

Research Notes

The Complete Mitochondrial Genome of the Parasitic Leech *Pterobdella arugamensis* (Annelida: Piscicolidae) from Malaysia and Phylogenetic Insights within Subclass Hirudinea

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Key words: Mitochondrial genome, marine leech, *Pterobdella arugamensis*, Piscicolidae

Article History

Accepted: 22 September 2026

First version online: 29 September 2026

Cite This Article:

Ghazali, S.Z., Madiran, N.A., Zaidi, N.A., Ismail, N., Mohamad Zamri, S.N.N., Han, M.G. & Kamarudin, A.S. 2026. The complete mitochondrial genome of the parasitic leech *Pterobdella arugamensis* (Annelida: Piscicolidae) from Malaysia and phylogenetic insights within subclass Hirudinea. Malaysian Applied Biology, 55(3): 191-195. <https://doi.org/10.55230/mabjournal.v55i3.3577>

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Pterobdella arugamensis (De Silva 1963), formerly known as *Zeylanicobdella arugamensis*, is a parasitic marine leech belonging to the family Piscicolidae. It is widely distributed across Indo-Pacific coastal waters, including Brunei Darussalam (Azmey *et al.*, 2020), China (Huang *et al.*, 2021), Indonesia (Murwantoko *et al.*, 2018), Iran (Polgar *et al.*, 2009), Japan (Nagasawa & Uyeno, 2009), Malaysia (Ravi & Yahaya, 2017), Philippines (Cruz-Lacierda *et al.*, 2000) and South Africa (Hayes *et al.*, 2006). In Malaysia, this species is commonly reported infesting commercially important teleost fishes such as groupers (*Epinephelus* spp.), snappers (*Lutjanus* spp.), and Asian seabass (*Lates calcarifer*) in the aquaculture industry (Kua *et al.*, 2010; Ravi & Yahaya, 2017; Wan Norhana *et al.*, 2021). Infestations by *P. arugamensis* can cause significant tissue damage, anemia, and secondary infections, often leading to high fish mortality rates and considerable economic losses in mariculture (Cruz-Lacierda *et al.*, 2000; Erazo-Pagador *et al.*, 2024).

Leeches in the family Piscicolidae are hematophagous ectoparasites that inhabit both freshwater and marine environments (Kearn, 2004; Nakano, 2017). Despite their ecological and economic importance, genomic data for marine piscicolid leeches remain scarce. Notably, only one complete mitochondrial genome of *P. arugamensis*, collected from China (Wang *et al.*, 2018), has been published to date. However, no mitogenomic data are yet available for other populations from Southeast Asia, where the species is also prevalent and poses a major threat to aquaculture. Given that mitochondrial genomes can vary among geographically distinct populations,

comparative mitogenomic studies are essential for understanding potential population-level divergence and local adaptation.

This study aims to (1) characterize and annotate the complete mitochondrial genome of *P. arugamensis* collected from Malaysia, (2) conduct a comparative analysis with the previously published mitogenome from China, focusing on genetic distance and gene arrangement, and (3) perform phylogenetic analyses with robust mitogenomic methodologies to elucidate the evolutionary position of *P. arugamensis* within the subclass Hirudinea.

Specimens of *P. arugamensis* parasitizing hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) were collected from a commercial hybrid grouper hatchery in Pantai Air Tawar, Besut, Terengganu. Samples were preserved in 95% ethanol, and genomic DNA was extracted using the ZymoBIOMICS™ DNA Extraction Kit (Zymo Research Corporation, Irvine, California, USA).

A total of 100 ng of genomic DNA was quantified using the Qubit High Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). The DNA was then sheared to approximately 300 base pairs using the Bioruptor system (Diagenode, Denville, NJ). Library preparation was carried out using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs). In summary, the fragmented DNA was end-repaired to generate blunt ends with 5' phosphates and 3' hydroxyl groups, followed by the addition of a single adenine (A) nucleotide to the 3' ends. Subsequently, Illumina sequencing adapters were ligated to the A-tailed fragments. Indexing PCR was performed to attach unique barcode sequences

