Journal of Helminthology

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A report on the complete mitochondrial genome of the trematode *Azygia robusta* Odhner, 1911, its new definitive host from the Russian Far East, and unexpected phylogeny of Azygiidae within Digenea, as inferred from mitogenome sequences

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Research Paper

Cite this article: Atopkin DM, Semenchenko AA, Solodovnik DA and Ivashko YI (2023). A report on the complete mitochondrial genome of the trematode *Azygia robusta* Odhner, 1911, its new definitive host from the Russian Far East, and unexpected phylogeny of Azygiidae within Digenea, as inferred from mitogenome sequences. *Journal of Helminthology*, **97**, e69, 1–12
https://doi.org/10.1017/S0022149X23000500

Received: 29 May 2023
Revised: 18 July 2023
Accepted: 03 August 2023

Keywords:
Azygia robusta; Azygiidae; complete mitochondrial genome; phylogenetic relationships; next-generation sequencing

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Abstract

New data on the complete mitochondrial genome of *Azygia robusta* (Azygiidae) were obtained by the next-generation sequencing (NGS) approach. The mitochondrial DNA (mtDNA) of *A. robusta* had a length of 13 857 bp and included 12 protein-coding genes, two ribosomal genes, 22 transfer RNA genes, and two non-coding regions. The nucleotide sequences of the complete mitochondrial genomes of two *A. robusta* specimens differed from each other by 0.12 ± 0.03%. Six of 12 protein-coding genes demonstrated intraspecific variation. The difference between the nucleotide sequences of the complete mitochondrial genomes of *A. robusta* and *Azygia hwangtsiyui* was 26.95 ± 0.35%; the interspecific variation of protein-coding genes between *A. robusta* and *A. hwangtsiyui* ranged from 20.5 ± 0.9% (*cox*1) to 30.7 ± 1.2% (*nad*5). The observed gene arrangement in the mtDNA sequence of *A. robusta* was identical to that of *A. hwangtsiyui.* Codon usage and amino acid frequencies were highly similar between *A. robusta* and *A. hwangtsiyui.* The results of phylogenetic analyses based on mtDNA protein-coding regions showed that *A. robusta* is closely related to *A. hwangtsiyui* (belonging to the same suborder, Azygiida) that formed a distinct early-diverging branch relative to all other Digenea. A preliminary morphological analysis of paratypes of the two azygiid specimens studied showed visible morphological differences between them. The specimen extracted from Sakhalin taimen (*Parahucho perryi*) was most similar to *A. robusta.* Thus, we here provide the first record of a new definitive host, *P. perryi*, for *A. robusta* and also molecular characteristics of the trematode specimens.

Introduction

Trematodes of the family Azygiidae Lühe, 1909 parasitise stomachs or body cavities in elasmobranchs and stomachs in freshwater teleosts and holosteans (Gibson 2002). Representatives of the type genus of this family, *Azygia* Looss, 1899, possess a characteristic medium- or large-sized, elongated body, a small oral sucker, a large ventral sucker, and two large tandem testes. In the Russian Far East, these worms are known to infect mainly freshwater fishes, including such species as *Esox reicherti* Dybowski, 1869, *Perccottus glenii* Dybowski, 1877, *Hucho taimen* (Pallas 1773), and *Channa argus* (Cantor, 1842) (Mamaev, Oshmarin, 1971; Dvoryadkin 1977; Ermolenko *et al.* 1998; Besprozvannykh 2005; Vainutis *et al.* 2023). The phylogenetic position of Azygiidae among other members of the Hemiurata is currently under debate. As a consequence, the taxonomic status of this family still remains unresolved. Taxonomists previously considered this group of trematodes as a separate suborder, Azygiata La Rue, 1957, or the order Azygiida Odening, 1963 (La Rue 1957; Skrjabin & Guschanskaya 1958; Nagasawa *et al.* 1987; Littlewood 2008; Sokolov & Zhukov 2016). At present, most authors, based on the results of molecular phylogenetic analyses using ribosomal DNA gene sequence data (Olson *et al.* 2003; Pérez-Ponce de León & Hernández-Mena 2019), recognize the status of this trematode group as a separate superfamily, Azygioidea Lühe, 1909 (Gibson 2002; Olson *et al.* 2003; Kostadinova & Pérez-del-Olmo 2014; Pérez-Ponce de León & Hernández-Mena 2019), and as a member of the suborder Hemiurata Skrjabin & Guschanskaja, 1954. A phylogenetic analysis of Digenea based on complete mitochondrial sequence data and also using the whole mitochondrial DNA (mtDNA) genome of an azygiid representative, *A. hwangtsiyui* Tsin, 1933, obtained for the first time, has shown that Azygiidae represents a distinct branch, basal for most of trematode groups except Schistosomatidae Stiles & Hassall, 1898 (Wu *et al.* 2020). In our opinion, these results provide sufficient grounds for revising the taxonomic status of Azygiidae through further phylogenetic studies using mtDNA complete sequence data for

different azygiid species. In our present study, we provide new data on the complete mtDNA sequence, inferred by the next-generation sequencing (NGS) approach, from two adult specimens of the trematode *Azygia robusta* Odhner, 1911 extracted from two salmonid species, the taimens *Hucho taimen* and *Parahucho perryi*, which were caught in two rivers of Primorsky Krai, Russia. This trematode species was earlier characterised morphologically by Besprozvannykh (2005), who provided a detailed description of its life cycle. Our study aimed mainly to compare the structures and variations in the complete mitochondrial genomes of two azygiid species, analyse phylogenetic relationships using the new complete mtDNA sequence data on *A. robusta*, and interpret the obtained results to clarify the Azygiidae systematics.

