alignments were concatenated with PhyloSuite v1.2.1 (Zhang et al., 2020). The best-fit partitioning scheme and substitution models were selected with PartitionFinder v2 (Lanfear, Calcott, Ho, & Guindon, 2012). A Maximum Likelihood (ML) phylogenetic tree was conducted in RAxML v8.2.0 with 1000 bootstrap replicates (Stamatakis, 2014). Then, the Bayesian Inference (BI) phylogenetic tree

was performed in MrBayes v3.2.2 with two independent Markov chain Monte Carlo (MCMC) runs of 10 million generations, sampling every 1000 generations, and the initial 25% of samples were discarded as burn-in (Ronquist & Huelsenbeck, 2003). The resulting phylogenetic trees were visualized in FigTree v1.4.4 (Rambaut, 2018).

RESULTS AND DISCUSSION

General features of the mitochondrial genome

The genome of *E. validus* is 15,948 bp in length (GenBank accession: PX503950), which falls within the size range reported for other members of Tessaratomidae, ranging from 15,350 bp in *Dalcantha dilatata* to 17,198 bp in *Pycanum ochraceum*. This variation is mainly attributable to differences in the length of the control region (Fig. S1). It contains the typical 37 genes annotated in metazoan mitogenomes, including 13 PCGs, 22 tRNAs, and two rRNAs, as well as a non-coding control region (Fig. 1, Table 1). Among these, 23 genes (nine PCGs and 14 tRNAs) are situated on the heavy strand (H-strand), while the remaining genes (four PCGs, two rRNAs, and eight tRNAs) are positioned on the light strand (L-strand). The gene composition and arrangement are identical to those of the ancestral insect *Drosophila yakuba* (Clary & Wolstenholme, 1985). Several gene overlaps and intergenic spacers were found in the mitochondrial genome, with lengths ranging from -18 bp to 17 bp (Fig. 1, Table 1). The longest overlap occurs between trnK and trnD, while the largest intergenic spacer is located between cytb and trnS2. Notably, the overlaps between trnW and trnC as well as between nad6 and cytb are highly conserved, sharing identical 8 bp nucleotide fragments (5′-AAGCTTTA-3′ and 5′-ATGAATAA-3′) with other tessaratomid species (Wang et al., 2021).

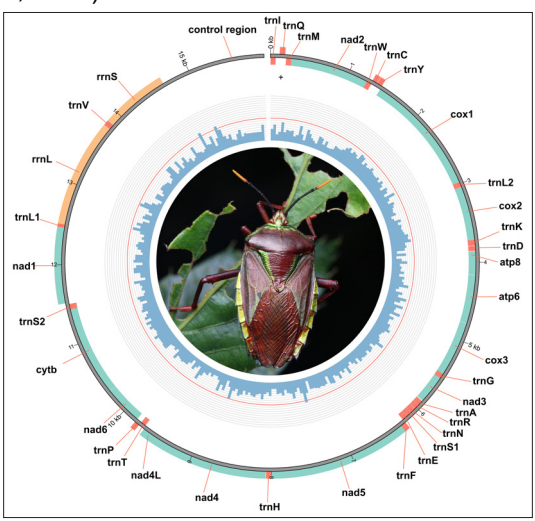

Figure 1. Complete mitogenome map of *E. validus*. Genes are transcribed anticlockwise outside and clockwise inside. The blue histogram on the inside indicates the GC content.

Table 1. Annotation and gene organization in the mitogenome of *Eurostus validus*.

