Open Access Library Journal
2025, Volume 12, e13399
ISSN Online: 2333-9721
ISSN Print: 2333-9705

Analysis of the *atp8* Gene and Discovery of a Cryptic Species in Geoplanoidea

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How to cite this paper: Feng, Y.F. and Sun, H.X. (2025) Analysis of the *atp*8 Gene and Discovery of a Cryptic Species in Geoplanoidea. *Open Access Library Journal*, **12**: e13399.
https://doi.org/10.4236/oalib.1113399

Received: April 7, 2025
Accepted: May 23, 2025
Published: May 26, 2025

Abstract

The absence of the *atp8* gene in the mitochondrial genomes of Platyhelminthes and certain other mollusks has garnered significant attention in recent years. Growing evidence suggests that this gene loss may be associated with the high divergence and length variability of *atp8*, making it particularly challenging for automated annotation. Analysis of putative *atp8* genes in the families Dugesiidae and Dendrocoelidae (superfamily Geoplanoidea) revealed substantial differences in gene structure and hydrophobic patterns of amino acid sequences. These variations may result from divergent selective pressures on *atp8*, driven by distinct ecological niches and survival strategies. This superfamily warrants further in-depth investigation. Phylogenetic tree reconstruction of sampled specimens indicated that Dugesia japonica forms a sister clade to the studied population, albeit with distant genetic relatedness, suggesting the latter may represent a cryptic species within Dendrocoelidae. These findings contribute to expanding and refining the genetic database of Dendrocoelidae.

Subject Areas

Genetics

Keywords

Mitochondrial Gene, *atp8*, Gene Analysis, Phylogenetic Relationships, Cryptic Species

1. Introduction

The superfamily Geoplanoidea belongs to the phylum Platyhelminthes, class Turbellaria, and order Tricladida, primarily comprising two major lineages: Dugesiidae and Dendrocoelidae. These organisms are globally distributed, found across most regions worldwide. Studies by Daisuke *et al.* support the hypothesis of *atp8*

presence in flatworm mitochondrial genomes [1], contrasting with the traditional view that *atp8* is absent in triclad mtDNA. Subsequent studies have identified *atp8* in certain triclad species [2] [3].

ATP synthase, the final enzyme complex in the respiratory chain, couples with the electrochemical gradient across the mitochondrial membrane to generate ATP. The *atp8* gene encodes the F0 subunit of *ATP8* synthase [4]. Notably, *atp8* exhibits high divergence in sequence length and structure across species. Its frequent absence in Platyhelminthes and some mollusks may stem from these variable features, complicating annotation. Automated pipelines often fail to detect *atp8*, necessitating manual validation via cross-species sequence comparison [5].

Since 1850, species delineation in free-living freshwater triclads has relied on morphological traits (e.g., copulatory apparatus anatomy), supplemented occasionally by karyological data. However, this approach overlooks asexual populations lacking reproductive structures [6]. Recent discoveries of cryptic species in Brazil's Atlantic Forest, Sardinia, and Mexico highlight this limitation [7]-[9]. In China, despite efforts by Liu Dezeng and Chen Guangwen to address knowledge gaps in freshwater triclad diversity, molecular studies remain scarce [10].

The selected species (Dugesiidae and Dendrocoelidae) represent the two major lineages of Geoplanoidea, encompassing contrasting habitats (aquatic vs. terrestrial) and feeding strategies. These taxa were prioritized due to their unresolved *atp8* annotation status in prior studies and their ecological divergence, which facilitates testing hypotheses linking gene structure to niche specialization. The *atp8* gene was targeted given its debated presence in Tricladida and its functional role in ATP synthase, a key energy metabolism component likely under differential selection across lifestyles.

2. Materials and Methods

2.1. Sample Collection and Morphological Identification

More than 200 Dendrocoelum planarians were collected from Zhejiang Province as experimental samples, ensuring the collected planarians were basically identical in morphology, size, sex, age, and health status. For convenience of description, they were designated as Dugesia sp. Subsequently, the collected samples were observed and photographed using an SMZ-1500 optical stereo microscope (Nikon, Tokyo, Japan) and TSView7 digital camera (Tucsen, Fujian, China), with focus on their anterior morphology, eye spot morphology, ventral cilia, mouth position and body color. After being cultured in a laboratory constant temperature incubator at 20˚C for 7 days, three samples were randomly selected for DNA extraction, and their cox1 gene sequences were amplified. The genetic distance differences among the three samples were then determined by comparing them with sequences in the NCBI database. The Dugesia sp. samples continued to be cultured.