Material and methods

Sample collection and DNA extraction

Adult worms were collected from the intestines of two naturally infected salmonids, a common taimen (*H. taimen*) caught in the Armu River (Besprozvannykh 2005) and a Sakhalin taimen (*P. perryi* (Brevoort, 1856)) caught in the Samarga River (unpublished, collected in 1987) (Table 1). The trematodes were killed with hot water and then fixed in 96% ethanol. Total DNA was extracted from the two specimens separately using a Qiamp Investigator kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Amount of total DNA was measured on a Qubit 3.0 fluorometer (Invitrogen, Waltham, MA, 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, MA, USA) with pre-fragmentation on a Covaris M220 Focused-ultrasonicator (Covaris, LLC, Woburn, MA, USA). The preparation of polymerase chain reaction (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) 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 mtDNA data were manually assembled in the MEGA X software (Kumar *et al.* 2018).

Mitochondrial genome annotation was performed using the MITOS2 on-line software (Donath et al., 2009, available at http://mitos2.bioinf.uni-leipzig.de), and then the mitochondrial genome was manually assembled and aligned with that of *A. hwangtsiyui* (Wu *et al.* 2020) in MEGA X. Tandem repeats were searched using the Tandem Repeat Finders software (https://tandem.bu.edu/trf/trf.html). Search and analysis of the transfer RNA (tRNA) gene structure were performed in the ARWEN software (http://130.235.244.92/ARWEN/).

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).

Table 1. List of Digenea sequences from GenBank used in phylogenetic analysis

Species	GenBank accession number	Reference
Xiphidiata		
Brachycladium goliath	KR703278	Briscoe *et al.* 2016
Carassotrema koreanum	ON59838	Ivashko *et al.* 2022
Dicroroelium chinensis	KF318786	Liu *et al.* 2014a
Dicrocoelium dendriticum	KF318787	Liu *et al.* 2014a
Eurytrema pancreaticum	KP241855	Chang et al. 2016
Paragonimus heterotremus	MH059809	Qian et al. 2018
Paragonimus kellicotti	MH322000	Wang *et al.* 2018
Paragonimus ohirai	KX765277	Le et al. 2019
Paragonimus westermani	KX943544	Biswal *et al.* 2014
Parasaccocoelium mugili	MW846232	Atopkin *et al.* 2021
Plagiorchis maculosus	MK641809	Suleman *et al.* 2019
Prosthogonimus cuneatus	MT586127	Guo *et al.* 2020
Echinostomata		
Artyfechinostomum sufrartyfex	KX943545	Biswal *et al.* 2016, unpublished
Echinostoma caproni	AP017706	Holroyd *et al.* 2016, unpublished
Echinostoma hortense	KR062182	Liu *et al.* 2016
Echinostoma miyagawai	MH393928	Fu *et al.* 2019a
Echinostoma revolutum	MN116706	Ran *et al.* 2020
Echinochasmus japonicus	KP844722	Le *et al.* 2016
Fasciola hepatica	AF216697	Le *et al.* 2000
Fasciola gigantica	KF543342	Liu *et al.* 2014b
Fasciola sp.	KF543343	Liu *et al.* 2014b
Fasciolopsis buski	KX169163	Ma *et al.* 2016, unpublished
Fascioloides magna	KU060148	Ma *et al.* 2016, unpublished
Hypoderaeum conoideum	KM111525	Yang *et al.* 2015
Tamerlania zarudnyi	MW334947	Suleman *et al.* 2021
Pronocephalata		
Acanthoparyphium sp.	MG792058	Kandari *et al.* 2018, unpublished
Tracheophilus cymbius	MK355447	Li et al. 2019
Uvitellina sp.	MK227160	Suleman *et al.* 2019
Calicophoron microbothrioides	KR337555	Ma *et al.* 2015, unpublished
Explanatum explanatum	KT198989	Ma *et al.* 2015, unpublished
Fischoederius cobboldi	KX169164	Ma *et al.* 2016, unpublished
Fischoederius elongatus	KM397348	Fang, 2014, Unpublished

(*Continued*)

https://doi.org/10.1017/S0022149X23000500 Published online by Cambridge University Press

Table 1. (*Continued*)

Species	GenBank accession number	Reference
Gastrothylax crumenifer	KM400624	Yang *et al.* 2016
Homalogaster paloniae	KX169165	Ma *et al.* 2016, unpublished
Ogmocotyle sikae	KR006934	Ma *et al.* 2015, unpublished
Orthocoelium streptocoelium	KM659177	Yang, 2014, Unpublished
Notocotylus intestinalis	MT560390	Xu *et al.* 2021
Paramphistomum cervi	KF475773	Yan et al. 2013
Hemiurata		
Azygia robusta	**OR350239**	**This study**
A. robusta	**OR350240**	**This study**
Azygia hwangtsiyui	MN844889	Wu *et al.* 2020
Opisthorchiata		
Amphimerus sp.	MK238506	Ma *et al.* 2019
Clonorchis sinensis	FJ381664	Shekhovtsov *et al.* 2010
Haplorchis taichui	KF214770	Lee *et al.* 2013
Metagonimus yokogawai	KC330755	Jeon *et al.* 2012, unpublished
Metorchis orientalis	KT239342	Na *et al.* 2016
Opisthorchis felineus	EU921260	Shekhovtsov *et al.* 2010
Diplostomata		
Clinostomum complanatum	KM923964	Chen, 2015, Unpublished
Cyathocotyle prussica	MH536510	Locke *et al.* 2018
Orientobilharzia turkestanicum	HQ283100	Wang *et al.* 2011
Postharmostomum commutatum	MN200359	Fu *et al.* 2019b
Schistosoma bovis	CM014335	Oey et al. 2019
Schistosoma curassoni	AP017708	Kikuchi *et al.* 2019, unpublished
Schistosoma haematobium	DQ157222	Littlewood *et al.* 2006
Schistosoma indicum	AF215860	Le *et al.* 2000
Schistosoma japonicum	MN637821	Jones *et al.* 2020
Schistosoma mansoni	HE601612	Protasio *et al.* 2012
Schistosoma mekongi	AF217449	Le *et al.* 2000
Schistosoma spindale	DQ157223	Littlewood *et al.* 2006
Trichobilharzia regenti	DQ859919	Webster *et al.* 2007
Trichobilharzia szidati	MF136777	Semyenova *et al.* 2017
Outgroup (Cestoda)		
Diphyllobothrium latum	DQ985706	Park *et al.* 2007