Gene	Strand	Nucleotide no.	Size (bp)	IN	Anticodon	Start codon	Stop codon
trnI	J	1-64	64	0	GAT		
trnQ	N	112-181	70	47	TTG		
trnM	J	195-262	68	13	CAT		
ND2	J	263-1246	984	0		ATA	TAA
trnW	J	1,245-1,308	64	-2	TCA		
trnC	N	1,301-1,366	66	-8	GCA		
trnY	N	1,369-1,433	65	2	GTA		
COX1	J	1,438-2,973	1536	4		TTG	TAT
trnL2	J	2,973-3,039	67	-1	TAA		
COX2	J	3,040-3,736	679	0		ATA	T
trnK	J	3,719-3,794	76	-18	CTT		
trnD	J	3,796-3,858	63	1	GTC		
ATP8	J	3,863-4,021	159	4		ATG	TAA
ATP6	J	4,015-4,695	681	-7		ATG	TAG
COX3	J	4,682-5,470	789	-14		ATG	TAT
trnG	J	5,469-5,533	65	-2	TCC		
ND3	J	5,534-5,887	354	0		ATC	TAA
trnA	J	5,896-5,960	65	8	TGC		
trnR	J	5,960-6,023	64	-1	TCG		
trnN	J	6,024-6,093	70	0	GTT		
trnS1	J	6,094-6,164	71	0	GCT		
trnE	J	6,165-6,227	63	0	TTC		
trnF	N	6,226-6,292	67	-2	GAA		
ND5	N	6,292-8,004	1,713	-1		ATA	TAA
trnH	N	8,006-8,068	63	1	GTG		
ND4	N	8,072-9,397	1,326	3		ATG	TAA
ND4L	N	9,391-9,681	291	-7		ATT	TAA
trnT	J	9,684-9,748	65	2	TGT		
trnP	N	9,749-9,812	64	0	TGG		
ND6	J	9,814-10,299	480	1		TTG	TAA
CYTB	J	10,301-11,437	1,137	1		ATG	TAG
trnS2	J	11,436-11,504	69	-2	TGA		
ND1	N	11,522-12,451	930	17		ATA	TAA
trnL1	N	12,449-12,514	66	-3	TAG		
rrnL	N	12,515-13,790	1,276	0			
trnV	N	13,791-13,857	67	0	TAC		
rrnS	N	13,858-14,654	797	0			
CR	J	14,655-15,948	1,294	0			

The nucleotide composition of the *E. validus* mitogenome exhibits a strong bias toward A and T, a feature typical of other Pentatomoidea species (Wang, Yang, Zhu, Zheng, & Bu, 2023). Its overall A + T content is 73.13%. The A + T content of the PCGs is 72.3%, with a higher value in the third codon positions (PCG3, 83.22%) than in the first and second codon positions (PCG12, 66.84%). The A + T contents of the rRNA and tRNA are 76.17% and 74.66%, respectively, both higher than that of the whole mitogenome. The predominance of AT bases is primarily driven by asymmetric mutational pressure during replication and transcription, which tends to convert GC pairs into AT pairs through processes such

as cytosine deamination (Lu, Huang, & Deng, 2023). Differences in energetic costs of nucleotide biosynthesis may also contribute to the relatively lower abundance of GC within cells (Hamilton et al., 2017). Compositional asymmetry showed that AT-skew values are positive for tRNAs but negative for other regions, whereas GC-skew values are positive in tRNAs and negative in the remaining regions. The biased base composition of mitogenomes is thought to result from strand-specific asymmetry in replication and transcription and selection pressures associated with these processes (Hassanin, Léger, & Deutsch, 2005).

To further explore the compositional characteristics of *E. validus* in an evolutionary context, we compared its nucleotide features with those of other tessaratomid species (Fig. 2A, Table S2). Across them, a pronounced A+T bias was uniformly detected, with the overall genome A+T content ranging from 72.7% (*M. splendidus*) to 75.7% (*D. dilatata*). Among genomic regions, PCGs (71.8-75.0%) exhibited lower A+T contents compared with rRNAs (75.5-78.9%) and tRNAs (74.66-78.0%). Consistent with the canonical compositional pattern of insect mitogenomes, the PCG3 (81.4-87.7%) displayed substantially higher A+T contents than the PCG12 (66.84-68.7%) (Fig. 2A). Notably, unlike the typically elevated AT content in the control region (CR) observed in many insect groups, the CR AT content of these species remains at a relatively moderate level (72.05-74.67%). Consistent positive and negative skew patterns were observed across most genomic regions, except for the CR (Fig. 2B, Table S3). The distinct skew values in the CR may be attributed to its highly variable length among species. AT-skews were generally positive for the whole mitogenome and tRNAs but negative for PCGs and rRNAs, whereas GC-skews were predominantly positive except in the whole mitogenome. Collectively, these conserved compositional patterns across tessaratomid species suggest that nucleotide bias and strand asymmetry are evolutionarily stable features of their mitogenomes, potentially shaping codon usage and substitution patterns over evolutionary time (Zhang et al., 2024a).

