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Characterization of the complete mitochondrial genome of *Acanthogyrus* (*Acanthosentis*) *bilaspurensis* Chowhan, Gupta & Khera, 1987 (Eoacanthocephala: Quadrigyridae), the smallest mitochondrial genome in Acanthocephala, and its phylogenetic implications

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Abstract

The phylum Acanthocephala is an important group of parasites with more than 1,300 species parasitizing intestine of all major vertebrate groups. However, our present knowledge of the mitochondrial genomes of Acanthocephala remains very limited. In the present study, we sequenced and annotated the complete mitochondrial genome of *Acanthogyrus* (*Acanthosentis*) *bilaspurensis* (Gyracanthocephala: Quadrigyridae) for the first time based on the specimens recovered from the intestine of common carp *Cyprinus carpio* Linnaeus (Cyprinidae) in Pakistan. The mitochondrial genome of *A. bilaspurensis* is 13,360 bp in size and contains 36 genes, representing the smallest mitogenome of acanthocephalans reported so far. The mitogenome of *A. bilaspurensis* also has the lowest level of overall A+T contents (59.3%) in the mitogenomes of Eoacanthocephala, and the non-coding region 3 (NCR3) lies between *trn*S2 and *trn*I, which is different from all of the other acanthocephalan species. Phylogenetic analyses based on concatenating the amino acid sequences of 12 protein-coding genes using maximum likelihood (ML) and Bayesian inference (BI) methods revealed that the family Pseudoacanthocephalidae is a sister to the Arhythmacanthidae rather than the Cavisomatidae, and the families Rhadinorhynchidae and Cavisomatidae showed sister relationships.

Introduction

Acanthocephalans of the class Eoacanthocephala mainly parasitize fishes and contain two orders, Gyracanthocephala (including only one family Quadrigyridae) and Neoechinorhynchida (including three families: Dendronucleatidae, Tenuisentidae, and Neoechinorhynchidae) (Amin 2013). However, the monophyly and phylogenetic relationships of the two orders and their included families/subfamilies remain unclear due to the scarcity and inaccessibility of genetic data for some taxa. The family Quadrigyridae contains two subfamilies Quadrigyrinae and Pallisentinae (Amin 2013). However, some previous molecular phylogenetic studies suggested that the family Quadrigyridae and the subfamily Pallisentinae are both non-monophyletic (Song *et al.* 2016; Muhammad *et al.* 2019a, 2020a, b; Ru *et al.* 2022).

Although approximately 250 species belonging to over 30 genera have been described in the class Eoacanthocephala, the mitogenomes of the Eoacanthocephala are currently available for only four species, namely, *Acanthogyrus cheni* Amin, 2005; *Neoechinorhynchus violentum* Van Cleave, 1928; *Paratenuisentis ambiguus* Van Cleave, 1921; and *Pallisentis celatusi* Van Cleave, 1928 (Song *et al.* 2016; Gazi *et al.* 2016; Pan and Nie 2013; Weber *et al.* 2013; Pan and Jiang 2018). Our current knowledge of the mitochondrial genomes of acanthocephalans, especially the class Eoacanthocephala, remains very limited.

The poorly known species *A. bilaspurensis* Chowhan, Gupta & Khera, 1987 was originally reported from the reba carp *Cirrhinus reba* (Hamilton) in India (Chowhan *et al.* 1987; Naidu

2012). Recently, this species was redescribed based on specimens collected from *Cyprinus carpio* (Linnaeus) in Pakistan (Ru *et al.* 2022). In the present study, the complete mitochondrial genome of *A. bilaspurensis* (Gyracanthocephala: Quadrigyridae) was sequenced and annotated for the first time. In addition, in order to assess the phylogenetic relationships of some families or subfamilies in Acanthocephala, phylogenetic analyses were performed using maximum likelihood and Bayesian inference (BI) based on concatenating the amino acid sequences of 12 protein-coding genes (PCGs) of mitogenomes.

Materials and methods

Parasites collection and species identification

Acanthocephalans were collected from the intestine of *C. carpio* in the Indus River, district Swabi (34°07′07.23″N, 72°36′32.38″E), Khyber Pakhtunkhwa, Pakistan. The acanthocephalans were identified as *A. bilaspurensis* according to some previous studies (Ru *et al.* 2022; Chowhan *et al.* 1987).

Molecular procedures

The genomic DNA was extracted for molecular studies using Wizard_SV Genomic DNA Purification System (Promega, Madison, USA) following the manufacturer's protocol. The extracted DNA was preserved at −20°C for further molecular studies. The overall mitochondrial DNA of *A. bilaspurensis* was amplified by PCR using the primers shown in Table 1. PCR reactions were conducted in a 50 µl reaction mixture, containing 22.5 µl dd H_2O, 22.5 µl PrimeSTAR Max DNA polymerase, 2 µl DNA template, and 1.5 µl of each primer. Long PCR amplification was conducted with 2 min denaturation at 96°C, then 15 cycles of denaturation at 98°C for 20 s, annealing at 50–65°C for 30 s, and extension at 68°C for 3 to 5 min, followed by 96°C denaturation for 2 min, plus 30 cycles of 96°C for 20s (denaturation), 52–65 °C for 30 s (annealing) and 65°C for 3 to 5 min, and a final extension at 72° C for 10 min. The positive amplified fragments, size up to 2 kb, were cloned in pMD18-T vector, and size above 2 kb were sequenced directly using primer walking strategy.