Phylogenetic analysis was performed on the basis of concatenated amino acid sequences by the Maximum likelihood (ML) algorithm available in the PhyML 3.1 software (Guindon & Gascuel 2003) and by the Bayesian Inference (BI) method available in the MrBayes 3.2.6 software (Ronquist *et al.* 2012). The ML algorithm was performed using an LG evolutionary model (Lee & Gascuel 2008), Subtree Pruning and Regrafting (SPR) tree topology search, and random sequence addition. 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. The Monte Carlo Markov chains algorithm was performed with 1 000 000 generations during two independent runs, with sampling each 1000th generation and burning the first 25% of all generations. The average standard deviation of split frequencies was 0.000865, and that was enough for phylogenetic reconstruction. Significance of phylogenetic relationships was estimated with *a posteriori* 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 was calculated for concatenated protein-coding gene sequence data in MEGA X. Analysis of correlation between the number of variable sites and the gene length was performed using Pearson's correlation coefficient in Statistica 13 software (TIBCO Software Inc. 2017).

Phylogenetic relationships were inferred using sequences of our samples and other trematode species accessed from the NCBI GenBank database (Table 1). The two annotated mitochondrial genomes have been deposited in GenBank under accession numbers OR350239 and OR350240, while raw Sequence Read Archive (SRA) sequencing data are available under accession numbers SAMN36469092–SAMN36469093.

Results

Brief visual morphological identification of the species

In this study we first performed a brief visual morphological analysis of the paratypes of trematodes used for the NGS analysis. The general view of the azygiid worms from *H. taimen* caught in the Armu River and from *P. perryi* caught in the Samarga River can be seen in Figures 1 and 2, respectively. Both specimens possess the main diagnostic characteristics of *A. robusta*, including the round pharynx and vitellaria extending beyond the posterior end of the second testis to half the distance between the second testis and the posterior end of the body (Skrjabin & Guschanskaja 1958; Bauer 1987). These morphological characteristics were observed clearly in both specimens (Figures 5, 6). For the NGS analysis, we used the mature trematode specimens that had been found in *H. taimen* from the Armu River cultivated from cercariae, identified as *A. robusta*, and published in the study of Besprozvannykh (2005), who described the life cycle of this trematode species. Thus, we keep the name *Azygia robusta* for both trematode specimens used for the NGS analysis.

Sequence quality and coverage

We obtained 3.5–4.5 million reads for two specimens of *A. robusta*. The sequence quality after FastQC was acceptable. Phred 33 values 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 GC content and numbers of duplications and adapters

https://doi.org/10.1017/S0022149X23000500 Published online by Cambridge University Press

Figure 1. General view of *Azygia robusta* extracted from *Hucho taimen* inhabiting the Armu River (the microscope slide and the photograph were kindly provided by V.V. Besprozvannykh).

Figure 2. General view of *Azygia robusta* extracted from *Parahucho perryi* inhabiting the Samarga River (the microscope slide and the photograph were kindly provided by V.V. Besprozvannykh).

did not exceed the norm. The mean coverage across the mitochondrial DNA was 103X and 204X for the two specimens of *A. robusta*.

General characteristics of the Azygia robusta mitochondrial genome

The mitochondrial genome of *A. robusta* had a length of 13 857 bp and contained 12 protein-coding genes, two ribosomal genes, 22 tRNA genes, and two non-coding regions: short (SNCR) and long (LNCR) (Figure 3, Table 2). Alternative read variants were absent from the NGS raw data, and no intraspecific variable positions were observed. The nucleotide composition in the *A. robusta* mitochondrial genome was as follows: A, 16.5%; T (U), 40.9%; C, 14.4%; and G, 28.2%. The nucleotide pair frequency was 57.4% for the AT-content and 42.6% for the GC-content, showing a bias towards T over A (AT skew = -0.43) and G over C (CG skew = 0.32), respectively.

Protein-coding genes of mitochondrial genome

The total length of the sequences of 12 protein-coding genes in the complete mitochondrial genome was 10 110 bp. The arrangement of protein-coding genes was as follows: *cox*3–*cyt*b–*nad*4L–*nad*4–*atp*6–*nad*2–*nad*1–*nad*3–*cox*1–*cox*2–*nad*6–*nad*5. The start-codons for

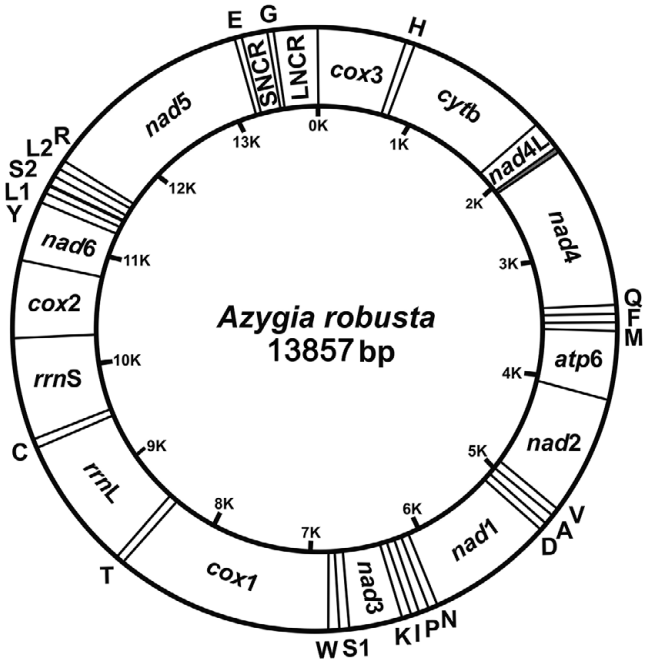

Figure 3. Organization of the complete mitochondrial genome in *Azygia robusta*.