2.2. DNA Extraction, PCR Amplification and Sequencing

Whole Dugesia sp. samples were cut into pieces, and total genomic DNA was iso-

lated using the Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech Company, Shanghai, China). The amplified PCR products were bidirectionally sequenced using the primer walking method by Sango Biotech (Shanghai, China).

2.3. Gene Annotation and Sequence Analysis

Contiguous and overlapping nucleotide fragments were manually proofread, assembled and analyzed using DNASTAR Package v.7.1 (Burland, Totowa, NJ, USA) [11], and tRNA genes were identified through the online MITOS server (http://mitos.bioinf.uni-leipzig.de/index.py) [12]. Subsequently, two mitochondrial genomes (AB618487, AB618488) were downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/) as references. The amino acid sequences of 12 PCGs and 2 rRNAs (12S and 16S rRNA) were identified and aligned using the Clustal W program in MEGA v.7.0 (Sudhir K., Philadelphia, PA, USA) [13]. The circular mt map of newly identified sequences was drawn using the CG View v.1.0 online server (Grant, Alberta, Canada) (http://cgview.ca/). The AT and GC skews were calculated as follows: AT skew = (A−T)/(A + T), GC skew = (G−C)/(G + C) [14]. The Ka/Ks ratios of 13 PCGs were calculated using KaKs Calculator v.2.

Mitochondrial genome sequences were searched in NCBI and data were obtained after screening. Attempts were made to locate the *atp8* gene together with the obtained Dugesia sp. whose taxonomic status remained to be determined. Manual annotation was primarily adopted by translating all unannotated blank regions with reference to the annotation information in ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). All translation and search work was based on the codon table "Echinoderm and Flatworm Mitochondrial Code" [15]. Uncertain annotations were temporarily abandoned. The annotation of Girardia tigrina had been completed by Cheng *et al.* and was labeled as a hypothetical protein. Nucleic acid translation was performed on the ExPASy website (https://web.expasy.org/translate/) [16]. Each translated ORF was tested for possible domains on the SMART website (http://smart.embl-heidelberg.de/) [17] as a verification method. On the ExPASy ProtScale tool (https://web.expasy.org/protscale/), hydrophobicity analysis of possible *atp8* gene amino acids was performed with the window size set to 9 and other parameters as default options. Excel software was used for data visualization analysis. Specific primers were designed based on the *atp8* gene of Dugesia sp. for PCR and RT-qPCR reactions.

2.4. Phylogenetic Analysis

A newly sequenced mitochondrial genome and 17 previously published mitochondrial genomes of Geoplanoidea superfamily, including Dugesiidae and Dendrocoelidae, were subjected to phylogenetic analysis. For outgroup selection, three ancient planarian species (KP208776.1, MW703985.1, NC_050050.1) were down-

loaded from NCBI for phylogenetic analysis. The dataset was divided into two types: PCG123 dataset (first, second and third codon positions of 13 PCGs) and PCG12 dataset (first and second codon positions of 13 PCGs). Based on the nucleotide sequence dataset of 13 PCGs, substitution saturation was detected using DAMBE v.4.2 [18]. Since the third codon position was not saturated, we used the first, second and third codon positions of 13 PCGs (PCG123 dataset) for phylogenetic analysis. The MAFFT v.7 program and Gblocks 0.91b with default settings were used to align the PCG123 dataset and screen conserved regions [19] [20]. Afterwards, the alignment results were imported into PhyloSuite v.1.2.2, with format conversion performed using Geneious v.8.1.6 [21]-[22]. Partition Finder v.2.2.1 was used to select the optimal partitioning scheme and best substitution model for the first, second and third codon positions of the 13 PCGs dataset for Bayesian Inference (BI) and Maximum Likelihood (ML) analyses [23]. Seven partitions were identified in the PCG123 dataset. The GTR + I + G model was used for subsequent phylogenetic analysis. BI analysis was run for 10 million generations in MrBayes v.3.2 program, with average standard deviation of split frequencies below 0.01 considered as convergence [24]. ML analysis was implemented in RAxML v.8.2 software, with rapid bootstrap analysis of 1000 replicates to assess nodal support [25]. To improve the accuracy of phylogenetic analysis results, the first 25% of data were discarded as burn-in. Tracer v.1.7.1 and FigTree v.1.4.0 were used to check convergence and visualize trees [26] [27].

