

The complete mitochondrial genome of *Aspidogaster ijimai* (Platyhelminthes: Trematoda: Aspidogastrea): gene content and phylogenetic inference

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ABSTRACT: *Aspidogastrea* is a species-poor group of the Neodermata, but it is important for understanding the evolution of the class Trematoda. Using the high-throughput sequencing method on the Ion S5 platform, data on the primary nucleotide sequence of the complete mitochondrial genome of a representative of *Aspidogastrea* (*Aspidogaster ijimai*) from the common carp *Cyprinus carpio* s. lato, which was obtained from a canal near Lake Biwa, Japan. The length of the mitochondrial genome of *A. ijimai* was 13432 bp; its sequence included 12 protein-coding genes, 22 tRNA genes, two rRNA genes, and a non-coding region (NCR). The location of protein-coding genes did not differ from that of the rest of Digenea, with the exception of some Schistosomatidae. The relative location of the tRNA genes for *A. ijimai* were coincides with those of 48% of the digenean species available in the International Gene Bank. A unique feature reflected in the mt-genome structure was the specific structure of the *tRNA-Ser1* and *tRNA-Trp* genes. The phylogenetic relationships of *A. ijimai* with different members of the Neodermata, reconstructed from pooled amino acid sequences and pooled rRNA and tRNA genes, showed that *Aspidogastrea* is the sister group to Digenea, confirming results of previous studies.

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KEY WORDS: *Aspidogaster*, mitochondrial DNA, Digenea, Neodermata, phylogenetic relationships.

Полный митохондриальный геном *Aspidogaster ijimai* (Platyhelminthes: Trematoda: Aspidogastrea): геновый состав и филогенетический анализ

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РЕЗЮМЕ: *Aspidogastrea* — группа паразитических плоских червей Neodermata, обладающая небольшим видовым разнообразием, но важная для понимания эволюции класса Trematoda. Благодаря методу высокопроизводительного секвенирования на платформе Ion S5 получены данные о первичной структуре полноразмерного митохондриального генома представителя *Aspidogastrea* (*Aspidogaster ijimai*) от сазана *Cyprinus carpio* s. lato, пойманного в канале около озера Бива, Япония. Размер митохондриального генома *A. ijimai* составил 13432 п.н.; его последовательность включала 12 белок-кодирующих генов, 22 гена тРНК, два гена рРНК и один некодирующий регион (NCR — non-coding region). Порядок расположения белок-кодирующих генов не отличался от такового у остальных дигеней, за исключением некоторых представителей Schistosomatidae. Взаимное расположение генов тРНК *A. ijimai* сходно с таковым для митохондриальных геномов 48% видов дигеней, доступных в Международном геномном банке. Выявлены некоторые особенности вторичной структуры генов tRNA-Ser1 и tRNA-Trp. Филогенетические связи *A. ijimai* с разными представителями Neodermata, реконструированные по данным объединенных аминокислотных последовательностей, а также объединенных генов рРНК и тРНК митохондриального генома, показали, что *Aspidogastrea* представляет собой сестринскую группу по отношению к Digenea, подтверждая результаты предыдущих исследований.

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КЛЮЧЕВЫЕ СЛОВА: *Aspidogaster*, митохондриальная ДНК, Digenea, Neodermata, филогения.

Introduction

Aspidogastrea is a small group of neodermites, morphologically similar to digenean trematodes but strongly differing from them by life cycle peculiarities, namely the absence of multiplying intramolluscan stages and a dispersal tailed larva (Rohde, 1972, 1990, 1994). The taxonomic status and phylogenetic relationships of aspidogastreans have been repeatedly discussed based on morphological, biological, and molecular data (e.g., Monticelli, 1892; Faust, Tang, 1936; La Rue, 1957; Stunkard, 1963; Gibson, 1987; Brooks, 1989; Rohde, 2002; Lockyer *et al.*, 2003; Brabec *et al.*, 2023). According to the currently dominant opinion, the Aspidogastrea is a subclass of the class Trematoda (Rohde, 2002; Olson *et al.*, 2003). However, based primarily on the peculiarities of the life histories of aspidogastreans, La Rue (1957) and Timofeeva (1975) proposed to consider them as a class. *Aspidogaster* Baer, 1827 is one of the most species-rich genera of Aspidogastrea. This genus includes 12 valid species, which infect mollusks, bony fishes and less commonly turtles (Alves *et al.*, 2015; Sokolov *et al.*, 2019). *Aspidogaster ijimai* Kawamura, 1915 is one of six species of the genus *Aspidogaster* known for East Asia territory (Alves *et al.*, 2015; Atopkin *et al.*, 2018; Sokolov *et al.*, 2019). This species discovered in the Russian Primorye and Khabarovsk Regions (Atopkin *et al.*, 2018; Sokolov *et al.*, 2019), Japan (Kawamura, 1915; Shimazu, 2003), South Korea (Lee *et al.*, 2017) and China (e.g., Zhang *et al.*, 2006; Chen *et al.*, 2010).