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Table 2. The organization of mitochondrial genome of *Azygia robusta*

Gene	Position 5' to 3'	Length (bp)	Initiation codons	Termination codons	Anti-codons (tRNA)
*cox*3	1–660	660	GTG	TAG	
tRNA-His (H)	665–730	66			GTG
*cyt*b	732–1841	1110	ATG	TAG	
*nad*4L	1849–2109	261	GTG	TAG	
*nad*4	2070–3344	1275	GTG	TAG	
tRNA-Gln (Q)	3353–3415	63			TTG
tRNA-Phe (F)	3420–3488	69			GAA
tRNA-Met (M)	3488–3556	69			CAT
*atp*6	3557–4060	504	GTG	TAG	
*nad*2	4067–4924	858	GTG	TAA	
tRNA-Val (V)	4933–4999	67			TAC
tRNA-Ala (A)	5011–5075	65			TGC
tRNA-Asp (D)	5082–5150	69			GTC
*nad*1	5153–6055	903	ATG	TAG	
tRNA-Asn (N)	6057–6122	66			GTT
tRNA-Pro (P)	6133–6199	67			TGG
tRNA-Ile (I)	6205–6268	64			GAT
tRNA-Lys (K)	6269–6339	71			CTT
*nad*3	6340–6699	360	GTG	TAG	
*tRNA-Ser (S1)	6706–6768	63			GCT
tRNA-Trp (W)	6778–6841	64			TCA
cox1	6842–8389	1548	TTG	TAG	
**tRNA-Thr (T)	8405–8467	63			TGT
*rrn*L	8473–9441	969			
*tRNA-Cys (C)	9442–9501	60			GCA
*rrn*S	9502–10 247	746			
*cox*2	10 248–10 829	582	GTG	TAG	
*nad*6	10 834–11 283	450	GTG	TAG	
tRNA-Tyr (Y)	11 290–11 354	65			GTA
tRNA-Leu (L1)	11 355–11 421	67			TAG
tRNA-Ser (S2)	11 433–11 494	62			TGA
tRNA-Leu (L2)	11 496–11 563	68			TAA
tRNA-Arg (R)	11 567–11 631	65			TCG
*nad*5	11 638–13 236	1598	GTG	TAG	
tRNA-Glu (E)	13 238–13 295	58			TTC
SNCR	13 296–13 422	127			
tRNA-Gly (G)	13 423–13 487	65			TCC
LNCR	13 488–13 857	370			

LNCR, long non-coding region; SNCR, short non-coding region.
*tRNA missed the DHU-arm
**tRNA missed the T-stem

protein-coding genes were ATG or GTG, except the *cox1* gene that started with TTG codon, as well as those for *A. hwangtsiyui* and *nad*3 gene that started with GGT codon (Table 2). The nucleotide composition of the assembled protein-coding part of the mitochondrial genome sequence was as follows: A, 14.5%; T (U), 43.3%; C, 14.0%; and G, 28.2%. The nucleotide pair frequency was 57.8% for the AT-content and 42.2% for the GC-content, showing a bias towards T over A (AT skew = -0.5) and G over C (CG skew = 0.34).

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Codon usage statistics for *A. robusta* 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 9.79%), UUG (5.99%), GUU (5.88%), GGG (4.4%), UGU (3.82%), and GUG (3.39%).

A total of 3 386 amino acids were encoded by the mitochondrial protein-coding genes in *A. robusta*. Of these, a maximal frequency was observed for leucine (15.0%), valine (12.2%), and phenylalanine (11.7%); the frequencies for lysine (1.18%) and glutamine (1.21%) were minimal compared to other amino acids. The amino acid frequencies of the mitochondrial protein sequences of *A. hwangtsiyui* were similar to those of *A. robusta*, with no marked differences observed (Table 3).

Intra- and interspecific variation of complete mtDNA sequences

Overall, the nucleotide sequences of the complete mitochondrial genomes, including all genes and non-coding fragments, of the two *A. robusta* specimens differed from each other by 0.12 ± 0.03%. The concatenated protein-coding gene sequences between these two specimens differed by 0.11 ± 0.03%, and amino acid sequences by 0.06 ± 0.05%. Six of 12 protein-coding genes demonstrated intraspecific variation in *A. robusta* (Table 4); a total of 12 substitutions were revealed, with each gene containing from one to three variable sites, transitions T/C (67%) or A/G (25%), and a single transversion T/G in *nad*6 gene.

The difference between the nucleotide sequences of the complete mitochondrial genomes of *A. robusta* and *A. hwangtsiyui* was 26.95 ± 0.35%; between the concatenated protein-coding nucleotide sequences, 26.00 ± 0.43%; and between the amino acid sequences, 30.15 ± 0.82%. The interspecific variation of protein-coding genes between *A. robusta* and *A. hwangtsiyui* ranged from 20.5 ± 0.9% (*cox*1) to 30.7 ± 1.2% (*nad*5) (Table 4). The results of correlation analysis using Pearson's correlation coefficient showed a high positive correlation ($r = 0.95$) between the number of variable sites and the gene length for interspecific comparison of protein-coding gene variations in the two *Azygia* species (Figure 4).