3. Results and Analysis

3.1. Determination of Complete Mitochondrial Genome Sequence

In this study, the complete mitochondrial genome of the sample Dugesia sp. was determined to be 14,990 bp in length. As shown in **Figure 1**, the gene arrangement structure is consistent with that of the typical mitochondrial genome of Dugesia japonica, containing a total of 33 genes, including 13 protein-coding genes, 2 rRNA genes, and 19 tRNA genes, without an AT-rich control region. The base composition was A: 19.03%; T: 53.07%; C: 10.85%; G: 17.05%.

3.2. Structure of *atp8* Gene

As shown in **Figure 1**, the *atp8* genes of all selected species were found at nearly identical positions, located between the nad2 gene and rrnS. The relative positions of all other protein-coding genes and rRNA genes were consistent.

When roughly classified by family, significant differences in the *atp8* gene were observed between Geoplanidae and Dugesiidae. The N-terminal pattern of Bipaliinae (including Parakontikia atrata and Platydemus manokwari) was MVHV, while most species of Bipalium exhibited an N-terminal pattern of MVHS. In contrast, Dugesiidae and Geoplaninae showed higher diversity. All these planarian N-termini were markedly different from the traditional MPQL of metazoans [28].

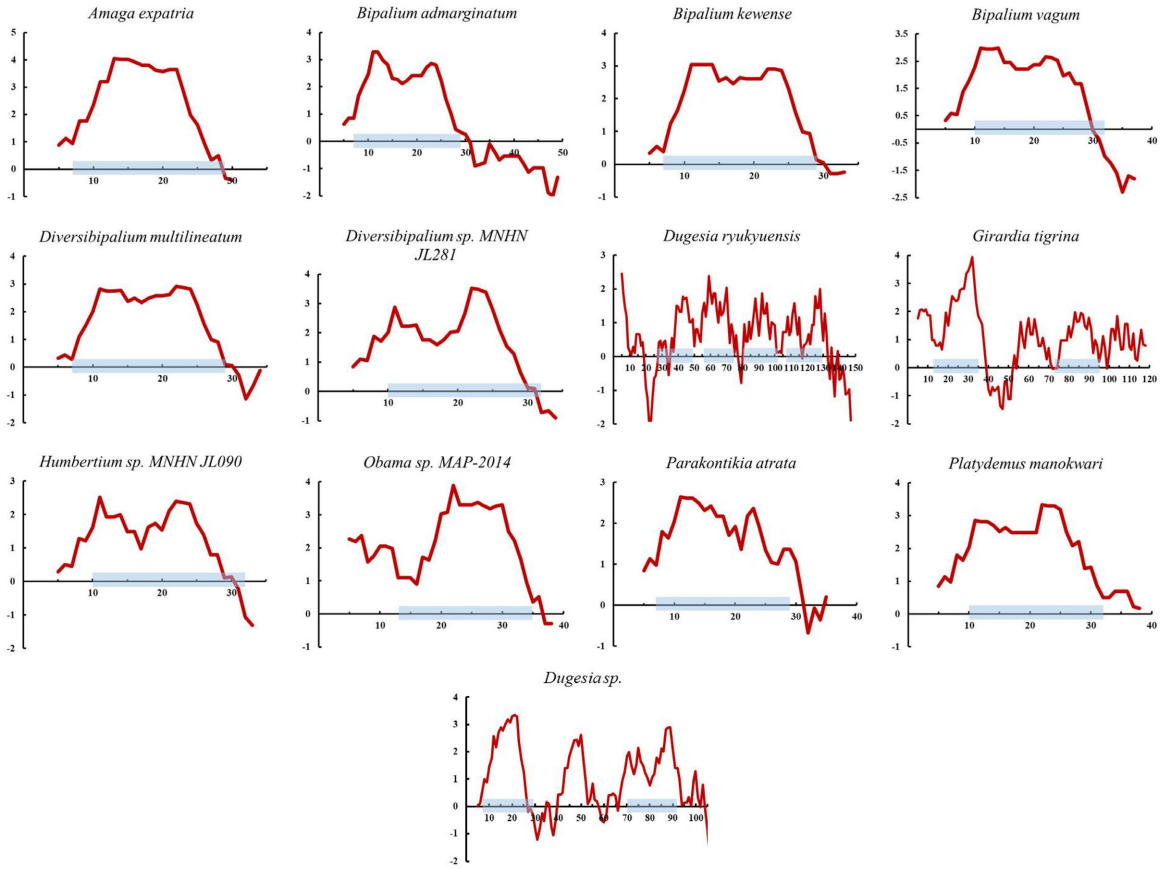

Figure 1. Hydrophobicity plot and transmembrane domains of predicted amino acid sequences.

Based on the predicted amino acid sequences, most species in Geoplanidae (except Bipalium admarginatum) contained 30-50 amino acids, while all three species in Dugesiidae contained more than 100 amino acids. Protein-protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis revealed no significantly similar protein sequences in the non-redundant protein sequence database.

In the predicted amino acid sequences, Geoplanidae species typically contained only one transmembrane domain, mostly appearing between the 7th-29th or 10th-32nd amino acids, with a low complexity region found between the 50th-60th amino acids in Bipalium admarginatum. In contrast, Dugesiidae species contained multiple transmembrane domains, with two or four domains present. In **Figure 2**, transmembrane domains are marked as light blue bars.

Analysis of the potential amino acid sequences revealed significant differences in hydrophobicity patterns. Geoplanidae species typically showed peak values around the 12th and 24th amino acids, forming a two-peak structure, with hydrophilicity scores reaching zero around the 30th amino acid. Dugesiidae species exhibited more complex and diverse peak structures. **Figure 2** displays the hydrophobicity analysis of each species, where the x-axis represents hydrophilicity scores (higher values indicate greater hydrophobicity) and the y-axis represents amino acid positions [28].