Table 1. Primers used for amplification and sequencing of the complete mitochondrial genome of *Acanthogyrus bilaspurensis* in the present study

Primers	Sequence (5′ → 3′)	Gene/region	Size (bp)
NSF1	CTAAGGTAGCGTAATCATTTGTC	16S	317
NSR1	GCTCACGCCGGTCTAAACTC		
NSF2	GGTAACAGGGCAATCTTTAGG	16S-*nad*5	4384
NSR2	CCAGAAAGCCTAGCAGAAAAG		
NSF3	GAGGTTTTGGGTGTTGTTAGG	*nad*5	281
NSR3	GAATGAACCAAAGAAGACAC		
NSF4	TGGAGTGGATGTTGGGTCTG	*nad*5-*cyt*b	1720
NSR4	TCGAGACAACCTAACAACC		
NSF5	GCGTTTATGGGGTATGTGTTGC	*cyt*b	429
NSR5	CTTGATGTGGCTGGGGGTAC		
NSF6	GTTGGTGTACTGATCTGG	*cyt*b-*nad*1	620
NSR6	CACTACCCCTAGAGTTAGGC		
NSF7	CTCGTAAGGGGCCTAACAAGG	*nad*1	498
NSR7	CAGACTCCCCCTCTAAGAAG		
NSF8	GACGCCTTTGGTGTTGAGG	*nad*1-16S	6075
NSR8	GTCTTTCCGTCTTATCCTAC		

The mitogenome of *A. bilaspurensis* was assembled manually in a stepwise manner using DNAstar v7.1 program (Burland 2000). The MEGA v7.0 (Kumar *et al.* 2016) was used for analyzing the nucleotides composition and codons usage. Protein-coding genes (PCGs) were identified using BLAST and ORF Finder tools by choosing the invertebrate mitochondrial code. The nucleotide alignment was checked against the genomes *A. cheni* and *P. ambiguus.* Most of the tRNAs were identified using MITOS web server (Bernt *et al.* 2013) and ARWEN (Laslett and Canback 2008). Ribosomal RNA genes (*rrn*L and *rrn*S) were identified by alignment comparison with other acanthocephalan species. The amino acid sequences of 12 PCGs were obtained using MEGA7, for which invertebrate mitochondrial code was chosen. Codons usage and relative synonymous codons usage (RSCU) for 12 PCGs were sorted out using PhyloSuite v1.1.15 (Zhang *et al.* 2020). The circular drawing of the mt genome of *A. bilaspurensis* was drawn out with MTVIZ, an online tool of mitochondrial visualization (available at http://pacosy.informatik.uni-leipzig.de/mtviz/). The RSCU figure was drawn using plugin ggplot2 (Wickham 2016).

Phylogenetic analyses

Phylogenetic analyses were performed based on concatenating the amino acid sequences of 12 PCGs of mitogenomes. *Rotaria rotatoria* Pallas, 1766 and *Philodina citrina* Ehrenberg, 1832 (Rotifera: Bdelloidea) were chosen as the out-group. Detailed information of representatives included in the phylogenetic analyses is provided in Table 2. Fasta files of the amino acid sequences of 12 PCGs were extracted from the GenBank, using PhyloSuite. Genes were aligned with MAFFT (Katoh *et al.* 2002) and integrated in PhyloSuite using normal-alignment mode. PhyloSuite was used to concatenate the generated alignments into a single alignment and then generate nexus and phylip format files for the phylogenetic analyses. The selection of best-fit models and partition strategy was done using PartitionFinder2 (Lanfear *et al.* 2017). Maximum likelihood analysis was carried out using IQ-TREE (Nguyen *et al.* 2015) with 50,000 ultrafast bootstraps (Minh *et al.* 2013). Bayesian inference analysis was generated using MrBayes v3.2, (Ronquist *et al.* 2012) by running two independent MC3 runs of four chains each for 5,000,000 generations and sampling tree topologies with every 1000 generations. 'Burn-in' periods were adjusted to one million generations following the standard deviation of split frequency value lower than (0.01). The phylograms were observed Using FigTree v.1.43 (Chen *et al.* 2014) and annotated in Adobe Illustrator®. Phylogenetic analyses ranked nodes with Bayesian posterior probabilities (BPP) and bootstrap support values (BS) ≥0.90/90 as strongly supported and ≥0.80/80 and < 0.90/90 as moderately supported.

Results and discussion

Organization of the mitochondrial genome of Acanthogyrus (Acanthosentis) bilaspurensis

The complete mitogenome of *A. bilaspurensis* is 13,360 bp in size and contains 36 genes, including 12 protein-coding genes (PCGs)

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Table 2. Detailed information of representatives included in the present phylogenetic analyses