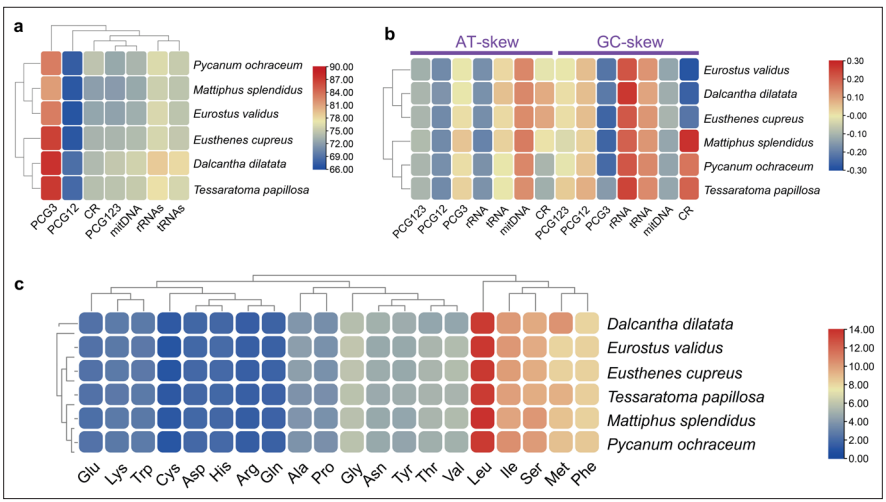

Figure 2. a) Nucleotide composition of different datasets of the six Tessaratomidae mitogenomes. b) AT- and GC-skew across various datasets of the six Tessaratomidae mitogenomes. c) Amino acid composition of the six Tessaratomidae mitogenomes.

Ribosomal RNAs, transfer RNAs and control region

The genome contains two ribosomal RNA genes and 22 transfer RNA genes (Fig. 1, Table 1). The large rRNA (rrnL, 1,276 bp) is situated between trnL1 and trnV, while the small rRNA (rrnS, 797 bp) occurs between trnV and the control region; both rRNA genes are encoded on the L-strand. Due to its high conservation, rRNA is a crucial molecular marker for classification and phylogenetic analysis in insect mitochondrial genomes (Ji, Jia, & Bai, 2024). The control region is located between the rrnS and trnI genes. All 22 tRNAs, ranging from 64 to 71 bp in length, were identified (Table 1). Fourteen are encoded on the H-strand and eight on the L-strand, consistent with other heteropteran mitogenomes. Most exhibit the typical cloverleaf structure, except trnS1, which lacks the DHU arm and forms a simple loop (Fig. 3). The absence of the DHU arm in trnS1 is a common feature in insect mitochondrial genomes and is observed in many other metazoans (Tian, Yang, Si, Guo, & Zhang, 2022). In addition to canonical Watson-Crick base pairing, 31 mismatched base pairs were detected, including 23 G-U, 3 C-U, 2 A-C, 2 U-U, and 1 G-A (Fig. 3). G-U mismatches were the most abundant. Although G-U pairs are capable of forming hydrogen bonds, they are weaker than A-U pairs due to their geometric configuration (Rousset, Pélandakis, & Solignac, 1991; Golden, Murrell, Martin, Pybus, & Hein, 2020). Nevertheless, they are commonly tolerated in RNA secondary structures and play important roles in various biological processes (Varani & McClain, 2000). The remaining mismatches occurred at much lower frequencies and are generally considered to have minimal impact on tRNA function (Xue et al., 2024).

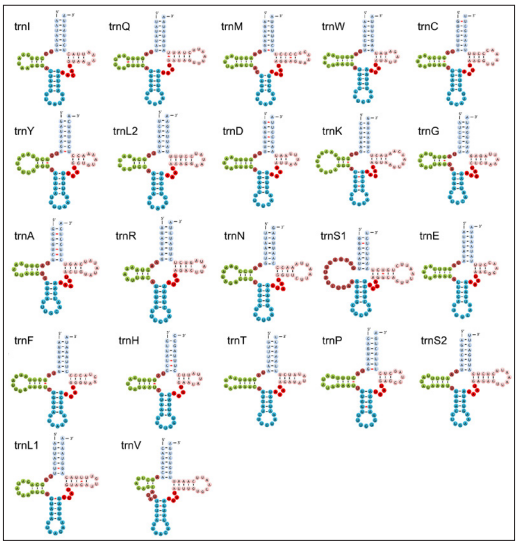

Figure 3. Secondary structures of the 22 tRNAs in the mitogenome of *E. validus*. Light blue: amino acid acceptor arm; Pink: TψC arm; Red: variable loop; Dark blue: anticodon arm; Green: dihydrouridine arm.