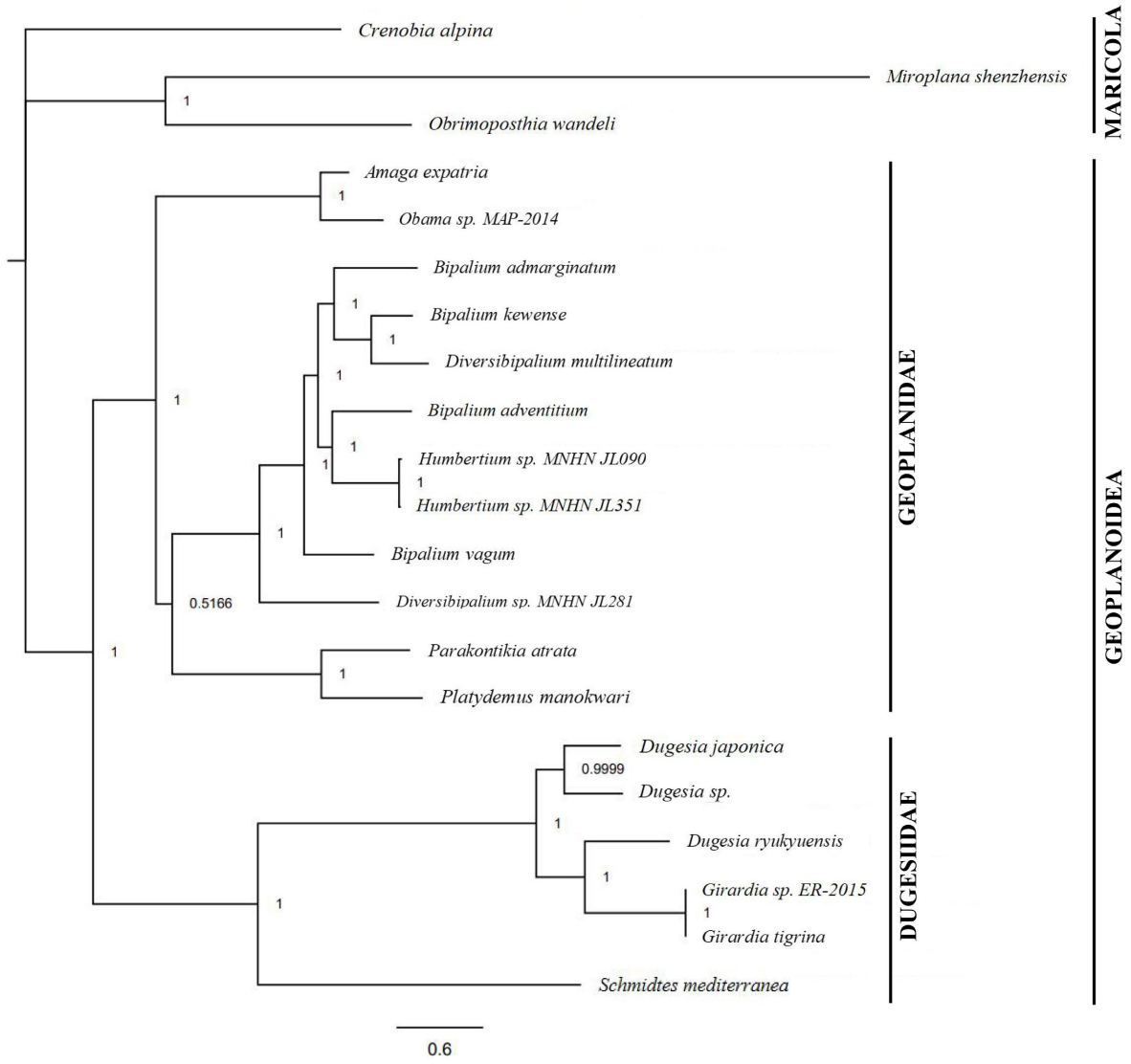

Figure 2. ML tree.

Table 1. Species selected for *atp8* gene study and structural information of *atp8* gene.

Family/ Subfamily	Species name	Number	Starting position	Termination position	The quantity of amino acids	The first four amino acids	Transmembrane region
Geoplani-nae	*Amaga expatria*	NC_057980	9213	9317	34	MIQI	7-29
Bipaliinae	*Bipalium admarginatum*	NC_072986	10694	10855	53	MVHS	7-29
Bipaliinae	*Bipalium kewense*	NC_045216	9297	9410	37	MVHS	7-29
Bipaliinae	*Bipalium vagum*	MZ561468	9439	9564	41	MVHS	10-32
Bipaliinae	*Diversibipalium multilineatum*	MZ561469	12971	13087	38	MVHS	7-29

Continued

Bipaliinae	*Diversibipalium sp.MNHNJL281*	MZ561470	9213	9329	38	MVHV	10-32
Dugesiidae	*Dugesia ryukyuensis*	AB618488	13648	14103	151	MFVL	27-49,56-75, 80-102,107-129
Dugesiidae	*Girardia tigrina*	MW972220	12332	12700	122	MCCY	13-35,73-95
Bipaliinae	*Humbertium sp.MNHNJL090*	MZ561471	9451	9564	37	MVHS	10-32
Geoplani-nae	*Obama sp. MAP-2014*	KP208777	11949	12074	41	IFLF	13-35
Rhyncho-deminae	*Parakontikia atrata*	NC_068631	10044	10163	39	MVHV	7-29
Rhyncho-deminae	*Platydemus manokwari*	MT081580	11548	11676	42	MVHV	10-32
Dugesiidae	*Dugesia sp.*	/	12181	12516	111	MSSL	7-29,70-92

Based on the predicted *atp8* gene of Dugesia sp., PCR products showed clear bands in gel electrophoresis, and RT-qPCR of cDNA yielded results with single peaks.

3.3. Phylogenetic Analysis

Prior to phylogenetic analysis, codon saturation was assessed. Since Iss< Iss.c and $p < 0.05$, the sequences were not saturated, allowing direct use of the PCG123 dataset for subsequent phylogenetic analysis. Figure 3 shows the analysis results of the PCG123 dataset from 21 species.