The metazoan mtDNA is an organelle genome, which represents a circular DNA molecule, characterized by compact gene arrangement, constancy of gene content, and presence of a main non-coding region (NCRs). The length of metazoan mtDNA has been reported in the range 13–19 kb and it shows a great variability in structure, gene content, organization and mode of expression in different organisms (Saccone *et al.*, 1999). Usually, mtDNA of Metazoa contains 13 protein-coding genes, 2 rRNA and 22 tRNA genes, and control region (D-loop). In trematodes, the protein-coding gene content reduced to 12 genes (the *atp8* gene is absent) and control region is absent too (Zhang *et al.*, 2017). Arrangement of protein-coding genes in trematodes commonly conservative, excluding

some species of the Schistosomatidae, for which protein-coding gene rearrangements have been reported (Webster, Littlewood, 2012). Sequences of complete mitochondrial genomes are highly popular as bases for phylogenetic studies for eukaryotes, including parasitic Platyhelminthes, because of the great number of molecular characteristics that can be analysed (Aguado *et al.*, 2016; Blasco-Costa *et al.*, 2016; Ross *et al.*, 2016; Vanhove *et al.*, 2018; Trevisan *et al.*, 2021; Zhang *et al.*, 2024). According to the NCBI database, 153 trematode species of order Diplostomida and different suborders of order Plagiorchiida are characterized with complete mitochondrial DNA sequence data. In a recent publication by Xiang *et al.* (2024) and Hu *et al.* (2024), it was stated that the authors first obtained data on complete mitogenomes of Aspidogastrea on the example of *Aspidogaster conchicola* Baer, 1827, and *A. ijimai*. However, the genomes of these aspidogastreans were not annotated, which makes it difficult to use these sequences for phylogenetic studies of Neodermata based on complete mitogenomic data. The mitochondrial DNA is presented by many copies in metazoan organisms. For this reason sequencing of whole mitochondrial genomes with the next-generation sequencing (NGS) technique is considered a perspective approach as it allows to generate big data on DNA sequences for a relatively short time with a high covering. This approach looks attractive to be applied for mitogenome interpretation of aspidogastreans. The aims of this study were the sequencing of the mitochondrial genome of the representative of Aspidogastrea, *Aspidogaster ijimai*, with the NGS approach, and to reconstruct the phylogeny of this species based on these new data.

Material and methods

SAMPLE COLLECTION AND DNA EXTRACTION. Specimens of *Aspidogaster ijimai* were collected from *Cyprinus carpio* s. lato caught in a canal near Lake Biwa and a fishing harbour at Harie in Lake Biwa, Takashima City, Shiga Prefecture, Japan (35°21'54"N, 136°03'16"E) during December 2 and 5, 2016. These specimens were characterised morphologically and genetically earlier by Sokolov *et al.* (2019). A single worm from this material was used for DNA extraction, followed by analysis with NGS sequencing. Total DNA was extracted from the two specimens separately using a Qiamp Investigator

kit (Qiagen, USA) according to the manufacturer's protocol. The amount of total DNA was measured on a Qubit 3.0 fluorometer (Invitrogen, Waltham, Massachusetts, USA) and then used for NGS sequencing in a final amount of 100 ng.

PREPARATION OF GENOME LIBRARY FOR NGS. Libraries were prepared using an Ion Plus Fragment Library kit and unique adapters from an Ion Xpress Barcode Adaptors kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) with pre-fragmentation on a Covaris M220 Focused-ultrasonicator (Covaris, LLC, Womurn, Massachusetts, USA). The preparation of PCR emulsion and templates was done on an Ion One Touch 2 System (ThermoFisher Scientific), followed by sequencing on an Ion S5 sequencing platform using an Ion 540 chip.

The sequence quality and length distribution of raw reads were checked using FastQC 0.11.9, Babraham Bioinformatics (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and then the reads were assembled using SPAdes 3.14.1 (Nurk *et al.*, 2013) with correction of IonTorrent data using the IonHammer tool available in the SPAdes software. The scaffolds containing mitochondrial DNA data were manually assembled in the MEGA X software (Kumar *et al.*, 2018).

Mitochondrial genome annotations were performed using the MITOS2 online software (<http://mitos2.bioinf.uni-leipzig.de>), and then the mitochondrial genome was manually assembled de novo in MEGA X. Search and analysis of the tRNA gene structure were performed in the ARWEN software (<http://130.235.244.92/ARWEN/>). Ambiguous fragments of the mitochondrial genome were sequenced with Sanger's sequencing technique using more than 50 unique oligonucleotide primers, constructed especially for this study (Table 1).

CODON USAGE, GENE VARIATIONS, AND PHYLOGENETIC ANALYSES. Alignments of nucleotide and amino acid sequences were performed by the ClustalW algorithm in MEGA X. Poorly aligned regions were removed using the Gblocks Server (http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=gblocks).

Phylogenetic analysis was performed on the basis of concatenated amino-acid sequences of twelve mitochondrial proteins, and all mitochondrial rRNA and tRNA concatenated gene sequences with the Maximum Likelihood (ML) algorithm available in the PhyML 3.1 software (Guindon, Gascuel, 2003) and Bayesian Inference (BI) available in the MrBayes 3.2.6 software (Ronquist *et al.*, 2012). For concatenated amino-acid sequences, the ML algorithm was performed using an LG evolutionary model (Lee, Gascuel, 2008) for SPRs tree topology search and random sequence addition, and the BI algorithm was performed using a protein model, a mixed set of substitution types, a mixed amino-acid model, and uninformative amino-

acid substitution rates. For the mitochondrial rRNA and tRNA concatenated gene sequence dataset, the best nucleotide substitution models, the GTR+I+G and TIM3+I+G (Posada, 2003), were estimated with jModeltest v. 2.1.5 software (Darriba *et al.*, 2012) for ML (AIC criterion) and Bayesian (BIC criterion) algorithms, respectively (Huelsenbeck *et al.*, 2001; Akaike, 1974). The Monte Carlo Markov chains algorithm was performed with 10000000 generations during two independent runs, with sampling each 1,000 generation and burning the first 25% of all generations. The average standard deviation of split frequencies was 0.000865, which was enough for phylogenetic reconstruction. The significance of phylogenetic relationships was estimated with posterior probabilities (Huelsenbeck *et al.*, 2001) for the BI algorithm and an approximate likelihood-ratio test (Anisimova, Gascuel, 2006) for the ML algorithm. Codon usage statistics were calculated for concatenated protein-coding gene sequence data in MEGA X.