Protein-coding genes

The 13 PCGs span 11,065 bp, accounting for approximately 71.7% of the entire genome. Nine PCGs (nad2, cox1, cox2, atp8, atp6, cox3, nad3, nad6, and cytb) are encoded on the

H strand, while the remaining four genes (nad5, nad4, nad4L, and nad1) are located on the L strand (Fig. 1, Table 1). Among the PCGs, atp8 is the shortest gene (159 bp), and nad5 is the longest (1,713 bp). Most PCGs start with conventional ATN initiation codons: five ATG, four ATA, one ATC and one ATT, except for cox1 and nad6, which initiate with the non-standard start codon TTG, a pattern frequently observed in other hemipteran mitogenomes (Xu et al., 2021; Yan et al., 2023; Wang et al., 2023; Ji et al., 2024). All PCGs terminate with complete stop codons (eight TAA, two TAG, and two TAT), except cox2 (T). Its incomplete stop codon is presumed to be completed via post-transcriptional polyadenylation (Li, Yan, Song, & Yang, 2025). Such variation in initiation and termination codons is commonly reported in insect mitogenomes (Despabiladeras & Bautista, 2024).

All Tessaratomidae species exhibited similar amino acid usage and RSCU patterns. Their amino acid composition showed that Leu, Ile, Phe, and Met were the most abundant in PCGs, whereas Cys and Arg were the least represented (Fig. 2C, Table S4). Among the 61 sense codons, *E. validus* exhibited the highest frequencies of UUA (Leu), UCU (Ser), and AUU (Ile), while CGC (Arg) and GCG (Ala) were least preferred; this pattern was consistent across all six mitogenomes (Fig. 4, Tables S5-S10). This conserved codon usage pattern further supports their close phylogenetic relationships (Wang et al., 2021). Notably, codons ending with adenine (A) or uracil (U) were strongly preferred, whereas those ending with cytosine (C) or guanine (G) were largely avoided. This pronounced A/U-ending bias closely corresponds to the overall high A+T content of the mitochondrial genome (Aksın & Taylan, 2026).

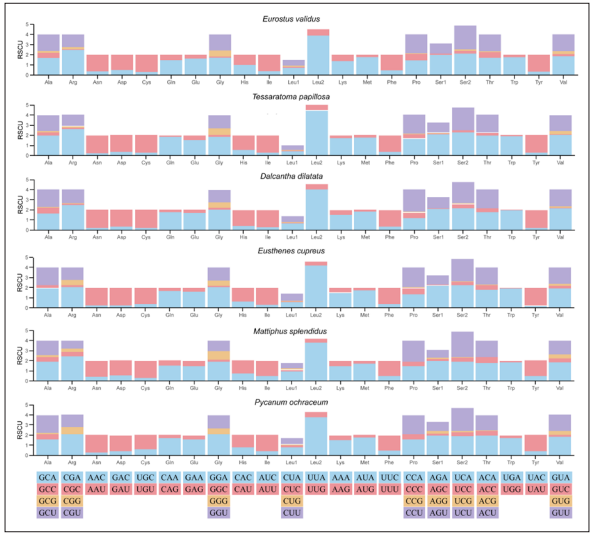

Figure 4. Relative synonymous codon usage (RSCU) of the six Tessaratomidae mitogenomes.

Evolutionary rate and selection pressure

In this study, the Pi values of 13 PCGs of the Pentatomoidea species were calculated (Fig. 5A). Among these genes, atp8 shows the highest Pi value, indicating the greatest

sequence variability, whereas cox1 displays the lowest Pi value, consistent with its well-recognized evolutionary conservation across insects (Fig. 5A, Table S11) (Shah et al., 2024). To further assess selective constraints, we examined the Ka and Ks substitution rates, which serve as indicators of selective pressure (Zhang et al., 2024b). The resulting Ka/Ks ratios ranged from 0.07 for cox1 to 0.73 for atp8, demonstrating that purifying selection acts on all PCGs (Ka/Ks < 1) (Fig. 5B, Table S11). Notably, atp8 exhibits the highest Ka/Ks ratio, suggesting relatively relaxed purifying selection, which may be attributed to its extremely short sequence, while cox1 shows the lowest ratio, reflecting strong functional constraints and high evolutionary conservation within Pentatomoidea.