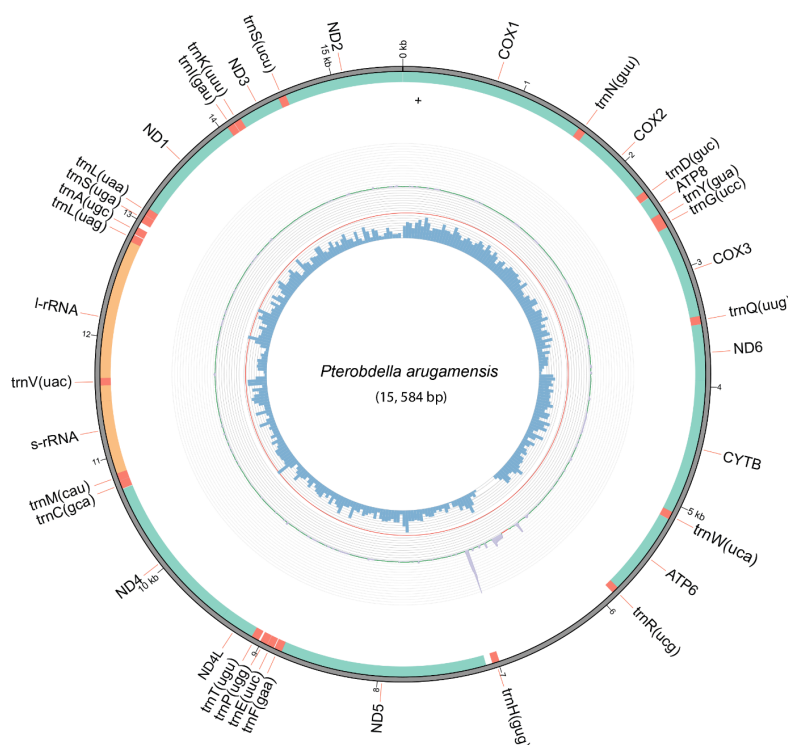


Fig. 1. Mitochondrial genome map of the sequenced specimen. The innermost ring represents GC content, with a 5% GC content increment per ring. The second ring represents read depth distribution with a 5x read depth increment per ring. High or uneven depth typically encountered in the non-coding control region may be due to the repetitive nature of the region leading to non-specific alignment. Uneven depth is also common in genomic regions with extreme AT or GC content and is more commonly encountered in standard library prep compared to PCR-free library prep. The outermost circle shows gene arrangements, with green for PCG fragments, orange for rRNAs, and red for tRNAs. Genes inside the outer circle are transcribed in a clockwise direction; otherwise, in a counterclockwise direction.

and to enrich DNA fragments flanked by adapters on both ends. The resulting library was evaluated for concentration and fragment size distribution using the Fragment Analyzer system (Agilent, Santa Clara, CA). Sequencing was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA) using a 2×150 bp paired-end configuration.

Adapter trimming of the paired-end reads was carried out using fastp v0.21 (<https://github.com/OpenGene/fastp.git>) (Chen *et al.*, 2018). The cleaned reads were then assembled de novo using the MEGAHIT assembler v1.29 (Li *et al.*, 2015) using its default settings. Mitogenome-derived contigs were identified through the MitoZ v3.5 assembly pipeline (<https://github.com/linzhi2013/MitoZ.git>) (Meng *et al.*, 2019). The resulting mitochondrial contig was circularized and reoriented according to the typical starting gene for the respective animal taxon. Gene annotation, including protein-coding genes, transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs), was conducted using the MitoZ v3.5 annotation module (Meng *et al.*, 2019).

Uncorrected pairwise genetic distances among species were calculated using the software MEGA 7 (Kumar *et al.*, 2016). Then, to investigate the phylogenetic relationship among the subclass Hirudinea, a total of 40 additional complete mitochondrial genomes were extracted from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>), including the outgroup, *Lumbriculus variegatus* (Clitellata: Lumbriculida). Multi-gene phylogenetic analysis was conducted using PhyloSuite v1.2.3 (Zhang *et al.*, 2020). The complete mitochondrial gene sequences were retrieved from GenBank and imported into the software in GenBank format. These sequences were first standardized to ensure consistent gene naming and to identify any annotation issues. The extracted sequences were aligned using the MAFFT option integrated into the PhyloSuite

software (Zhang *et al.*, 2020). The alignments were cleaned and concatenated into a single dataset, and a partition file was generated to define the boundaries of each gene. Phylogenetic tree reconstruction was carried out using the Maximum Likelihood method in RAxML-HPC v.8 (Stamatakis, 2014) through the CIPRES Science Gateway (Miller *et al.*, 2010). Finally, the resulting phylogenetic tree was visualized and annotated using the Interactive Tree of Life (iTOL) platform (<https://itol.embl.de/>) (Letunic & Bork, 2007).

The complete mitochondrial genome of *P. arugamensis* was successfully sequenced and deposited in GenBank under the accession number PV340640. The mitogenome is a circular mitochondrial DNA with a total length of 15,584 base pairs (bp), encoding the standard set of 37 genes typically found in metazoan mitochondrial genomes: 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes (Figure 1). Notably, two tRNAs, tRNA-Leu and tRNA-Ser, are duplicated.