The phylogenetic relationships were inferred using sequences of our samples and other trematode species accessed from the NCBI GenBank database (Supplementary Table S1). The annotated mitochondrial genome and ribosomal operon have been deposited in GenBank under accession nos. PP999303 and PP392593, respectively.

Results

SEQUENCE QUALITY AND COVERAGE. We obtained 2.4 million reads for the *A. ijimai* specimen. The sequence quality after FastQC was acceptable. Values of Phred 33 were 20–30 (mode 26) and decreased slightly in long reads. The sequence length was 25–449 bp; for most reads, the length was 120–240 bp. The mean coverage across the mitochondrial DNA was 33X.

ASPIDOGASTER IJIMAI NUCLEAR RIBOSOMAL OPERON ANNOTATION. The ribosomal operon of *A. ijimai* was 9732 bp in length, including the external transcribed spacer 1 (ETS1, 1785 bp), 18S rRNA gene (1991 bp), ITS1 rDNA (731 bp), 5.8S rRNA gene (154 bp), ITS2 rDNA (650 bp), 28S rRNA gene (3985 bp) and ETS2 (436 bp). The nucleotide composition of ribosomal operon of *A. ijimai* was: A — 22.5%, T (U) — 26.9%, C — 21.5%, and G — 29.1%.

GENERAL CHARACTERISTICS OF THE ASPIDOGASTER IJIMAI MITOCHONDRIAL GENOME. The mitochondrial genome of *A. ijimai* had a length of 13432 bp and contained 12 protein-coding genes, two ribosomal genes, 22 tRNA genes, and a non-

Table 1. Oligonucleotide primers used for PCR and Sanger's sequencing of the mitochondrial DNA of *Aspidogaster ijimai*. Annealing temperature ranged from 46° to 56 °C.
Таблица 1. Олигонуклеотидные праймеры, используемые для ПЦР и секвенирования митохондриальной ДНК *Aspidogaster ijimai*. Температура отжига варьировала от 46° до 56 °C.

Primer's name	Sequence 5' ... 3'
AI2FOR	CTT TTT TTA TAT TGG GGG AA
AI2REV	ATT CGT TTC ACA CAG CAT TC
AI12.1FOR	TAT TTT TTC TTT TTC GGC TA
AI12.1REV	ATA ACC TAA CAC CTT TCG TT
AI13.1FOR	TGG GTA TTG GTT TAT TCC TT
AI13REV	GCA TTA CAA AAG CAA CAT TC
AI14FOR	AGT ACG AAA GGA TTA TAG GA
AI14.1REV	CTA AAC CGC CCC CAT ATC TT
AI16FOR	TGT TTG GTC ATT TTA GTT TT
AI16REV	AAA GTT ACA CTC ACA CTC AT
AI17FOR	ATG AGT GTG AGT GTA ACT TT
AI17REV	GGC GAT ACC AAC AAA AAA AA
AI18FOR	TGT TTT TTT TGT TGG TAT CG
AI18REV	TCT AAT ATA ATC AGG CAT CT
AI19FOR	TGA TTA TAT TAG AAA ACC TA
AI19REV	CTT AGC CGA AAA AGA AAA AA
AI20FOR	TAT GGG AGT CTT TGC GAG TT
AI20REV	GCT AAC AGG ATG GGT AAT AA
AI21FOR	TTA TTC TTA TGT TGG GAC TA
AI21REV	AAA AAA AAC CTA AAA AAA AC

coding region (Fig. 1, Table 2). Alternative read variants were absent from the NGS raw data, and no intraspecific and/or intraspeciment variable positions were observed. The nucleotide composition in the *A. ijimai* mitochondrial genome was as follows: A, 18.1%; T, 41.1%; C, 13.7%; and G, 27.1%. The nucleotide pair frequency was 59.2% for the AT content and 40.8% for the GC content, showing a bias towards T over A (AT skew = -0.39) and G over C (CG skew = 0.33), respectively (Fig. 1).

PROTEIN-CODING GENES OF MITOCHONDRIAL GENOME. The total length of the sequences of 12 protein-coding genes in the complete mitochondrial genome of *A. ijimai* was 10560 bp. All protein-coding genes located at the same DNA chain, as it reported for all other trematodes. The arrangement of protein-coding genes was as follows: *cox3*–*cytb*–*nad4L*–*nad4*–*atp6*–*nad2*–*nad1*–*nad3*–

cox1–*cox2*–*nad6*–*nad5*. The start codons for protein-coding genes were ATG or GTG, except, and the *nad5* gene, which started with the GTT codon (Table 2). The nucleotide composition of the assembled protein-coding part of the mitochondrial genome sequence was as follows: A, 16.8%; T, 43.1%; C, 13.1%; and G, 27.0%. The nucleotide pair frequency was 59.9% for the AT content and 40.1% for the GC content, showing a bias towards T over A (AT skew = -0.44) and G over C (CG skew = 0.35).