Taxa	Family	Species	Accession nos	Size	AT%	References
Ingroup						
Class Archiacanthocephala						
Oligacanthorhynchida	Oligacanthorhynchidae	*Oncicola luehei*	NC_016754	14,281	60.2	Gazi *et al.* (2012)
		Macracanthorhynchus hirudinaceus	NC_019808	14,282	65.2	Weber *et al.* (2013)
Moniliformida	Moniliformidae	*Moniliformis* sp. XH-2020	OK415026	14,066	66.2	Dai *et al.* (2022)
Class Palaeacanthocephala						
Echinorhynchida	Arhythmacanthidae	*Heterosentis pseudobagri*	OP278658	13,742	62.5	Gao *et al.* (2023)
	Rhadinorhynchidae	*Micracanthorhynchina dakusuiensis*	OP131911	16,309	56.8	Gao *et al.* (2022)
	Pseudoacanthocephalidae	*Pseudoacanthocephalus bufonis*	MZ958236	14,056	58.4	Zhao *et al.* (2023)
	Echinorhynchidae	*Echinorhynchus truttae*	FR856883	13,659	63.1	Weber *et al.* (2013)
	Cavisomatidae	*Cavisoma magnum*	MN562586	13,594	63.0	Muhammad *et al.* (2020a)
	Rhadinorhynchidae	*Leptorhynchoides thecatus*	NC_006892	13,888	71.4	Steinauer *et al.* (2005)
	Iliosentidae	*Brentisentis yangtzensis*	MK651258	13,864	68.3	Song *et al.* (2019)
	Pomphorhynchidae	*Pomphorhynchus bulbocolli*	NC_060483	13,915	59.9	Unpublished
	Pomphorhynchidae	*Pomphorhynchus rocci*	NC_060484	13,845	60.7	Unpublished
	Pomphorhynchidae	*Pomphorhynchus tereticollis*	JQ809451	13,965	56.9	Unpublished
	Pomphorhynchidae	*Pomphorhynchus laevis*	JQ809446	13,889	57.1	Mauer *et al.* (2020)
Polymorphida	Plagiorhynchidae	*Plagiorhynchus transversus*	KT447549	15,477	61.1	Gazi *et al.* (2016)
	Polymorphidae	*Southwellina hispida*	KJ869251	14,742	63.9	Gazi *et al.* (2015)
	Polymorphidae	*Polymorphus minutus*	MN646175	14,149	64.4	Sarwar *et al.* (2021)
	Centrorhynchidae	*Centrorhynchus clitorideus*	MT113355	15,884	55.5	Muhammad *et al.* (2020b)
	Centrorhynchidae	*Centrorhynchus milvus*	MK922344	14,314	54.5	Muhammad *et al.* (2019b)
	Centrorhynchidae	*Centrorhynchus aluconis*	KT592357	15,144	54.5	Gazi *et al.* (2016)
	Centrorhynchidae	*Sphaerirostris picae*	MK471355	15,170	58.1	Muhammad *et al.* (2019a)
	Centrorhynchidae	*Sphaerirostris lanceoides*	MT476588	13,478	58.0	Muhammad *et al.* (2020c)
Class Eoacanthocephala						
Gyracanthocephala	Quadrigyridae	*Acanthogyrus bilaspurensis**	MT476589	13,360	59.3	Present Study
	Quadrigyridae	*Acanthogyrus cheni*	KX108947	13,695	65.3	Song et *al.* (2016)
	Quadrigyridae	*Pallisentis celatus*	JQ943583	13,855	61.5	Pan & Nie (2013)
Neoechinorhynchida	Tenuisentidae	*Paratenuisentis ambiguus*	FR856885	13,574	66.9	Weber *et al.* (2013)
	Neoechinorhynchidae	*Neoechinorhynchus violentum*	KC415004	13,393	59.4	Pan & Nie (2014)
Class Polyacanthocephala						
Polyacanthorhynchida	Polyacanthorhynchidae	*Polyacanthorhynchus caballeroi*	KT592358	13,956	56.3	Gazi *et al.* (2016)
Outgroup						
Bdelloidea	Philodinidae	*Philodina citrina*	FR856884	14,003	77.7	Weber *et al.* (2013)
Bdelloidea	Philodinidae	*Rotaria rotatoria*	GQ304898	15,319	73.1	Min & Park (2009)

*The mitogenome labeled as *Neoechinorhynchus* sp. (MT476589) in the GenBank actually corresponds to *Acanthogyrus (Acanthosentis) bilaspurensis.*

(*cox*1–3, *atp*6, *nad*1–6, *nad*4L, and *cyt*b, lacking *atp*8), 22 tRNAs and two rRNAs genes (*rrn*L and *rrn*S), plus three non-coding regions (NCR1, NCR2, and NCR3) (Figure 1) (Table 3), which represent the smallest mitogenome of acanthocephalans reported so far (Table 2). All genes are encoded on the heavy strand and transcribed in the same direction. According to the previous studies, most of the acanthocephalan species lack *atp*8 gene in their mitogenomes, except for *Leptorhynchoides thecatus* Linton, 1891 (Steinauer *et al.* 2005). The nucleotide contents in the complete mitogenome of *A. bilaspurensis* were 17.0% A (2276 bp), 30.1% G (4027 bp), 42.3% T (5652 bp), and 10.5% C (1405 bp) (Table 4). The level of overall A+T contents (59.3%) is lower than that in the