Phylogenetic analysis

The maximum likelihood (ML) and Bayesian Inference (BI) algorithms were based on alignment of 2 280 amino acids available after Gblocks processing. Overall, mitochondrial genomes of 62 species, including 61 digenean and one cestode species, *Diphpyllobothrium latum* (Linnaeus, 1758) Lühe, 1899, were incorporated into the phylogenetic analysis. As the BI tree topology showed, the digeneans could be subdivided into three highly supported clades (Figure 5). The first clade was early divergent and consisted of three *Azygia* specimens: one *A. hwangtsiyui* (GenBank accession no. MN844889) and two *A. robusta* (our material). The second clade represented the order Diplostomida, including species of the families Schistosomatidae Stiles & Hassal, 1898, Clinostomidae Lühe, 1901, Cyathocotylidae Mühling, 1898, and Brachylaimidae Joyeux & Foley, 1930. The third clade comprised 47 species from 18 families, representing seven suborders. The topology of this subclade completely agreed with the previous phylogenetic reconstructions of Digenea (Ivashko *et al.* 2022). The suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003 was polyphyletic and appeared as two independent groups. The first group consisted of *Brachycladium goliath* (van Beneden, 1858) and species of the genus *Paragonimus* Braun, 1988

Table 3. Amino acid frequencies in concatenated protein sequences of mitochondrial protein-coding region of *Azygia robusta* (1, ex *Hucho taimen*, Amu River (Besprozvannykh, 2005) 2002; 2, ex *Parahucho perryi*, Samarga River, 1987) and *A. hwangtsiyui*

		Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
1	***Azygia robusta***	3.80	5.28	1.87	1.93	11.7	10.0	1.84	2.87	1.18	15	3.20	1.63	3.29	1.21	2.05	9.12	2.9	12.2	3.59	4.89
2	***A. robusta***	3.86	5.28	1.87	1.93	11.7	10.1	1.84	2.87	1.18	15	3.20	1.63	3.29	1.21	2.05	9.09	2.9	12.2	3.59	4.89
3	***Azygia hwangtsiyui***	3.58	5.24	2.02	2.05	12.5	9.64	1.78	2.80	0.99	15	3.73	1.69	2.47	1.23	2.20	8.89	2.6	13.0	4.01	5.03

https://doi.org/10.1017/S0022149X23000500 Published online by Cambridge University Press

Table 4. Variation of mitochondrial protein-coding genes of *Azygia robusta* and between *A. robusta* and *A. hwangtsiyui.*

Gene	Intraspecific values for *A. robusta*			Interspecific values	
	Variable sites/Gene length, bp	Variation, %	Substitutions ($type_{site\ number}$)	Variable sites/Gene length, bp	Variation, % ± std.err.
cox3	2/660	0.3	T/C_{85}, C/T_{369}	161	24.2 ± 1.5
cytb	0/1110	0	–	254	22.9 ± 1.2
nad4L	0/261	0	–	61	23.4 ± 2.6
nad4	0/1275	0	–	356	28.0 ± 1.2
atp6	0/504	0	–	150	30.1 ± 2.1
nad2	0/858	0	–	243	28.8 ± 1.5
nad1	3/903	0.33	C/T_{191}, A/G_{556}, T/C_{819}	237	26.3 ± 1.4
nad3	1/360	0.28	A/G_{288}	97	27.3 ± 2.4
cox1	1/1548	0.07	C/T_{582}	318	20.5 ± 0.9
cox2	0/582	0	–	123	21.1 ± 1.7
nad6	2/450	0.44	T/G_{9}, T/C_{255}	129	29.4 ± 2.1
nad5	3/1599	0.19	T/C_{185}, A/G_{1042}, T/C_{1594}	486	30.7 ± 1.2

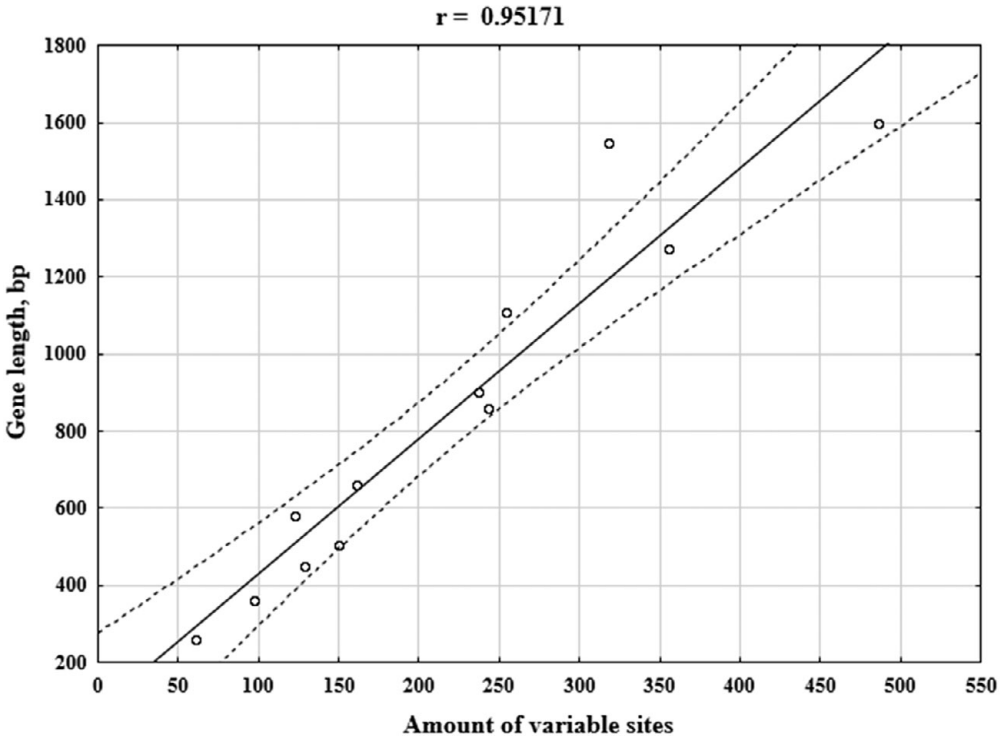

Figure 4. Results of an analysis based on Pearson's coefficient of correlation between gene length and number of variable sites with pairwise comparison of mitochondrial protein-coding genes of *A. robusta* and *A. hwangtsiyui. r* is the Pearson's correlation coefficient.