The mitochondrial genome of *A. ijimai* possesses several gene overlaps that are usually not observed in the mt-genomes of other trematode species. The cytochrome oxidase subunit 3 gene (*cox3*) overlaps with the complete *tRNA-His* gene (71 bp) and with 28 bp of the 5'-end of the *cytb* gene. The *nad5* gene overlaps with the complete *tRNA-Gly* gene at 1806–1815 bp positions of the *nad5* gene (13034–13100 bp on a whole mt-

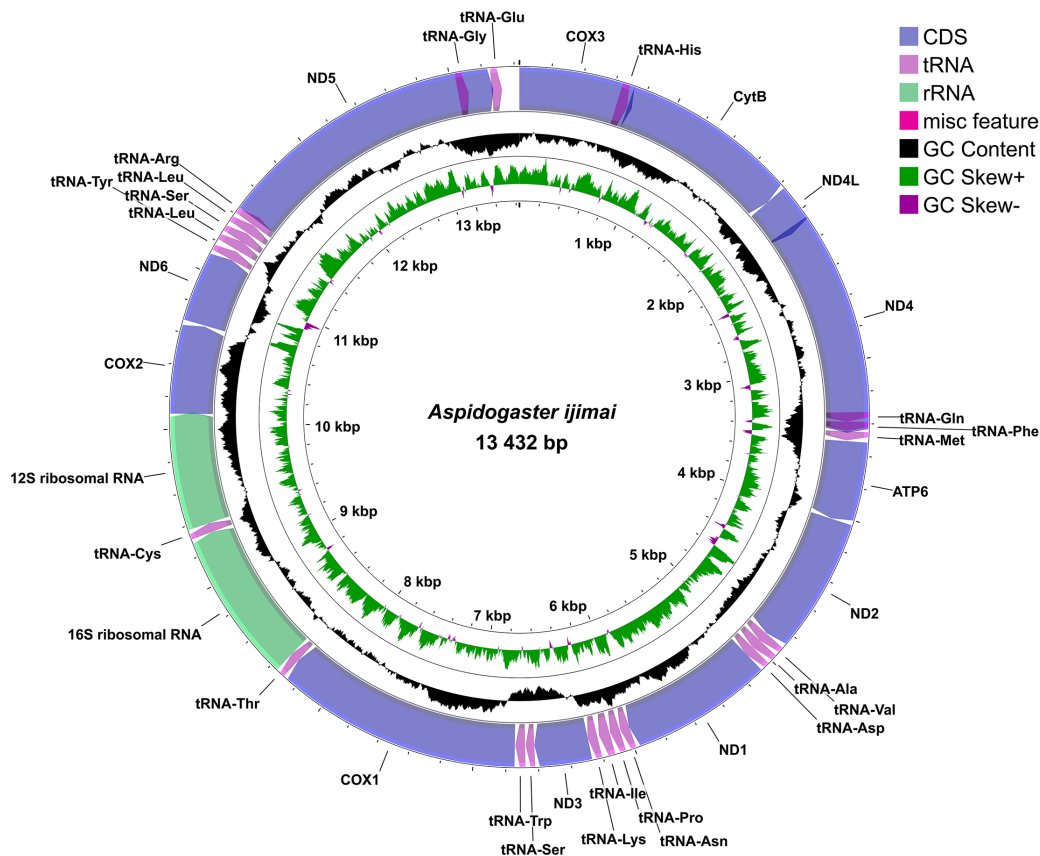


Fig. 1. Organization of the mitochondrial genome of *Aspidogaster ijimai*.

Рис. 1. Структура митохондриального генома *Aspidogaster ijimai*.

genome) and, partially, with the *tRNA-Glu* gene at the 3'-end; the length of overlapping is 10 bp.

The codon usage statistics for *A. ijimai* were consistent with the proportions in the nucleotide composition: the most common triplets contained T (U) and/or G bases, namely UUU (with a frequency of 11.24%), UUG (6.54%), GUU (6.77%), and GGG (3.78%). Also, two codons with an adenine base have a relatively high frequency, namely AUU (4.23%) and UUA (3.55%).

A total of 3,508 amino acids were encoded by the mitochondrial protein-coding genes in *A. ijimai*. Of these, a maximal frequency was observed for leucine (15.69%), phenylalanine (12.37%), and valine (11.79%); the frequency for glutamine (1.08%) was minimal compared to other amino acids.

The tRNA gene arrangement in the mitochondrial genome of *A. ijimai* consists of 48%

of all available mt-genomes in Digenea (Fig. 2). The most frequent profile of tRNA gene arrangement is observed for all Pronocephalata, Ecinostomata (except *Fasciola gigantica* Cobbold, 1855, and *Tracheophilus cymbius* (Diesing, 1850)), and some Xiphidiata sensu Perez-Ponce de Leon and Hernandez-Mena (2019), including *Paragonimus* spp. and *Eurytrema pancreaticum* (Janson, 1899). In a whole, overall gene arrangement of *A. ijimai* completely agree with that for *Ogmocotyle sikae* (Yamaguti, 1933) Ruiz, 1946 from the pattern 1a of the set of unique gene orders of Neodermata, provided by Zhang *et al.* (2017). Most of the tRNA genes of *A. ijimai* possess secondary structures, which are common for all Digenea, including *tRNA-Ser1* (S1) with a truncated D-arm (Fig. 3A) and the short T-stem in the secondary structure observed for the *tRNA-Thr* (T) gene of *A. ijimai* (Fig. 3B).

Table 2. The organization of mitochondrial genome of *Aspidogaster ijimai*.
Таблица 2. Организация митохондриального генома *Aspidogaster ijimai*.