The results of the ENC-plot and neutrality plot analyses provided insights into the factors shaping codon usage bias. In the neutrality plot (Fig. 5C, Table S12), the GC12 values ranged from 0.271 to 0.581, while the GC3 values ranged from 0.198 to 0.587. A significant positive correlation was observed between GC12 and GC3; however, the regression slope (0.3107) was far below 1, indicating that natural selection, in addition to mutation pressure, substantially contributes to codon usage variation (Lu et al., 2023). In the ENC-plot (Fig. 5D, Table S12), all the mitochondrial genes from the eight analyzed families were distributed below the expected standard curve, with ENC values ranging from 35.81 to 50.58. Such a distribution implies that codon usage is not solely determined by mutation pressure but is also strongly influenced by natural selection, especially translational selection (Sueoka, 1988). The relatively low ENC values further indicate a moderate to strong codon preference among the PCGs (Wright, 1990). Overall, the combined results from both analyses demonstrate that both mutation pressure and natural selection jointly shape the codon usage bias of the mitochondrial genome, with natural selection playing a more dominant role in determining codon preference.

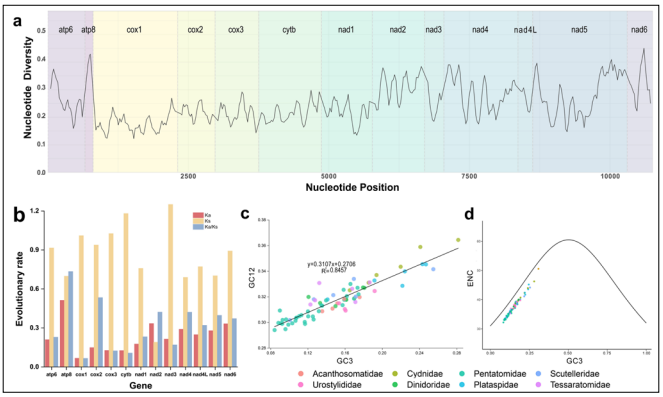

Figure 5. Sliding window analysis, selection pressure assessment, and codon preference analysis of the 13 PCGs across the 74 Pentatomoidea mitogenomes. a) Nucleotide diversity (Pi) calculated using a sliding window approach across PCGs, with the black line indicating variability among different genomic regions. b) The ratio of non-synonymous (Ka) to synonymous (Ks) substitution rates (Ka/Ks) for the PCGs, illustrating differences in evolutionary rates and selective pressures among genes. c) The GC content trend line and R value of codon 1 and 2 (GC12) and codon 3 (GC3) of the PCGs. (D) The scatter plot of the correlation between ENC value and GC3, where the black curve represents the expected functional relationship between ENC and GC3 under mutation pressure alone (without selection pressure).

Phylogenetic analysis

Substitution saturation analyses indicated that transitions and transversions across most PCGs in Pentatomoidea had not reached saturation. Nevertheless, at the third codon positions, the transversion curve showed a weak saturation signal, suggesting that substitutional noise may partially obscure deep phylogenetic signal at this site (Fig. S2; Table S13). To minimize the potential impact of saturation on phylogenetic inference, subsequent analyses were therefore conducted using the PCG12RNA dataset, which excludes third codon positions of PCGs and combines first and second codon positions with ribosomal RNA genes. This strategy is widely adopted in mitogenomic phylogenetics and is considered effective for resolving deep-level relationships within Heteroptera.

Both Bayesian Inference (BI) and Maximum Likelihood (ML) analyses yielded highly congruent topologies, with strong nodal support across most internal branches (Fig. 6). All sampled families within Pentatomoidea were robustly recovered as monophyletic, providing strong mitogenomic support for their current taxonomic delimitations. At the family level, the recovered topology can be summarized as: ((((Dinidoridae + Tessaratomidae) + (Cydnidae + Scutelleridae)) + (Pentatomidae + Plataspidae)) + (Acanthosomatidae + Urostylididae)). This topology is supported by high posterior probabilities and bootstrap values at nearly all backbone nodes, indicating a stable and well-resolved phylogenetic framework for Pentatomoidea based on mitochondrial genomic data.

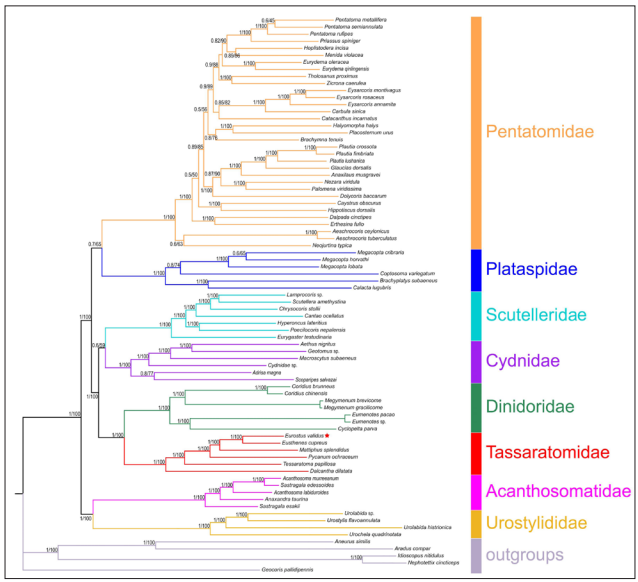

Figure 6. Phylogenetic trees obtained from ML and BI analysis. Numbers on nodes are the bootstrap value (BV) and posterior probability (PP).