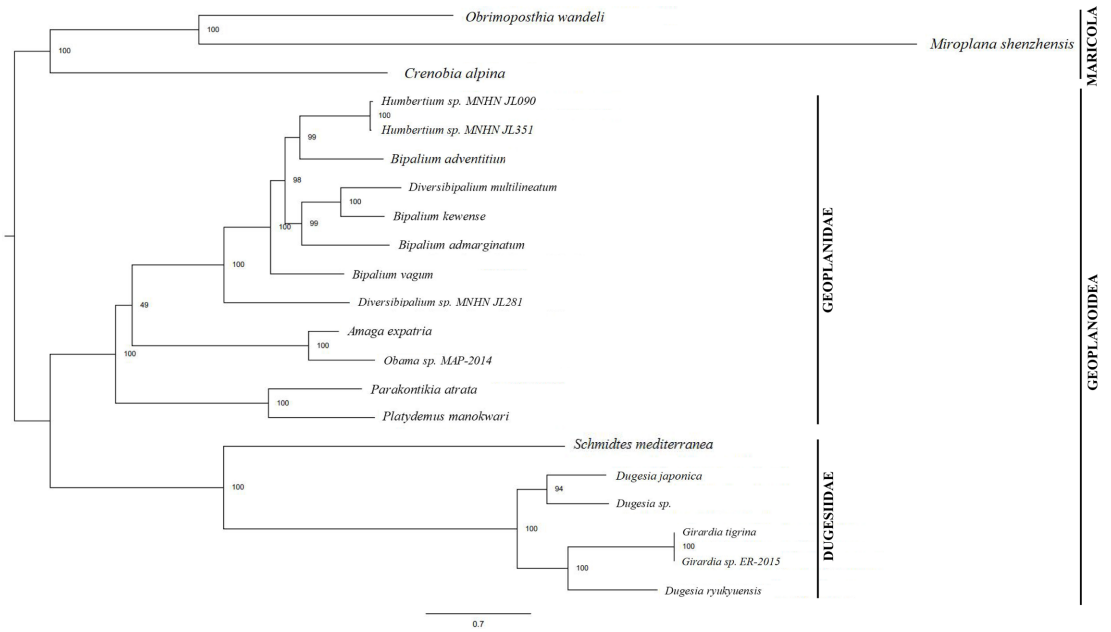

Figure 3. BI tree.

In this study, both ML and BI analyses produced well-supported phylogenetic trees, showing Dugesia japonica as the sister clade to Dugesia sp. The results indicate that Dugesia sp. is distantly related to Dugesia japonica, occupying a distant phylogenetic position within Dugesiidae.

The genetic distance between Girardia tigrina and Girardia sp. was 0.0%, indicating they belong to the same species within the genus Girardia, while their genetic distance from Dugesia ryukyuensis was 16.5%. Although Dugesia ryukyuensis and Dugesia japonica both belong to the genus Dugesia, their genetic distance reached 17.9%, and they did not cluster together. The genetic distances between Dugesia sp. and the other four species were all above 15.5%.

Williams *et al.* found that Baetis rhodani populations from different geographic locations showed genetic distances of 8-19%, leading to the identification of some populations as cryptic species [29]. Based on these molecular data, our results suggest the presence of a cryptic species within the genus Dugesia. Although Dugesia sp. and Dugesia japonica form sister clades, they are not conspecific. While Dugesia japonica is one species within the genus Dugesia, the sample Dugesia sp. represents another distinct species. Therefore, we speculate that the sample Dugesia sp. likely represents a cryptic species within the genus Dugesia.

4. Discussion

4.1. Analysis of *atp8* Gene Divergence

Animal mitochondrial genomes are known to contain 13 protein-coding regions that constitute the oxidative respiratory chain, providing energy for life activities. Within the superfamily Geoplanoidea, the *atp8* gene exhibits intriguing structural differences. In Geoplanidae, the *atp8* gene is more conserved, with relatively uniform structure, shorter sequences, and more compact arrangements among other mitochondrial protein-coding genes. In contrast, Dugesiidae displays more open *atp8* gene architecture, greater diversity, longer sequences, and extended gaps between genes. These differences may correlate with distinct lifestyles and feeding strategies between the two families.

Geoplanidae species predominantly inhabit moist terrestrial environments, exhibit larger body sizes, and employ active foraging strategies, with some species known to prey on snails [30] [31]. Conversely, Dugesiidae species primarily inhabit freshwater ecosystems, featuring smaller body sizes, reduced pigmentation, and a sessile lifestyle attached to submerged rocks or other substrates, adopting passive feeding behaviors with limited mobility.