Gene	Position 5' to 3'	Length (bp)	Initiation codon	Termination codon	Anti-codon (tRNA)
<i>cox3</i>	1–747	747	ATG	TAG	
tRNA-His (H)	646–716	71			GTG
<i>cytb</i>	720–1835	1116	ATG	TAG	
<i>nad4L</i>	1836–2096	261	GTG	TAG	
<i>nad4</i>	2057–3460	1404	GTG	TAA	
tRNA-Gln (Q)	3328–3388	61			TTG
tRNA-Phe (F)	3389–3449	61			GAA
tRNA-Met (M)	3452–3510	59			CAT
<i>atp6</i>	3516–4028	513	ATG	TAG	
<i>nad2</i>	4032–4892	861	GTG	TAG	
tRNA-Val (V)	4897–4958	62			TAC
tRNA-Ala (A)	4959–5022	64			TGC
tRNA-Asp (D)	5024–5085	62			GTC
<i>nad1</i>	5084–5989	906	GTG	TAG	
tRNA-Asn (N)	5989–6049	61			GTT
tRNA-Pro (P)	6055–6117	63			TGG
tRNA-Ile (I)	6123–6192	70			GAT
tRNA-Lys (K)	6206–6268	63			CTT
<i>nad3</i>	6269–6619	351	ATG	TAG	
*tRNA-Ser (S1)	6621–6676	56			GCT
tRNA-Trp (W)	6681–6743	63			TCA
<i>cox1</i>	6748–8295	1548	ATG	TAG	
**tRNA-Thr (T)	8297–8355	59			TGT
<i>rrnL</i>	8356–9312	957			
tRNA-Cys (C)	9313–9372	60			GCA
<i>rrnS</i>	9373–10104	732			
<i>cox2</i>	10097–10672	576	ATG	TAA	
<i>nad6</i>	10680–11141	462	ATG	TAG	
tRNA-Tyr (Y)	11143–11203	61			GTA
tRNA-Leu (L1)	11216–11279	64			TAG
tRNA-Ser (S2)	11278–11341	64			TGA
tRNA-Leu (L2)	11347–11408	62			TAA
tRNA-Arg (R)	11410–11471	62			TCG
<i>nad5</i>	11448–13262	1815	GTT	TAG	
tRNA-Gly (G)	13034–13100	67			TCC
tRNA-Glu (E)	13253–13319	67			TTC
NCR	13320–13432	113			

* — truncated D-arm stem in the secondary structure;

** — short T-stem in the secondary structure.

<i>Aspidogaster ijimai</i>	H	Q	F	M	V	A	D	N	P	I	K	S1	W	T	C	Y	L1	S2	L2	R	G	E
<i>Ogmocotyle sikae</i>	H	Q	F	M	V	A	D	N	P	I	K	S1	W	T	C	Y	L1	S2	L2	R	G	E
<i>Carassotrema koreanum</i>	H	Q	F	M	V	A	D	N	P	I	K	S1	W	T	C	Y	L1	S2	L2	R	E	G
<i>Cyathocotyle prussica</i>	H	Q	F	M	A	V	D	N	P	I	K	S1	W	T	C	Y	L1	S2	L2	R	E	G
<i>Brachycladium goliath</i>	H	Q	F	M	V	A	D	N	P	I	K	S1	W	T	C	L1	S2	L2	R	G	Y	E
<i>Schistosoma japonicum</i>	H	Q	F	M	A	D	N	P	I	K	W	Y	S1	T	C	Y	L1	S2	L2	R	G	E
<i>Schistosoma curassoni</i>	H	Q	K	D	V	P	M	W	S1	T	C	Y	L1	S2	N	I	F	A	L2	R	G	E
<i>Trichobilharzia regenti</i>	H	Q	F	M	A	D	N	P	I	K	V	W	S1	T	C	Y	L1	S2	L2	R	G	E
<i>Orientobilharzia turkestanicum</i>	C	W	F	A	N	P	I	K	Q	V	M	S1	T	Y	L1	H	D	L2	R	S2	G	E

Fig. 2. Mutual alignment of tRNA genes in *Aspidogaster ijimai* and several species of Digenea in their mitochondrial genomes.

Рис. 2. Взаимное расположение генов тРНК у *Aspidogaster ijimai* и нескольких видов Digenea в митохондриальных геномах.

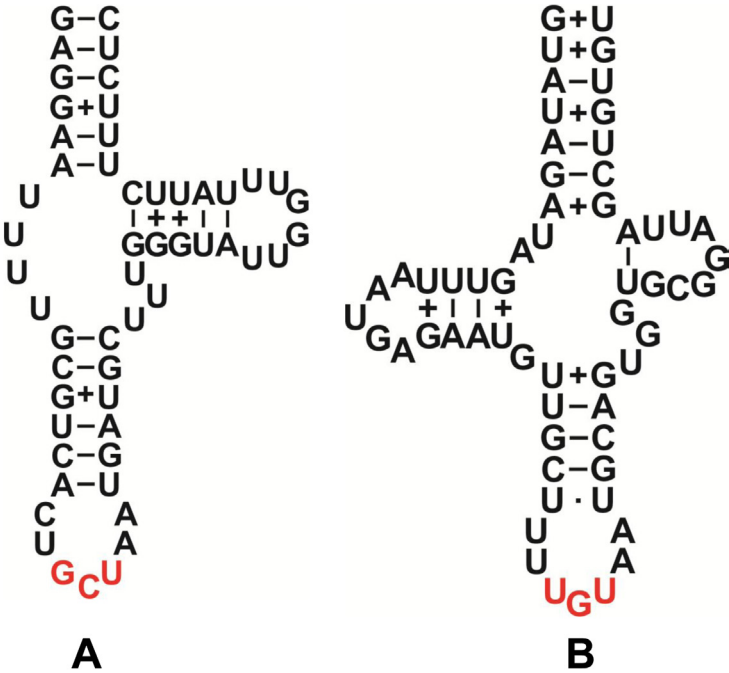


Fig. 3. Predicted secondary structures of the mitochondrial tRNA of *Aspidogaster ijimai*. A — *tRNA-Ser1*; B — *tRNA-Thr*.