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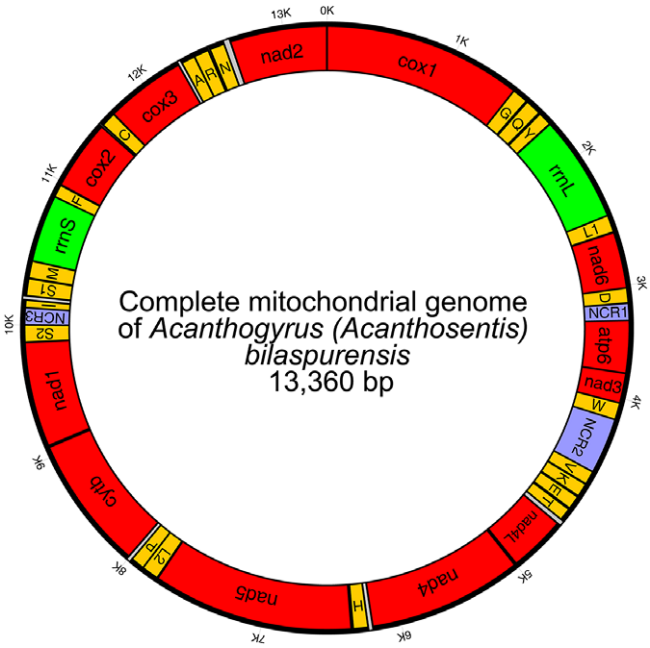

Figure 1. Organization of the complete mitogenome of *Acanthogyrus* (*Acanthosentis*) *bilaspurensis*. The 12 protein-coding genes (PCGs) (red), 22 tRNA genes (yellowish-brown), 2 rRNA genes (green), and three non-coding regions (NCR) (purple) shown in different colors. All genes are transcribed in the clockwise direction on the same strand.

mitogenomes of the other Eoacanthocephala species (59.4–66.9%) (Song *et al.* 2016; Pan and Nie 2013, 2014; Weber *et al.* 2013). The low level of overall A+T contents in the mitogenome of *A. bilaspurensis* is due to the low use of A-rich codons in the PCGs, especially in the second and third codon positions, which were only 13.5% and 11.6%, respectively (Table 4).

Protein-coding genes and codon usage

The size of 12 PCGs of *A. bilaspurensis* is 9,786 bp, containing 3,262 codons excluding termination codons, with average A+T content of 58.6% (Table 4). The size of 12 PCGs varied from 246 bp (*nad*4L) to 1545 bp (*nad*5) (Table 3). In the 12 PCGs of the mitogenome of *A. bilaspurensis*, the high level of T contents (44.2%) is consistent with high frequency of T-rich codons, which has been reported in the other acanthocephalans (Gazi *et al.* 2016; Muhammad *et al.* 2020a). TTG for lecine (9.23%) is the most frequently used codon, followed by TTT for phenylalanine (8.22%) and GTT for valine (7.69%), while CGC is the most unfrequently used codon (only 0.03%) (Table 5). Leucine (17.3%) is the most persistent amino acid in the PCGs of *A. bilaspurensis* (Table 5). The overall codon usage and RSCU for the construction of 12 PCGs are displayed in Figure 2. The highly frequent usage of leucine has also been reported in *Cavisoma magnum* Southwell, 1927 [16.7%], *Sphaerirostris picae* Rudolphi, 1819 [14.0%], and *Plagiorhynchus transversus* Rudolphi, 1819 [15.0%] (Muhammad *et al.* 2019a, 2020a; Gazi *et al.* 2016). The most frequent start codon is GTG, used for seven PCGs (*cox*1, *nad*6, *atp*6, *nad*3, *nad*4L, *cox*3, and *nad*2), whereas *cyt*b and *cox*2 used ATG as start codon. The remaining PCGs (*nad*4, *nad*5, and *nad*1) used ATT, ATA, and TTG as start codons, respectively (Table 3). The most frequently used complete stop codons are TAG and TAA. The stop codon TAG was used for five PCGs (*nad*3, *nad*4L, *nad*5, *nad*1, and *nad*2), while the codon TAA was used for three PCGs (*atp*6, *cytb*, and *cox*2). The remaining four PCGs (*cox*1, *nad*6, *nad*4, and *cox*3) were inferred to terminate with incomplete stop codon T. The incomplete stop codon T was also reported in the mitogenomes of some other acanthocephalan species (Muhammad *et al.* 2019a, b, 2020a). The detailed information of codons of 12 PCGs of *A. bilaspurensis* is provided in Table 3.

Ribosomal and transfer RNA genes

Two ribosomal RNAs, *rrn*L and *rrn*S, are 879 bp and 591 bp in size, with A+T (61.1%) and (60.2%), respectively. The *rrn*L lies between *trn*Y and *trn*L1, which is the same as most of the acanthocephalan species reported so far, except for *Macracanthorhynchus hirudinaceus* (Pallas, 1781) (*rrn*L lies between *trn*Y and *trn*L2) (Weber *et al.* 2013). The *rrn*S is located between *trn*M and *trn*F, which is also the same as most of acanthocephalan species reported so far, except for *P. celatus* and *L. thecatus* (*rrn*S located between *trn*S1 and *trn*F) (Muhammad *et al.* 2019a, b, 2020a; Pan and Nie 2013; Steinauer *et al.* 2005). In the complete mitogenome of *A. bilaspurensis*, the size of 22 transfer RNAs ranges from 49 bp (*trn*Q) to 66 bp (*trn*I), lacking TΨC arm but having TV-replacement loop. The two tRNAs, *trn*S1 and *trn*S2, both lack (DHU) arm, and the other tRNAs are predicted to fold into a 'cloverleaf-like' secondary structure (Muhammad *et al.* 2019a, b, 2020a; Gazi *et al.* 2016). The size, location, and anticodons of all the 22 tRNAs are presented in Table 3.