Gene arrangement comparisons across 40 leech taxa (Figure 2) reveal both conserved and variable patterns within subclass Hirudinea. The overall order of protein-coding genes (e.g., *cox1-3*, *atp6*, *atp8*, *cytb* & *nad* genes) is largely conserved across families. In contrast, substantial variation is observed in the positioning of ribosomal RNA (rRNA) and transfer RNA (tRNA) genes. Within the family Piscicolidae, *Piscicola geometra* consists of only 36 genes, lacking the *nad2* gene that is otherwise consistently present in other Piscicolidae members, including *P. arugamensis*. Meanwhile, the family Hirudinidae shows the most conserved group and shares identical mitochondrial gene orders.

A comparison between the newly sequenced *P. arugamensis* from Malaysia and the previously published mitogenome from China (Wang *et al.*, 2018) revealed overall high similarity, including a shared high A+T content, a typical hallmark of annelid mitochon-



Fig. 2. Mitochondrial gene arrangements of *Pterobdella arugamensis* and other selected leech species within subclass Hirudinea. Protein-coding genes (PCGs), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) are shown in their respective order and orientation.

drial genomes (Bolbat *et al.*, 2021; Chae *et al.*, 2022). The newly sequenced genome has a slightly lower A+T content (78%), compared to 79.2% in the specimen from China. Both mitogenomes share an identical gene order, and the pairwise genetic distance between them is low (1.2%), consistent with intraspecific variation between geographically separated populations. According to Azmey *et al.* (2022), who conducted a population genetic study on *P. arugamensis* using mitochondrial cytochrome c oxidase subunit I (*cox1*) gene sequences, specimens from Peninsular Malaysia and China belong to the same haplogroup, indicating close genetic relatedness within the species despite geographical separation.

The phylogenetic tree constructed using concatenated mitochondrial protein-coding genes (PCGs) from 40 leech taxa provides robust support for the placement of *P. arugamensis* within the family Piscicolidae (Figure 3). This placement is well-supported by high bootstrap values, indicating strong phylogenetic confidence.

The tree topology reveals three main clades that correspond to the current classification of leeches into the orders Rhynchobdellida, Arhynchobdellida, and Acanthobdellida (Tessler *et al.*, 2018; Phillips *et al.*, 2019). The order Rhynchobdellida is a group characterized by jawless leeches possessing a protrusible proboscis (proboscis-bearing leeches). This group forms a monophyletic group and is supported by relatively moderate bootstrap values (BS<80%). This group comprises the families Piscicolidae, Ozobranchidae, and Glossiphoniidae. The maximum likelihood tree reveals a close evolutionary relationship between the family Piscicolidae and Ozobranchidae, which form a well-supported sister group (BS = 94%) and a distinct lineage separate from Glossiphoniidae. Within Piscicolidae, *P. arugamensis* is closely related to *Piscicola geometra* and *Codonobdella* sp. This tree topology is consistent with previous studies (Tessler *et al.*, 2018; Phillips *et al.*, 2019).

The use of mitochondrial PCGs provides high-resolution phylogenetic signal and new insight into phylogeny, thus allowing for new insight into the evolutionary diversification of parasitic leeches. To further elucidate the dynamics of gene rearrangements and

lineage diversification within the subclass Hirudinea, expanded mitogenomic data from other species across all major orders (Rhynchobdellida, Arhynchobdellida and Acanthobdellida) are needed. This study will enhance phylogenetic resolution and deepen our understanding of the evolutionary history, diversification, and ecological adaptations of parasitism within the subclass Hirudinea.

In aquaculture settings, *P. arugamensis* infestations remain a recurring problem. Heavy infestations by *P. arugamensis* have been associated with skin ulceration, fin erosion, secondary bacterial and fungal infections, anaemia, reduced growth, and, in severe cases, elevated mortality of cultured stock, particularly in high-density hatchery and grow-out systems for hybrid grouper and other high-value species (Cruz-Laciera *et al.*, 2000; Kua *et al.*, 2010; Murwantoko *et al.*, 2018; Azmey *et al.*, 2020). The leech's direct life cycle, without an intermediate host, and its ability to survive detachment from the host for extended periods, allow it to persist and re-infest fish within recirculating and net-cage aquaculture systems (Kua *et al.*, 2010). Several mitigation approaches have been reported to manage *P. arugamensis* infestations. Freshwater bathing and formalin treatment are commonly practised in hatcheries to dislodge attached leeches, although these methods offer only temporary relief and can cause stress to the host fish (Murwantoko *et al.*, 2018). Physical removal, improved water exchange, and stocking density management have also been used in efforts to reduce transmission risk (Azmey *et al.*, 2020). More recently, plant-derived extracts have shown anti-leech activity in vitro, which may offer a potentially safer, more environmentally friendly alternative to chemical treatments (Wan Norhana *et al.*, 2021), while ecological control techniques targeting the leech's cocoon-laying substrate and life-cycle stages have also been explored as a possible way to interrupt reinfestation cycles (Huang *et al.*, 2021). Given the scale of economic losses attributed to piscicolid leech infestations in South-east Asian mariculture (Erazo-Pagador *et al.*, 2024), the genetic resource generated in this study provides a foundation for future applied work. Mitochondrial markers, including the complete mitogenome reported here, can support the development of rapid,