Рис. 3. Предсказанные вторичные структуры митохондриальных тРНК *Aspidogaster ijimai*. A — *tRNA-Ser*; B — *tRNA-Thr*.

RESULTS OF PHYLOGENETIC ANALYSIS. The ML and Bayesian algorithms were realized on the basis of the alignment of 2252 amino acids available after Gblocks processing. Overall, mitochondrial genomes of 74 species, including 60 digeneans, three cestodes, seven monopisthocotylans, three polyopisthocotylans, and one planariid species, *Crenobia alpina* (Dana, 1766) as an outgroup, were incorporated into the phylogenetic analysis (Fig. 4). Additionally, phylogenetic analysis was performed with a

Bayesian algorithm based on a concatenated rRNA and tRNA gene sequence dataset, overall 3410 bp (2022 bp after Gblocks processing) in length (Fig. 5). This dataset includes five digenean species and the same species of other Neodermata and outgroups as for amino acid-based analysis. As the amino acid-based BI tree topology showed, *A. ijimai* appears to be a distinct branch, closely related to Digenea, with high statistical support (Fig. 4). In turn, the digeneans could be subdivided into three main clades (Fig. 4). The

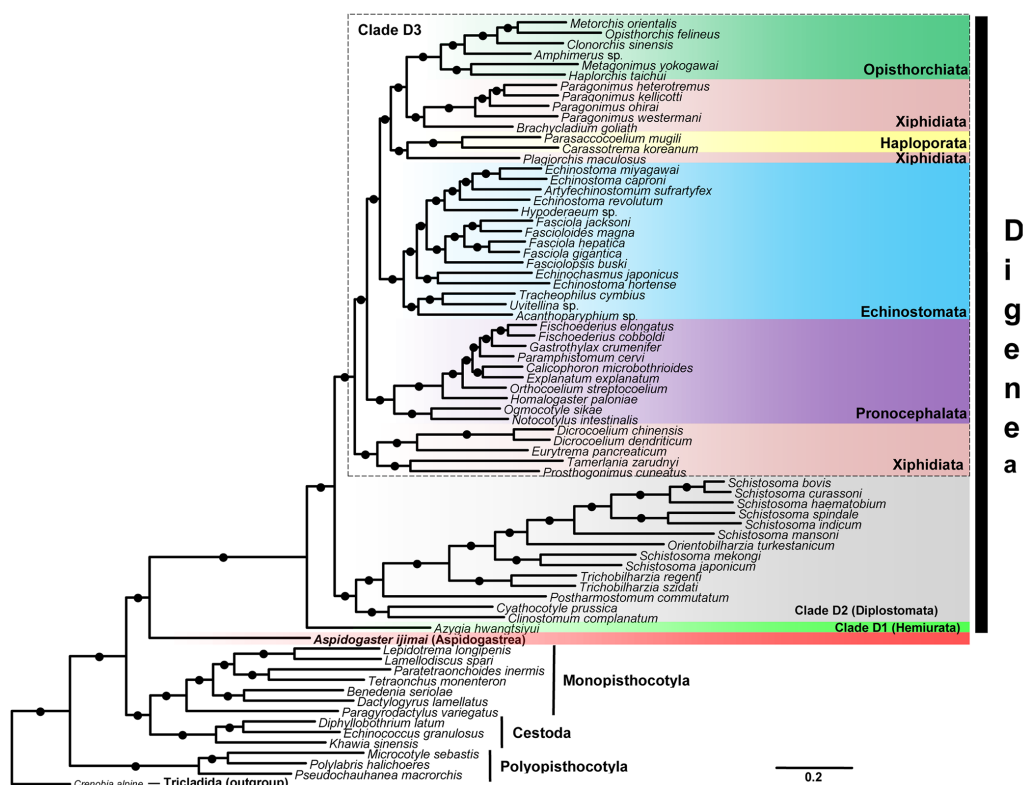


Fig. 4. Phylogenetic relationships of *Aspidogaster ijimai* and other representatives of Neodermata reconstructed on the basis of alignment of mitochondrial protein sequences containing 2,252 amino acids. Clade support with high values (0.9–1.0) for both ML and BI algorithms is shown with black points on the branches. Рис. 4. Филогенетические связи *Aspidogaster ijimai* и других представителей Neodermata, реконструированные на основании выравнивания митохондриальных аминокислотных последовательностей, содержащих 2252 остатка. Поддержка клад с высокими значениями (0,9–1,0) для алгоритмов ML и BI показана черными точками на ветви.

first clade (Clade D1) was the early divergent lineage of *Azygia hwangtsiyui* Tsin, 1933 (Hemiurata, Plagiorchida). The second clade (Clade D2) represented the suborder Diplostomata, including species of the families Schistosomatidae, Clinostomidae, Cyathocotylidae, and Brachylaimidae. The third clade (Clade D3) comprised 47 species from 18 families, representing five suborders of Plagiorchida recognized by Perez-Ponce de Leon and Hernandez-Mena (2019), four of which were monophyletic. One of the suborders, namely the Xiphidiata sensu Perez-Ponce de Leon and Hernandez-Mena (2019), was polyphyletic and subdivided into three monophyletic clades, one including representatives of the Microphalloidea and the Gorgoderoidea, a second of the Plagiorchioidea and the Haploporoidea (Haploporata), and a third of the Troglotrematoidea

and the Brachycladioidea. Among other classes of Neodermata, Monopisthocotyla and Cestoda were sisters relative to each other, and Polyopisthocotylea appears as a sister relative to these two classes; each of them was highly supported.