(Xiphidiata). This group was closely related to Opisthorchiata. *Plagiorchis maculosus* (Rudolphi, 1802) appeared as sister to the above-mentioned Xiphidiata and Opisthorchiata species. The second group of Xiphidiata included representatives of Dicrocoeliidae Odhner, 1911 (*Dicrocoelium dendriticum* (Rudolphi, 1819), *D. chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978, and *Eurytrema pancreaticum* (Janson, 1899)), Eucotylidae Skrjabin, 1924 (*Tamerlania zarudnyi* Skrjabin, 1924), and Prosthogonimidae Lühe, 1909 (*Prosthogonimus cuneatus* (Rudolphi, 1802) Braun, 1901). This group appeared as sister to the subclade that contained species of the suborder Pronocephalata Olson, Cribb, Tkach, Bray, Littlewood, 2003. The suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019, represented by *Parasaccocoelium mugili* Zhukov, 1971 and *Carassotrema koreanum* Park, 1938, formed a basal branch within the third clade.

In general, the ML tree topology was similar to that of the BI tree, demonstrating three main clades within Digenea (Figure 6). The marked differences from the BI tree topology were as follows:

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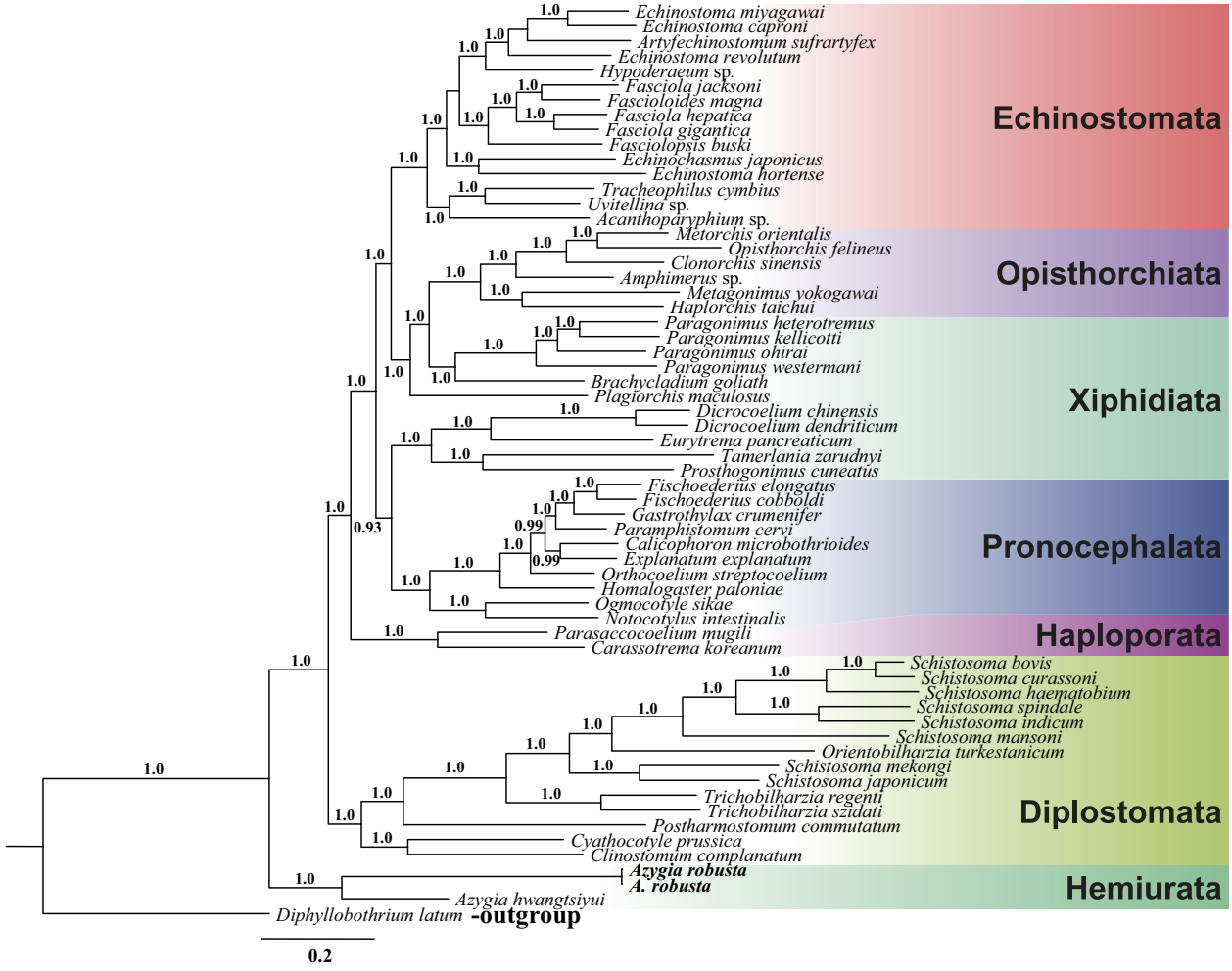

Figure 5. Phylogenetic relationships of *Azygia robusta* and other digenetic trematodes reconstructed by the Bayesian Inference (BI) algorithm on the basis of alignment of protein sequences containing 2280 amino acids, available after Gblock processing. Nodal support is shown with *a posteriori* probabilities calculated using the BI algorithm.