Non-coding regions

There are usually two non-coding regions (NCR) in the mitogenomes of acanthocephalans, but *A. cheni*, *Pomphorhynchus laevis* (Zoega in Müller, 1776), *Polymorphus minutus* (Zeder, 1800) and *Pomphorhynchus bulbocolli* have three non-coding regions in their mitogenomes (Song *et al.* 2016; Mauer *et al.* 2020; Sarwar *et al.* 2021). *Paratenuisentis ambiguus* and *Heterosentis pseudobagri* Wang & Zhang, 1987 have only one non-coding region (NCR) (Weber *et al.* 2013; Gao *et al.* 2023). Only *Neoechinorhynchus violentum* Van Cleave, 1928 has been reported with four non-coding regions (Pan and Nie 2014). Present study revealed *A. bilaspurensis* also has three non-coding regions, the total size of which is 974 bp. The NCR1 is 182 bp in length, located between *trn*D and *atp*6. The NCR2 is 415 bp in length, located between *trn*W and *trn*V, while the NCR3 is 104 bp, located between *trn*S2 and *trn*I. The position of NCR3 of the present species is different from all of the acanthocephalan species reported so far. Details of position and size of NCRs are provided in Figures 1 and 3 and Table 3.

Gene order

The gene arrangement of the mitogenomes can provide useful information for the phylogenetic relationships of metazoans (Song *et al.* 2019; Muhammad *et al.* 2020a). The tRNAs in the mitogenomes of acanthocephalans seem to have more variability in translocation (Song *et al.* 2019; Muhammad *et al.* 2020a). In the mitogenome of *A. bilaspurensis*, gene order of 12 PCGs and 2 rRNAs is in the following pattern: *cox*1, *rrn*L, *nad*6, *atp*6, *nad*3, *nad*4L, *nad*4, *nad*5, *cyt*b, *nad*1, *rrn*S, *cox*2, *cox*3, *nad*2, which is the same as the other acanthocephalans reported so far (Muhammad *et al.* 2019a, b, 2020a, b, c; Zhao *et al.* 2023). However, several tRNAs of the mitogenome of *A. bilaspurensis* (i.e., *trn*M, *trn*V, *trn*S1, *trn*S2, *trn*K, *trn*C, and *trn*R) exhibit variability in translocation (Figure 3).

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Table 3. Organization of the mitochondrial genome of *Acanthogyrus (Acanthosentis) bilaspurensis*

Gene/Region	Position 5′ to 3′	Size (bp)	No. of aa	Ini/Ter codons	Anti-codon	Int. seq.
*cox*1	1–1528	1528	509	GTG/T		0
tRNA-Gly (G)	1529–1580	52			TCC	–8
tRNA-Gln (Q)	1573–1621	49			TTG	–9
tRNA-Tyr (Y)	1613–1664	52			GTA	–2
*rrn*L	1663–2541	879				0
tRNA-Leu (L1)	2542–2598	57			TAG	–4
*nad*6	2595–3021	427	142	GTG/T		0
tRNA-Asp (D) NCR1	3022–3071 3071-3253	50 182			GTC	182 –1
*atp*6	3254–3727	474	157	GTG/TAA		0
*nad*3	3728–4051	324	107	GTG/TAG		0
tRNA-Trp (W)	4052–4112	61			TCA	0
NCR2	4113–4527	415				0
tRNA-Val (V)	4528–4579	52			TAC	3
tRNA-Lys (K)	4583-4632	50			CTT	–7
tRNA-Glu (E)	4626–4675	50			TTC	–9
tRNA-Thr (T)	4667–4725	59			TGT	37
*nad*4L	4763–5008	246	81	GTG/TAG		–13
*nad*4	4996–6220	1225	408	ATT/T		33
tRNA-His (H)	6254–6306	53			GTG	13
*nad*5	6320–7864	1545	514	ATA/TAG		5
tRNA-Leu (L2)	7870–7927	58			TAA	0
tRNA-Pro (P)	7928–7978	51			TGG	30
*Cyt*b	8009–9076	1068	355	ATG/TAA		12
*nad*1	9089–9949	861	286	TTG/TAG		–1
tRNA-Ser (S2)	9949–10001	53				0
NCR3	10002-10105	104				0
tRNA-Ile (I)	10106–10171	66			GAT	30
tRNA-Ser (S1)	10202-10264	63			ACT	4
tRNA-Met (M)	10269–10328	60			CAT	–1
*rrn*S	10328–10918	591				0
tRNA-Phe (F)	10919–10970	52			GAA	–4
*cox*2	10967–11596	630	209	ATG/TAA		15
tRNA-Cys (C)	11582–11640	59			GCA	1
*cox*3	11642–12326	685	228	GTG/T		27
tRNA-Ala (A)	12354–12406	53			TGC	1
tRNA-Arg (R)	12408–12468	61			TCG	14
tRNA-Asn (N)	12455–12511	57			GTT	47
*nad*2	12559–13359	801	266	GTG/TAG		1

NCR: non-coding region, bp: base pair, aa: amino acid, Ini/Ter: initial/terminal codons, Int. seq.: intergenic sequences