Phylogenetic relationships of Trematoda based on concatenated mitochondrial rRNA and tRNA gene sequences performed in our study for the first time. The topology of the main branches of the tree constructed on the basis of rRNA+tRNA gene sequences (Fig. 5) differs from that based on mitochondrial amino-acid sequences, except for the for the close relationships between Aspidogastrea and Digenea (Fig. 5). On this tree, Trematoda, Monopisthocotyla, and Polyopisthocotylea form a polytomy, whereas Cestoda appears as a distinct clade, sister to the above-mentioned polytomic clade.

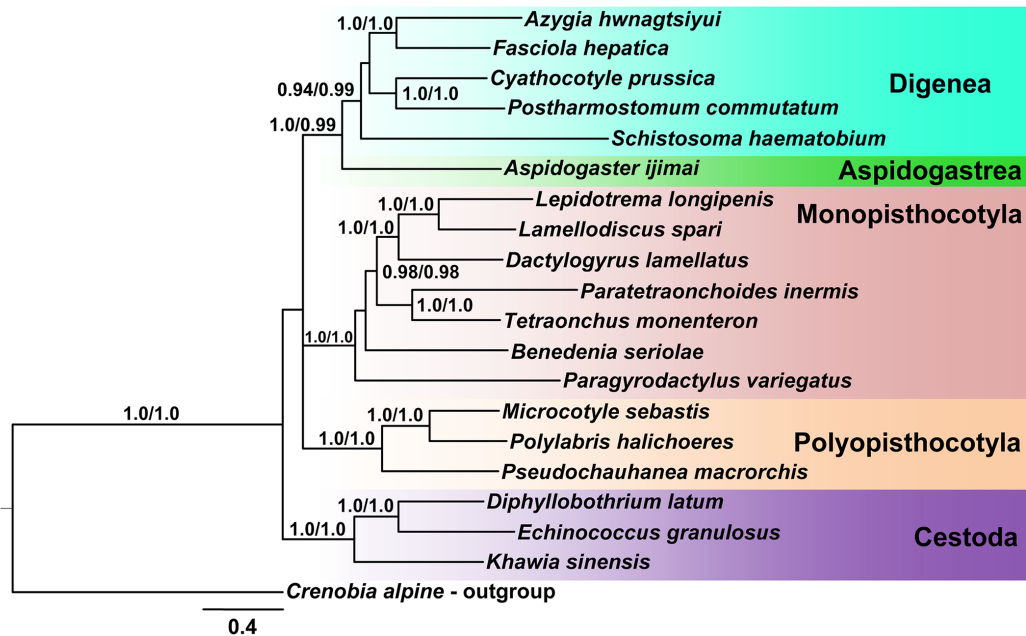


Fig. 5. Phylogenetic relationships of *Aspidogaster ijimai* and other representatives of Neodermata based on alignment of concatenated mitochondrial rRNA and tRNA gene sequences 2022 bp in length. Clade support is shown with a posteriori probabilities calculated for ML/BI algorithms.

Рис. 5. Филогенетические связи *Aspidogaster ijimai* и других представителей Neodermata, реконструированные на основании выравнивания конкатенированных митохондриальных генов рРНК и тРНК общей длиной 2022 п.н. Поддержка клад показана в виде значений апостериорных вероятностей для алгоритмов ML/BI.

Discussion

MITOCHONDRIAL GENOME STRUCTURE FEATURES. In general, the structure of the mitochondrial DNA of *Aspidogaster ijimai* is highly similar to that of most digenean species, except for some representatives of the Schistosomatidae, for which gene rearrangements have been revealed (Littlewood *et al.*, 2006; Webster, Littlewood, 2012). Alongside this, some features concerning the second structure of some tRNA genes that common for all known digenean mitochondrial genomes, are presented in *A. ijimai* mtDNA. The main features are the short loop of the T-stem in the *tRNA-Thr* (T) gene and truncated D-arm in the *tRNA-Ser1* (S1) gene secondary structures. Many mitochondrial tRNAs lack some conserved nucleotides involved in the formation of the secondary and tertiary structures of “classical” nuclear tRNAs. For example, the absence of T-stem was reported for the nematodes *Ascaris suum* Goeze, 1782 and *Caenorhabditis elegans* (Maupas, 1900) (Wolstenholme *et al.*,

1994). In addition, T- and D-loops in the tRNA of animal mitochondria can be shortened. The mitochondrial tRNAs of nematodes have such an unusual structure: they are shorter than the “classical” ones (54–65 nucleotides). In this case, the secondary structure may lack a D-arm or simultaneously have a T-arm and a V-loop (Favorova, 1998). For example, in the nematodes *C. elegans* and *A. suum*, the T-arm and variable loop can be replaced by a simpler loop of 6–12 nucleotides (in *A. suum*, 21 out of 22 tRNAs are subject to such modifications), and in the tRNA encoding serine, the D-arm is replaced by a loop of 5–8 nucleotides. There are also groups of animals in which both D- and T-arms disappear in tRNA at once; for example, modified tRNA-Ser in nematodes, acanthocephals, trematodes, mollusks, brachiopods, and arthropods (Arita, 2006; Sakurai, 2006; Jühling, 2018). Hypothetically, these structures disappear because they do not play an important role in the mechanism of protein biosynthesis. In this case, the most important for biosynthesis are the aminoacyl

and anticodon stems, which are necessary for interaction with aminoacyl-tRNA synthetase and ribosomes during translation. It is believed that without additional structures, the tertiary conformation of tRNA becomes more flexible and protein synthesis can proceed faster (Okimoto, Wolstenholme, 1990).