(1) Dicrocoeliidae (Xiphidiata) formed a separate basal subclade within the third clade, creating polyphyly for Xiphidiata, and (2) representatives of Haploporata, *C. koreanum* and *P. mugili*, were within a single subclade with *Plagiorchis maculosus*, with this subclade being sister to closely related representatives of Xiphidiata (*Paragonimus* spp. and *Brachycladium goliath*) and Opisthorchiata. These differences between the BI and ML tree topologies were also reported in previous studies (Atopkin *et al.* 2021; Ivashko *et al.* 2022).

Discussion

Mitochondrial genome variations in A. robusta and A. hwangtsiyui

The complete mitochondrial genome structure of *A. robusta* was highly similar to that of *A. hwangtsiyui* in gene arrangement, the existence of two non-coding regions separated from each other by tRNA-Gly (G) gene, and a higher level of AT content relative to GC content for both mitochondrial genome sequences and protein-coding genes. Also, the two azygiid species had the same most frequent codons and the same start-codon (TTG) for the *cox*1 gene. A difference was revealed in the start-codon of the *nad*3 gene, which started with GGT in *A. robusta* vs. ATG in *A. hwangtsiyui.* There were also differences in the lengths of some protein-coding genes between *A. robusta* and *A. hwangtsiyui*: 1275 vs. 1272 bp, respectively, for the *nad*4 gene; 504 vs. 513 bp for *atp*6; 903 vs. 906 bp for *nad*1; 1548 vs. 1564 bp for *cox*1; 450 vs. 444 bp for *nad*6; and 1598 vs. 1600 bp for *nad*5.

New definitive host of Azygia robusta from the Russian Far East

To date, four species of definitive hosts for trematodes *Azygia* spp. are known from the Russian Far East: the northern snakehead *Channa argus* (Cantor, 1842) and the Amur pike *Esox reicherti* Dybowski, which are freshwater fishes, and the common taimen *Hucho taimen* (Pallas, 1773) and the Chinese sleeper *Perccottus glenii* Dybowski, 1877, which are freshwater/brackish-water fishes (Mamaev, Oshmarin, 1971; Dvoryadkin 1977; Ermolenko *et al.* 1998; Besprozvannykh 2005; Vainutis *et al.* 2023). In this study, we have extended the list of definitive hosts for this region by adding the Sakhalin taimen, *Parahucho perryi* (Brevoort, 1856). This fish is one of the world's largest salmonids, reaching maturity at 6–8 years of age and living for more than 20 years. The species' geographic range is confined to the Sea of Japan, from the southern Kuril Islands and Primorsky Krai, Russia, to Hokkaido, Japan. *Parahucho perryi* occupy a variety of habitats including upper and lower reaches of rivers, lakes, brackish-water lagoons, estuaries,

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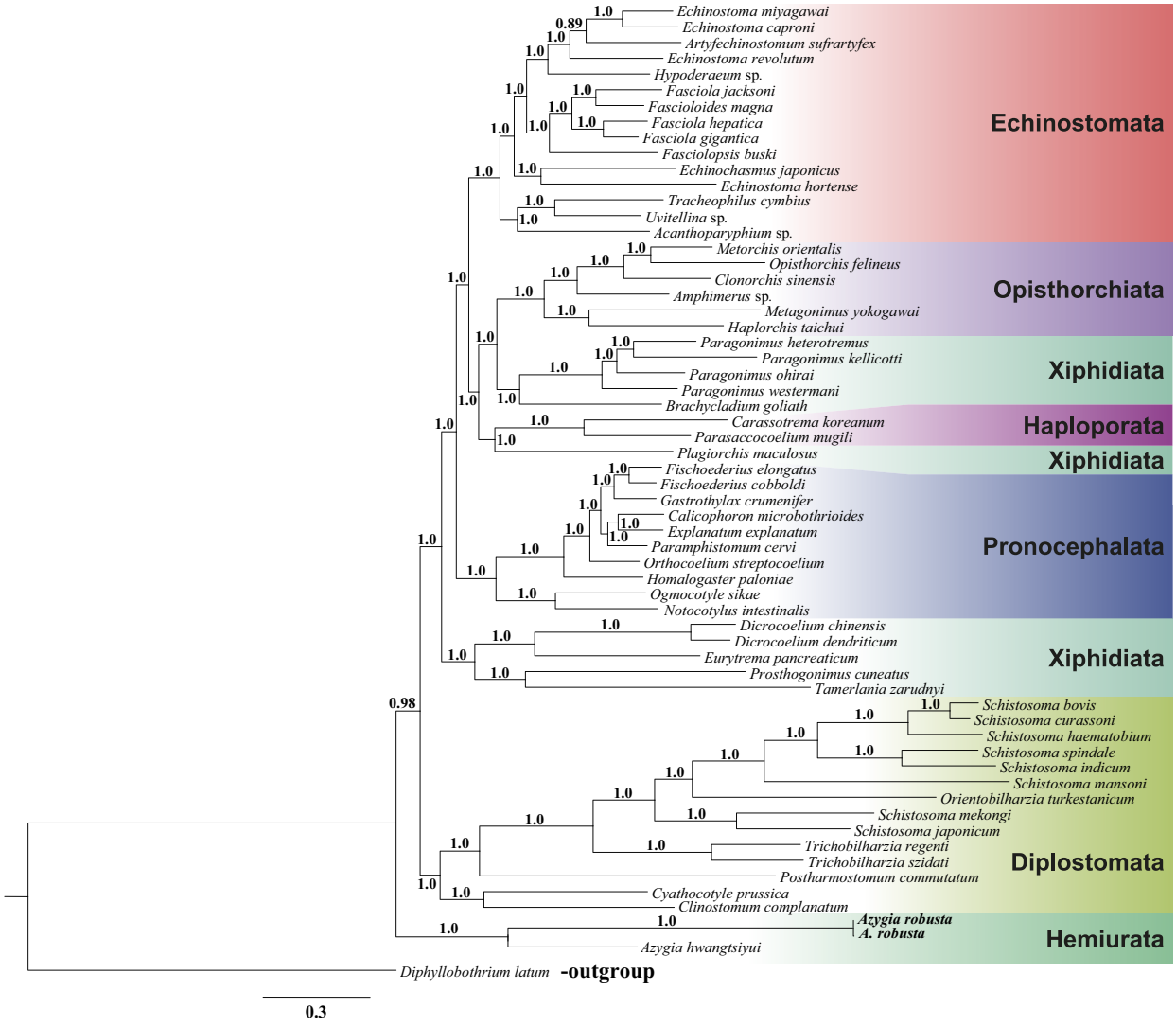