Empirical studies corroborate the link between mitochondrial gene evolution and energy demands. For example, Wang *et al.* showed that yak *atp8* genes underwent positive selection in high-altitude populations, reflecting adaptation to oxidative stress. Similarly, Boll *et al.* documented fecundity differences in Geoplanidae tied to dietary energy intake, indirectly supporting our hypothesis that terrestrial predators experience stronger *atp8* constraints. These parallels suggest that mitochondrial gene divergence is a broader evolutionary response to meta-

bolic requirements, with Geoplanoidea providing a tractable model for testing such mechanisms.

Our findings align with prior studies highlighting the high variability of *atp8* in Platyhelminthes. For instance, Egger *et al.* demonstrated that *atp8* is present but highly divergent in flatworm mitochondrial genomes, corroborating our observations of structural plasticity in Geoplanoidea. Similarly, Zhao *et al.* noted that *atp8*'s absence in certain mollusks stems from annotation challenges due to rapid sequence evolution, mirroring our manual annotation efforts for Dugesiidae. However, unlike the conserved *atp8* patterns reported in other metazoans, Geoplanoidea exhibits family-specific motifs, suggesting lineage-specific adaptations. This divergence underscores the need for taxon-specific annotation protocols, as automated pipelines often fail to detect *atp8* in non-model taxa.

The structural disparities in *atp8* between Geoplanidae and Dugesiidae directly reflect their ecological dichotomies. Terrestrial Geoplanidae, which actively hunt prey like snails, require sustained energy for locomotion and predation, favoring conserved *atp8* sequences with compact gene arrangements (e.g., single transmembrane domains) to optimize oxidative phosphorylation. Conversely, sessile freshwater Dugesiidae exhibit elongated *atp8* genes with multiple transmembrane domains, likely a consequence of relaxed selection pressures in stable, low-energy environments. This aligns with Sun *et al.*'s findings in mollusks, where reduced mobility correlated with mitochondrial gene diversification. Thus, *atp8*'s architectural openness in Dugesiidae may reflect trade-offs between energy efficiency and genomic flexibility in less demanding niches.

Generally, the *atp8* gene in mitochondrial genomes experiences relatively relaxed selective constraints [32]. The lifestyle and feeding strategies of Dugesiidae result in lower energy demands: reduced mobility decreases energy requirements, while stable aquatic temperatures minimize peak thermal energy needs. These factors likely contribute to relaxed selective pressures on *atp8* in Dugesiidae, permitting greater structural openness and diversity. In contrast, the terrestrial, actively foraging Geoplanidae experience stronger selective pressures on *atp8* due to higher energy demands, leading to greater gene conservation [33].

As two distinct taxonomic units within the same superfamily, these contrasting patterns make Geoplanoidea an excellent model for studying *atp8* gene divergence. Detailed investigations of selective pressures warrant further research.

4.2. Analysis of Cryptic Species

While our mitochondrial data strongly suggest cryptic speciation in Dugesia sp., integrative taxonomy would benefit from: (1) nuclear gene sequencing (e.g., ITS-1, 18S rRNA) to exclude mitochondrial introgression artifacts; (2) morphological reassessment of asexual traits (e.g., parenchyma ultrastructure); and (3) cross-breeding experiments to assess reproductive isolation, as employed for other Tricladida cryptic species. Such multilocus approaches would solidify the taxonomic status of Dugesia sp. beyond genetic distance thresholds.

5. Conclusions

This study molecularly confirms the presence of *atp8* in triclad mitochondrial genomes through experimental evidence. Comparative analysis of *atp8* in Geoplanoidea reveals that freshwater Dugesiidae exhibit more open *atp8* gene architecture compared to terrestrial Geoplanidae, potentially associated with habitat and feeding adaptations.

Based on mitochondrial genome characteristics, genetic distances, and phylogenetic relationships, the specimen Dugesia sp. was identified as likely representing a cryptic species within Dugesiidae. This work further establishes mitochondrial genomes as effective molecular markers for cryptic species identification.

Conflicts of Interest

The authors declare no conflicts of interest.

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