In our analyses, Cydnidae was recovered as the sister group to Scutelleridae, and this combined clade formed the sister lineage to (Dinidoridae + Tessaratomidae). This relationship is consistent with several recent mitogenomic studies (Wang et

al., 2023; Liu et al., 2019), reinforcing the hypothesis of a close evolutionary affinity between burrower bugs (Cydnidae) and jewel bugs (Scutelleridae). However, the phylogenetic position of Cydnidae has been shown to be sensitive to analytical framework. For example, analyses employing site-heterogeneous mixture models have recovered Cydnidae as the earliest-diverging lineage within Pentatomoidea, whereas site-homogeneous models and expanded taxon sampling often support a more derived placement, as observed here (Xu et al., 2021). These discrepancies likely reflect differences in model assumptions, dataset composition, and taxon coverage, underscoring the importance of model selection and comprehensive sampling in deep-level pentatomoid phylogenetics.

The sister-group relationship between Pentatomidae and Plataspidae recovered in this study is congruent with several mitogenomic analyses (Yan & Chen, 2023; Liu et al., 2019). Nevertheless, alternative topologies have been proposed, notably grouping Plataspidae with Scutelleridae (Wang et al., 2023; Xu et al., 2021). The persistence of these competing hypotheses suggests that the evolutionary history of these lineages may involve rapid radiation or incomplete lineage sorting, which can complicate phylogenetic reconstruction using mitochondrial data alone. At the base of the pentatomoid tree, Acanthosomatidae and Urostylididae formed a strongly supported sister group, representing the earliest-diverging lineage in our analyses. This result differs from studies that recovered Urostylididae alone as the basal lineage (Wang et al., 2023; Yan & Chen, 2023), but is consistent with analyses incorporating site-heterogeneous models and broader taxon sampling (Xu et al., 2021; Li et al., 2017; Liu et al., 2019). Our findings therefore lend additional support to the hypothesis of a close evolutionary relationship between Acanthosomatidae and Urostylididae.

Within Tessaratomidae, all sampled taxa were resolved with high support. *E. validus* and *E. cupreus* were consistently recovered as sister taxa, forming a well-supported clade. The internal relationships among tessaratomid species can be summarized as: (*Dalcantha dilatata* + (*Tessaratoma papillosa* + (*Pycanum ochraceum* + (*Mattiphus splendidus* + (*Eurostus validus* + *Eusthenes cupreus*))))). This topology differs from previous hypotheses that placed *E. cupreus* as the sister taxon to *M. splendidus* (Xu et al., 2021; Yan & Chen, 2023; Wang et al., 2023). The revised placement recovered here is supported by both BI and ML analyses and likely reflects the increased phylogenetic signal provided by expanded taxon sampling and complete mitogenomic data.

CONCLUSION

This study reports the first complete mitochondrial genome of *E. validus* (Tessaratomidae), thereby filling a key taxonomic gap in pentatomoid mitogenomic data. Comparative analyses revealed that the mitogenome exhibits the characteristic AT bias and strand asymmetry typical of Tessaratomidae, while evolutionary rate assessment across Pentatomoidea identified atp8 as the most variable and cox1 as the most conserved protein-coding gene. Phylogenetic reconstruction strongly supported the sistergroup relationship between Dinidoridae and Tessaratomidae, and within Tessaratomidae,

recovered *E. validus* and *E. cupreus* as closely related sister taxa. These results not only provide a foundational genomic resource for Tessaratomidae but also offer clearer phylogenetic insights into the relationships within Pentatomoidea. The data and analyses presented here will support future studies on molecular evolution, taxonomic revision, and comparative genomics in this economically and ecologically important group of true bugs.

Ethic statement

This study involved an insect species (*Eurostus validus*). In accordance with relevant institutional and national regulations, ethical approval was not required for studies involving insects. All procedures were conducted following accepted scientific and ethical standards, and no protected species were used.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The new mitogenome for *E. validus* has been deposited in GenBank with the accession number: PX503950.

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