Phylogeny

Phylogenetic trees using ML and BI methods have almost identical topologies, which supported the division of the phylum Acanthocephala into three large monophyletic clades (clades I, II, and III), representing the classes Palaeacanthocephala, Archiacanthocephala, and Eoacanthocephala, respectively (Figure 4). The clade I consists of *M. hirudinaceus*, *O. luehei*, and *Moniliformis* sp., all belonging to the class Archiacanthocephala, which further

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Table 4. Nucleotide composition of the mitochondrial genome of *Acanthogyrus (Acanthosentis) bilaspurensis*

Nucleotide sequence	Size (bp)	A (%)	C (%)	T (%)	G (%)	A+T (%)	G+C (%)
Overall	13360	17.0	10.5	42.3	30.1	59.3	40.6
Protein coding genes	9786	14.4	10.3	44.2	31.0	58.6	41.3
Codon position*							
1st	3262	18.2	10.0	37.5	34.4	55.7	44.4
2nd	3262	13.5	13.3	50.7	22.5	64.2	35.8
3rd	3262	11.6	7.7	44.5	36.2	56.1	43.9
Ribosomal RNA genes	1470	25.4	11.9	35.3	27.3	60.7	39.2
Transfer RNA genes	1218	23.0	10.7	38.3	28.1	61.3	38.8
Non-coding region 1	415	29.2	12.5	29.2	29.2	58.3	41.7
Non-coding region 2	104	37.5	3.9	45.2	13.5	82.7	17.3

*Termination codons were excluded.

Table 5. Genetic code and codon usage for 12 PCGs in the mitochondrial genome of *Acanthogyrus (Acanthosentis) bilaspurensis*

Codon	aa	No.	%	Codon	Aa	No.	%
TTT	Phe	268	8.22	TAT	Tyr	104	3.19
TTC	Phe	28	0.86	TAC	Tyr	35	1.07
TTA	Leu	117	3.59	TAA	*	0	0.00
TTG	Leu	301	9.23	TAG	*	0	0.00
CTT	Leu	77	2.36	CAT	His	32	0.98
CTC	Leu	5	0.15	CAC	His	9	0.28
CTA	Leu	18	0.55	CAA	Gln	10	0.31
CTG	Leu	46	1.41	CAG	Gln	13	0.40
ATT	Ile	91	2.79	AAT	Asn	32	0.98
ATC	Ile	10	0.31	AAC	Asn	11	0.34
ATA	Met	33	1.01	AAA	Lys	20	0.61
ATG	Met	120	3.68	AAG	Lys	37	1.13
GTT	Val	251	7.69	GAT	Asp	44	1.35
GTC	Val	25	0.77	GAC	Asp	18	0.55
GTA	Val	44	1.35	GAA	Glu	15	0.46
GTG	Val	219	6.71	GAG	Glu	60	1.84
TCT	Ser	139	4.26	TGT	Cys	64	1.96
TCC	Ser	18	0.55	TGC	Cys	11	0.34
TCA	Ser	14	0.43	TGA	Trp	22	0.67
TCG	Ser	23	0.71	TGG	Trp	78	2.39
CCT	Pro	39	1.20	CGT	Arg	24	0.74
CCC	Pro	6	0.18	CGC	Arg	1	0.03
CCA	Pro	10	0.31	CGA	Arg	10	0.31
CCG	Pro	16	0.49	CGG	Arg	9	0.28
ACT	Thr	36	1.10	AGT	Ser	48	1.47
ACC	Thr	5	0.15	AGC	Ser	19	0.58
ACA	Thr	9	0.28	AGA	Ser	28	0.86
ACG	Thr	11	0.34	AGG	Ser	83	2.54

(*Continued*)

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Table 5. (*Continued*)

Codon	aa	No.	%	Codon	Aa	No.	%
GCT	Ala	63	1.93	GGT	Gly	141	4.32
GCC	Ala	19	0.58	GGC	Gly	31	0.95
GCA	Ala	12	0.37	GGA	Gly	16	0.49
GCG	Ala	15	0.46	GGG	Gly	149	4.57

No.: number of copies; *: Stop (termination) codon; aa: amino acid; Average# codons: 3262