Mitochondrial genome of *A. ijimai*, generated in the present study is characterized by several overlappings of protein-coding and tRNA genes which are unusual for trematodes. First of all, overlapping of cytochrome oxidase subunit 3 gene (*cox3*) and the complete *tRNA-His* gene (71 bp) represents most interesting case. As mentioned above, the complete mitogenomes *Aspidogaster* spp. available in GeneBank (PP903627, PP903628) are not annotated and the comparative analysis of these mitochondrial genomes with that, provided in our study, is quite difficult. Brief analysis of these sequences indicate that there are a number of serious shortcomings, mainly concerning the primary structure of protein-coding genes, where internal stop codons are found everywhere when using both the code 9 — the Echinoderm and Flatworm Mitochondrial Code and the code 14 — the Alternative Flatworm Mitochondrial Code. Nevertheless, we propose that observed overlapping of 3'-end of the *cox3* gene and complete *tRNA-His* gene in our data caused by elongated C-end of the *cox3* gene overall gene length (747 bp). The same feature was observed earlier for trematodes *Hypoderaeum conoideum* (Bloch, 1782) Dietz, 1909, *Alaria americana* Hall et Wigdor, 1918, *Posthodiplostomum centrarchi* Hoiffman, 1958 and others (Yang *et al.*, 2015a, b; Locke *et al.*, 2018). As consequence, the presence of elongated C-end of *cox3* gene provides possible presence of an incomplete stop-codon (TA), well-known feature for mitochondrial genomes (Donath *et al.*, 2019).

PHYLOGENY. The results of phylogenetic analyses based on both amino acid and rRNA+tRNA gene sequence datasets show that *A. ijimai*, a member of Aspidogastrea, is a sister relative to Digenea. This concept for the phylogenetic position of aspidogastreans has been supported earlier by most researchers based on cladistics analysis of morphological features (e.g., Brooks, 1989; Rohde, 1990; Zamparo *et al.*, 2001; Zamparo, Brooks, 2002) and Sanger's sequencing data (e.g., Blair, 1993; Littlewood *et al.*, 1999; Lockyer *et al.*, 2003; Olson *et al.*,

2003) or on NGS data (Brabec *et al.*, 2023). In turn, this concept relies on the morphologically and molecularly supported monophyly of aspidogastreans (e.g., Zamparo, Brooks, 2002; Lockyer *et al.*, 2003; Chen *et al.*, 2007). However, molecular evidence for the hypothesis about the monophyly of aspidogastreans has only been obtained for members of the Aspidogastridae, Multicalycidae, and Rugogastridae (Lockyer *et al.*, 2003; Chen *et al.*, 2007) and requires confirmation with regard to Stychocotylidae. According to Gibson (1987), Aspidogastridae are morphologically closer to Digenea than to other aspidogastreans represented by Stychocotylidae, Multicalycidae, and Rugogastridae and have a paraphyletic origin with them. As stated above, this hypothesis is not confirmed in relation to Multicalycidae or Rugogastridae but requires testing in relation to Stychocotylidae.

The data provided here further demonstrate inconsistencies between the phylogenies of the suborders Xiphidiata sensu Pérez-Ponce de León and Hernández-Mena (2019) and Haploporata, and in overall order Plagiorchiida reconstructed on the basis of nuclear rRNA genes and complete mitochondrial genome sequences (e.g., Olson *et al.*, 2003; Pérez-Ponce de León, Hernández-Mena, 2019; Sokolov *et al.*, 2022; Le *et al.*, 2024; Suleman *et al.*, 2020, 2021; Atopkin *et al.*, 2021; Huang *et al.*, 2023; Solórzano-García *et al.*, 2024). This controversy is difficult to resolve with phylogenetic analysis based on complete mtDNA sequences with current data capacity. At present, 115 of the 18000 trematode species, including *Aspidogaster ijimai*, reported here, have been characterized with mitochondrial genome sequence data, representing 0.64% of all trematode species. This data amount is too small for representative phylogenetic reconstruction with this molecular marker. Obviously, more mtDNA data on species of the Plagiorchiida will be useful for resolving the problem of the polyphyly of this order as well as the suborder Xiphidiata. Moreover, the most of mtDNA phylogenetic studies of trematodes are based on amino-acid alignments, so their results may be affected by choosing of evolutionary model and mitochondrial DNA code. Additionally, different mitochondrial genes may possess different efficiency in phylogenetic resolution at the inter-familial level, which requires detailed testing of the variation of each gene and amino-

acid sequences with the following optimal combinations.

Phylogenetic relationships at the level of early-branching lineages such as Cestoda, Monopysthocotyla, Polyopisthocotyla, and Trematoda have been discussed repeatedly in the literature (e.g., Litvaitis, Rohde, 1999; Lockyer *et al.*, 2003; Park *et al.*, 2007; Perkins *et al.*, 2010; Hahn *et al.*, 2014; Egger *et al.*, 2015; Brabec *et al.*, 2023; Zhang *et al.*, 2024). The conclusions of different authors are controversial and depend on the set of genetic markers they use, the evolutionary model chosen, and the number of species involved in the analysis. One of the most discussed hypotheses concerns the sister relationship between Cestoda and Trematoda (e.g., Park *et al.*, 2007; Egger *et al.*, 2015). The results of our study are in agreement with reconstructions that do not support the sister relationship between these classes and, therefore, suggest more than a single transition to endoparasitism within Neodermata (International Helminth Genomes Consortium, 2019; Brabec *et al.*, 2023; Caña-Bozada *et al.*, 2023).

Supplementary data. The following Table is available online.

Supplementary Table S1. List of Digenea sequences from GenBank used in phylogenetic analyses.

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Compliance with ethical standards.

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