Figure 6. Phylogenetic relationships of *Azygia robusta* and other digenetic trematodes reconstructed by the Maximum Likelihood (ML) algorithm on the basis of alignment of protein sequences containing 2280 amino acids, available after Gblock processing. Nodal support is shown with *a posteriori* probabilities calculated using the approximate likelihood ratio test.

and coastal marine waters (Zolotukhin & Semenchenko 2008; Fukushima *et al.* 2011). The ecological features of *P. perryi* are favorable for the trematode *A. robusta* to infect this fish species. Moreover, one of the azygiid species, *A. perryi*, has been reported as a parasite for *P. perryi* from Japan (Nagasawa *et al.* 1987; Popiołek *et al.* 2013), while *A. robusta* is known to parasitise salmonids (Nikolić *et al.* 2018). Thus, we here provide the first record of a new definitive host, *Parahucho perryi*, for the trematode *Azygia robusta* from the Russian Far East.

Systematics and phylogenetic relationships of Azygiidae

The systematic position of Azygiidae is still unclear because of the lack of molecular data for representatives of this group, and, as a consequence, controversies arise between interpretations of morphological and molecular data. Most authors recognize the status of this trematode group as a separate superfamily (Gibson 2002; Olson *et al.* 2003; Kostadinova & Pérez-del-Olmo 2014; Pérez-Ponce de León & Hernández-Mena 2019). However, viewpoints on the membership of Azygioidea at a higher taxonomic level are different. These worms were considered as a separate suborder, Azygiata (La Rue 1957; Skrjabin & Guschanskaya 1958), or the order Azygiida Odening, 1963 (Littlewood 2008; Sokolov & Zhukov 2016). At present, this superfamily is recognized as a member of the suborder Hemiurata mainly on the basis of data inferred from molecular phylogenetic analyses using ribosomal DNA gene sequences (Olson *et al.* 2003; Pérez-Ponce de León & Hernández-Mena 2019). However, as the latest studies have shown, the Azygiida is a valid order (Ramilo *et al.* 2023). The first complete mitochondrial genome sequences for a representative of Azygiidae, *Azygia hwangtsiyui*, were obtained by Wu *et al.* (2020). These data were applied to the reconstruction of the phylogenetic position of Azygiidae within Digenea using a dataset of concatenated amino acid sequences representing 12 protein-coding genes. The position of *A. hwangtsiyui* (Azygiidae) was considered the 'most basal lineage of the Digenea'; however, in that study, Schistosomatidae rather than Azygiida was basal for Digenea (Wu *et al.* 2020). Nevertheless, the authors did not provide any definitive conclusion about the systematic position of Azygiidae and stated that 'the family Azygiidae still awaits investigation of relationships based on a much

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wider taxon sampling and more mitogenome datasets' (Wu *et al.* 2020). Our results clearly demonstrate that this statement is relevant. The introduction of one new azygiid species into the phylogenetic analysis based on concatenated amino acid sequences of 12 protein-coding mitochondrial genes has considerably changed the phylogenetic position of Azygiidae within Digenea. In contrast to the results from previous studies, the present phylogenetic tree consists of three main, highly supported digenean clades, including the early diverging clade Azygiidae and two sister clades, Diplostomida and other digeneans. On the one hand, this result supports the taxonomic status of Azygiidae as a separate order within Digenea, which largely agrees with the previous results by Wu *et al.* (2020) that showed a basal position of Azygiidae relative to other Digenea, except Schistosomatidae. On the other hand, our results do not confirm the hypothesis, advanced in our previous studies, about the consistency between phylogenetic relationships and gene rearrangement within mitochondrial genomes of Schistosomatidae and other trematodes (Atopkin *et al.* 2021; Ivashko *et al.* 2022). In particular, the basal position of Azygiidae relative to other trematodes and the emergence of *Cyathocotyle prussica* and *Clinostomum complanatum* within a single clade with Schistosomatidae are evidence against this hypothesis. However, in this respect, we agree with Wu *et al.* (2020), who indicated the need for additional data, with complete mitochondrial genome sequences obtained for more Azygiidae species and other unstudied digenean taxa, to provide a sufficient basis for conclusions about the systematics of this family.

Acknowledgements. We are deeply thankful to Vladimir V. Besprozvannykh, Dr. Sci. Biol., the head of the Department of Parasitology, Federal Scientific Center of East Asian Terrestrial Biodiversity FEB RAS, for providing the material on *Azygia robusta* (the specimens for the DNA extraction and the microscope slides of paratypes for the morphological analysis) and to Dr. Kirill A. Vinnikov, the Director of the Institute of the World Ocean, Far Eastern Federal University, for providing the laboratory equipment and constructive comments while performing NGS.

Financial support. This study was supported by the Russian Scientific Foundation, project number 22-24-00896.

Competing interest. None.

Ethical standard. All applicable institutional, national and internationalguidelines for the care and use of animals were followed.

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https://doi.org/10.1017/S0022149X23000500 Published online by Cambridge University Press