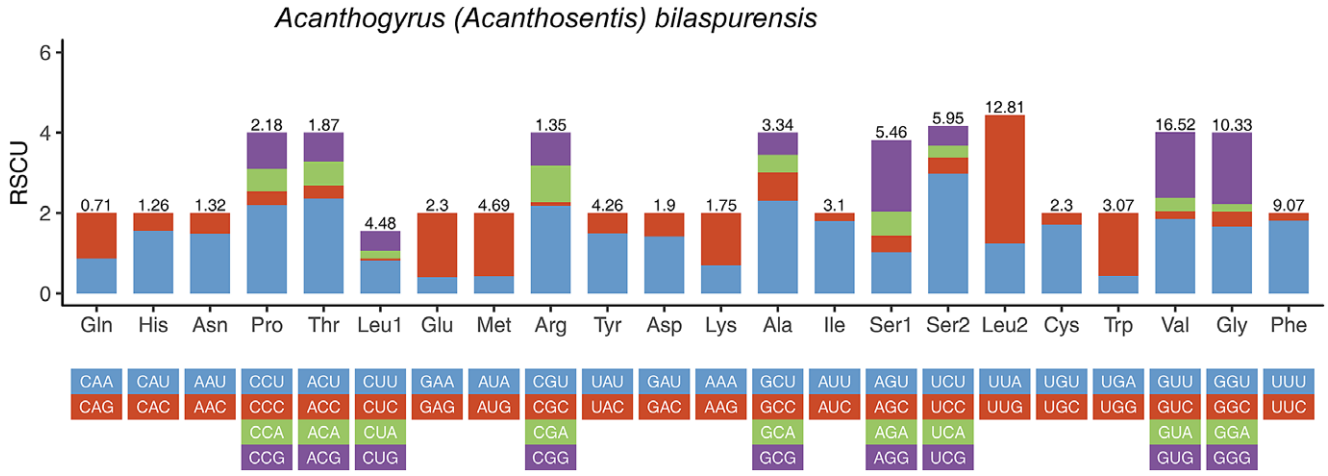

Figure 2. Relative synonymous codon usage (RSCU) of the 12 protein-coding genes (PCGs) of *Acanthogyrus* (*Acanthosentis) bilaspurensis.* The codon families are labelled on the x-axis. Values on the top of each bar represent amino acid usage in percentage.

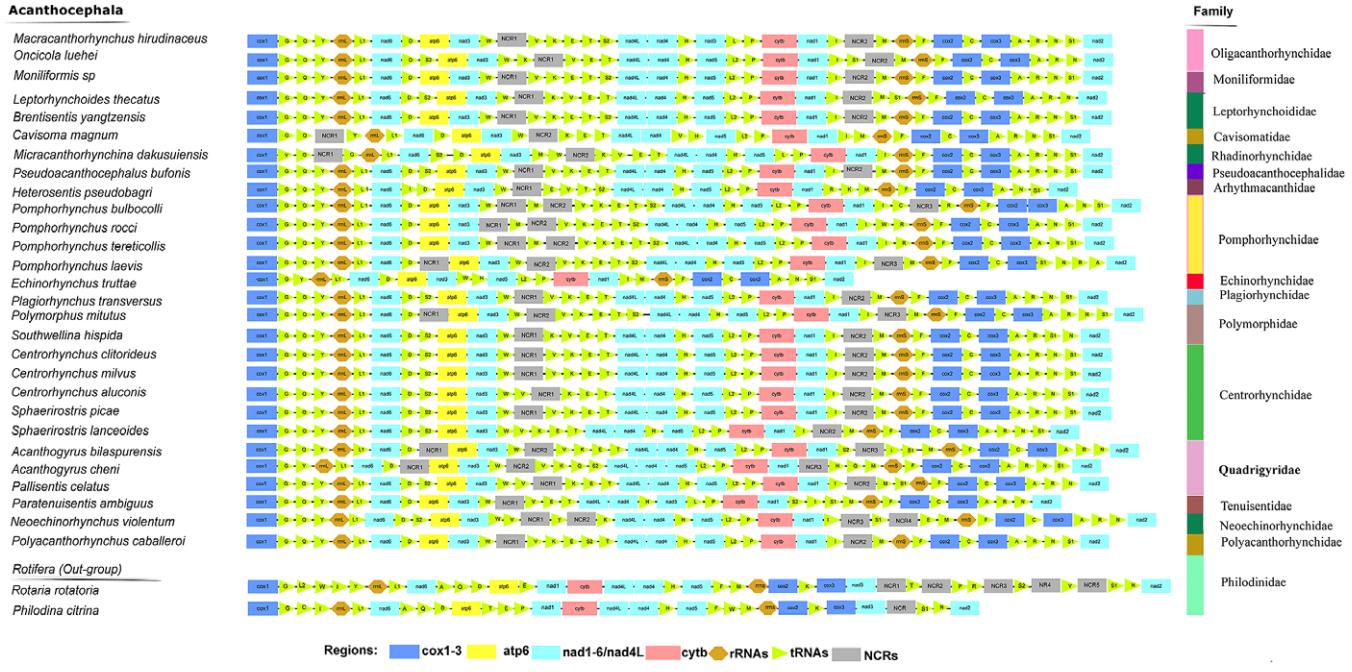

Figure 3. Linearized comparison of the mitochondrial gene arrangement for 28 acanthocephalan species and two rotifer species. All genes are encoded from left to right in the same direction. The tRNAs are labelled by single-letter code for the corresponding amino acid.