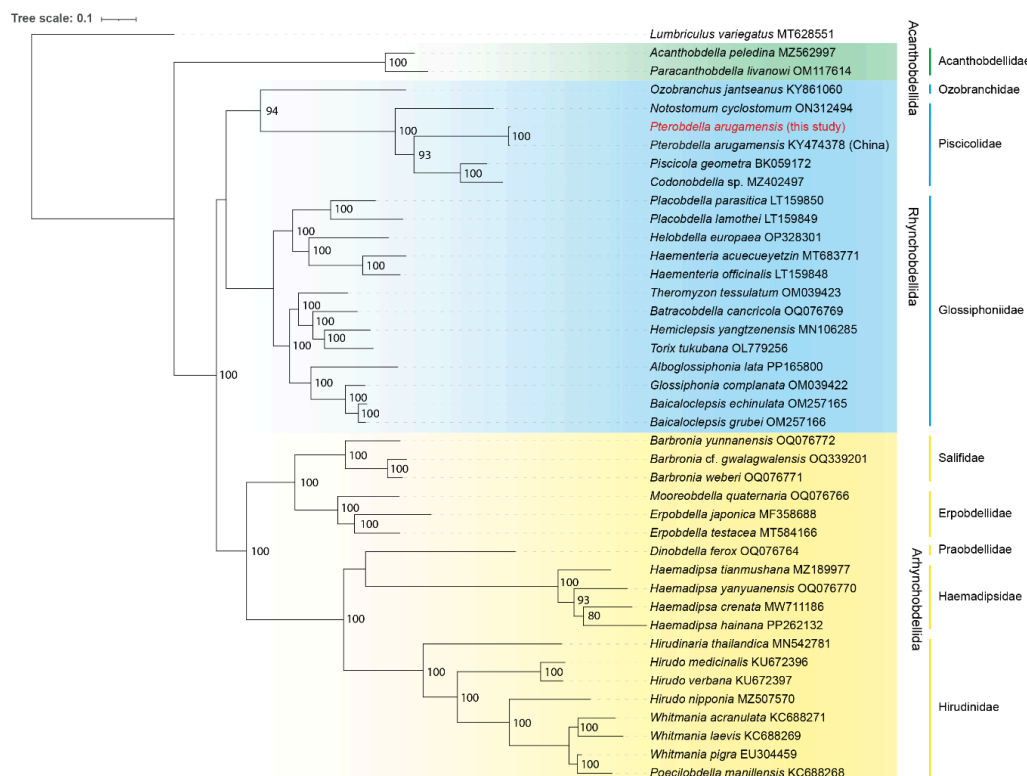


Fig. 3. Phylogenetic tree based on mitochondrial protein-coding genes (PCGs) from 40 leech mitogenomes, including *Pterobdella arugamensis* (this study), rooted with *Lumbriculus variegatus*. Bootstrap values $\geq 80\%$ are shown at nodes.

sensitive molecular diagnostic tools (e.g., species-specific PCR or eDNA-based detection) for early identification of *P. arugamensis* in aquaculture facilities before infestations become severe. Such tools would complement existing morphology-based identification, which can be difficult at early leech life stages, and could inform targeted, population-specific control strategies.

This study presents the mitochondrial genome of *Pterobdella arugamensis* from Malaysia, adding to the genomic resources for Piscicolidae. A comparison with a specimen from China showed gene arrangement and low genetic divergence, suggesting some variation between populations. Phylogenetic analysis further supports the placement of *P. arugamensis* within the family Piscicolidae. The genomic and comparative data generated from this study provide a molecular resource for developing accurate diagnostic and surveillance tools to detect *P. arugamensis* in aquaculture settings, supporting early intervention and more effective, targeted mitigation strategies against this economically damaging ectoparasite.

DATA AVAILABILITY

Sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra>) under BioProject ID PRJNA1184845, accession numbers SAMN44677341 to SAMN44677348. The complete mitogenome of *Pterobdella arugamensis* is available in GenBank under accession number PV340640.

ACKNOWLEDGEMENTS

The present study was supported by a scientific grant provided by the Universiti Sultan Zainal Abidin under the Geran Dalam Universiti (UniSZ/2023/DPU2.0/21).

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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