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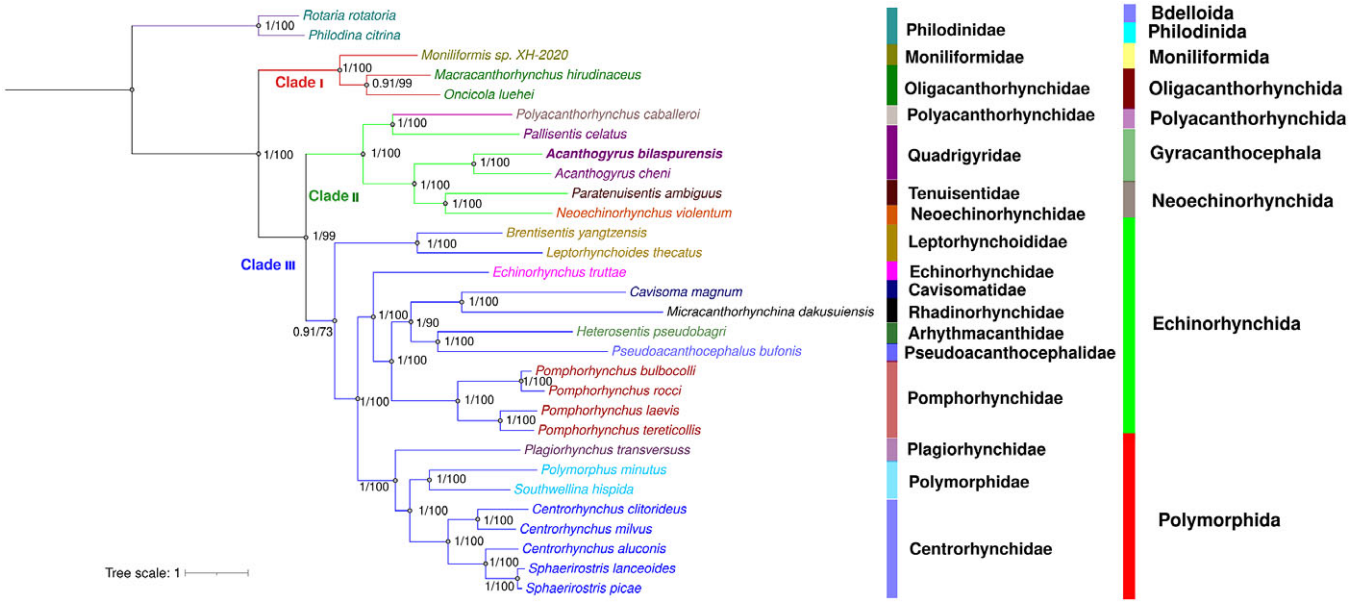

Figure 4. Phylogenetic relationships of acanthocephalans based on concatenating the amino acid sequences of 12 protein-coding genes (PCGs) of mitogenomes using Bayesian inference (BI) and maximum likelihood (ML) analyses. *Philodina citrina* and *Rotaria rotatoria* (Bdelloidea: Rotifera) were used as outgroups. Bootstrap values≥90 and Bayesian posterior probabilities values≥0.90 are shown in the phylogenetic trees.

confirmed the Archiacanthocephala as a sister to the remaining Acanthocephala. The results are consistent with the previous phylogenetic studies (Gao *et al.* 2022).

The clade II contains representatives of the classes Eoacanthocephala and Polyacanthocephala. Species of the class Polyacanthocephala (including only *Polyacanthorhynchus caballeroi* Diaz-Ungria & Rodrigo, 1960) nested in representatives of the class Eoacanthocephala, which is accordant with the previous mitogenomic phylogenies (Muhammad *et al.* 2019a, b, 2020a, b; Gazi *et al.* 2016; Song *et al.* 2019; Zhao *et al.* 2023; Dai *et al.* 2022). However, some phylogenetic analyses based on 18S, 18S+28S, and 18S+28S +*cox*1 sequence data supported that Polyacanthocephala is an independent class (Garey *et al.* 1996; García-Varela *et al.* 2002; García-Varela and Nadler 2006; Verweyen *et al.* 2011). Our phylogenetic analyses showed *A. bilaspurensis* clustered together with *A. cheni*, which displayed distant relationship to *P. celatus* in the family Quadrigyridae (Figure 4). The present phylogeny further confirmed the order Gyracanthocephala, the Quadrigyridae, and its subfamily Pallisentinae are not monophyletic, which is coincident with the previous phylogenetic studies based on mitogenomics and nuclear DNA data (Gazi *et al.* 2016; Song *et al.* 2019; Muhammad *et al.* 2019a, b, 2020a, b, c; Dai *et al.* 2022; Zhao *et al.* 2023).

The clade III includes species of the class Palaeacanthocephala. Phylogenetic results showed that the order Polymorphida is a monophyletic group, and the families Polymorphidae (including *Southwellina hispida* (Van Cleave, 1925) and *P. minutus*) and Centrorhynchidae (including *Centrorhynchus milvus* Ward, 1956, *Centrorhynchus clitorideus* Meyer, 1931, *C. aluconis* Müller, 1780, *Sphaerirostris lanceoides* Petrochenko, 1949, and *S. picae*) have a more close relationship than the family Plagiorhynchidae (including *P. transversus*) (Figure 4), which agreed well with some previous phylogenetic studies based on the mitogenomic data (Gazi *et al.* 2016; Song *et al.* 2019; Muhammad *et al.* 2019a, b, 2020a, b, c; Dai *et al.* 2022; Zhao *et al.* 2023) but conflicted with these phylogenies using 18S + 28S + *cox*1 data (García-Varela *et al.* 2013; Gazi *et al.* 2015) or some phylogenies based on the mitogenomic data (Muhammad *et al.* 2020a, b, c). Phylogenetic analyses also indicated the order Echinorhynchida is a paraphyletic group. However, the families Pseudoacanthocephalidae (including only *Pseudoacanthocephalus bufonis* Shipley, 1903) and Arhythmacanthidae (including only *H. pseudobagri*) displayed sister relationships, which is inconsistent with the previous phylogeny (Zhao *et al.* 2023).

Authors' contribution. NM and LL designed the study and analyzed mitogenomic data. NM performed the experiment, sequenced data, and wrote the manuscript. LL and RSS identified acanthocephalan specimens. NM and DXL performed the phylogeny. Suleman, DS, and MAA collected specimens. All authors read and approved the final manuscript.

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Competing interest. The authors declare that they have no competing interests.

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