



Characterization of the complete mitochondrial genome of *Miamiensis avidus* causing flatfish scuticociliatosis

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Abstract

Miamiensis avidus is a parasitic pathogen that causes the disease scuticociliatosis in teleost fish species. It is a ciliate and a free-living marine protozoan belonging to the order Philasterida, subclass Scuticociliatida, class Oligohymenophorea, and phylum Ciliophora. The complete mt-genome of *M. avidus* was linear and 38,695 bp in length with 47 genes, including 40 protein-coding genes, two ribosomal RNA (rRNA) genes, and five transfer RNA (tRNA) genes. Of these, 20 genes typically belong to the clusters of orthologous groups, playing roles in energy production and conversion, translation, ribosomal structure and biogenesis, and defense mechanisms. This is the first report of sequencing and characterization of the mt-genome of *M. avidus*, which was observed to be linear and possessing the typical ciliate mitochondrial genome organization and phylogenetic relationships. Remarkable differences were observed between *M. avidus* and other ciliates in the mitochondrially encoded rRNAs, extensive gene loss in ribosomal genes and tRNAs, terminal repeat sequences, and stop codon usage. A comparative and phylogenetic analysis of *M. avidus* and *Uronema marinum* of the order Hymenostomatida, which is most closely related to the order Philasterida, signified the promise of the mitogenome data of *M. avidus* as a valuable genetic marker in species detection and taxonomic research. The present study has potential applications in epidemiological studies and host-parasite interaction investigations facilitating disease control.

Keywords Mitochondrial genome · Parasite · *Miamiensis avidus* · Scuticociliatosis, clusters of Orthologous Groups · Alternative start codon

Introduction

Scuticociliatosis, a parasitic disease of fish and crustaceans is caused by invasive scuticociliates, which are free-living marine protozoa belonging to the subclass Scuticociliatida of the phylum Ciliophora (Gao et al. 2016). In the last decade, scuticociliate infection became a prominent parasitological problem in mariculture worldwide. There have been many reports of severe disease outbreaks caused by several scuticociliates species including *Uronema marinum*, *Philasterides dicentrarchi*, *Pseudocohnilembus persalinus*,

Anophryoides haemophila, and *Miamiensis avidus*, which have led to serious economic loss (Ragan et al. 1996; Kim et al. 2004a, b; Harikrishnan et al. 2012; Stidworthy et al. 2014; Iglesias et al. 2018; Li et al. 2018). Scuticociliates are characterized by their high potential for systemic invasion, infecting not only the surface of the body but also internal organs, including the brain, kidney, spleen, and the spinal cord, leading to high host mortality rates (Puig et al. 2007; Harikrishnan et al. 2010). The major clinicopathological manifestations of scuticociliatosis in infected fish are dark coloration, loss of scales, excessive body mucus, hemorrhagic and bleached spots on the skin, and severe dermal necrotic lesions that ultimately destroy tissues, leading to mortality (Jung et al. 2007; Jin et al. 2009; Moustafa et al. 2010).

Previous reports of scuticociliate infection, including pathological and chemotherapeutic studies, have focused on the prevention and control of scuticociliatosis, species identification for taxonomy, investigation on diversity, and on the diagnosis of scuticociliatosis. Little attention has been

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paid to the molecular mechanisms of pathogenicity, mainly due to a lack of basic research on areas such as the life cycle, genetics, and the genome of scuticociliates. Although morphological analysis of scuticociliates performed by a series of methods such as scanning electron microscopy, protein silver staining, and silver impregnation is an useful approach for identification, it is time-consuming and laborious, and thus, is not suitable for the identification of closely related species and for scuticociliatosis treatment (Jung et al. 2011; Huang et al. 2021).

Although molecular identification of scuticociliate species, such as *Pseudocohnilembus persalinus* (Jones et al. 2010), *Phiasterides dicentrachi* (DE Felipe et al. 2017), *Uronema marinum* (Smith et al. 2009) and *Miamiensis avidus* (Jung et al. 2005), has been performed through the DNA identification of mitochondrial cytochrome oxidase c subunit I (COI) sequences, mitochondrial small subunit ribosomal DNA (mtSSU-rDNA) sequences, and nuclear small subunit ribosomal DNA (nSSU-rDNA) sequences (Whang et al. 2013; Zhang et al. 2019), and through phylogenetic analyses based on SSU rDNA or the ITS1-5.8 S-ITS2 region sequences of related parasite species, these methods are limited in their identification of closely related species (Jung et al. 2011). In addition, due to the presence of different serotypes of scuticociliate species (Piazzón et al. 2008; Song et al. 2009), molecular detection in hosts with low parasite numbers and vaccine-mediated controls is difficult (Jung et al. 2011). Mitochondrial (mt) genomes are increasingly used to study the evolution and molecular epidemiology of scuticociliates such as *Pseudocohnilembus persalinus* isolated from Turbot (*Scophthalmus maximus* L.) (Gao et al. 2018) and *Uronema marinum* isolated from *Takifugu rubripes* (Li et al. 2018). The mt-genome of *Miamiensis avidus* causing flatfish scuticociliatosis has been largely uninvestigated.

Here, we sequenced and annotated the first complete mt-genome of *Miamiensis avidus*, in order to determine the nucleotide and amino acid similarities between the mt-genomes of *Miamiensis avidus* and other related ciliates.

Materials and methods

Ciliates isolation and cultivation

M. avidus strain was obtained from the Pathology Research Division of the National Institute of Fisheries Science, which were identified as *M. avidus* using species-specific oligonucleotide primers (Seo et al. 2013) were isolated from the ascitic fluids of olive flounders in a local farm (Pohang-si, Gyeongsangbuk-do, 2008). In a recent publication we isolated 32 clones harboring peptidase gene sequences from 1,265 EST clones of the *M. avidus* cDNA library that

related to infection of *M. avidus* by comparison of expression level between the cell-fed and the starved ciliates. Chinook salmon epithelia-214 cells were incubated at 20 °C in Eagle's minimum essential medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum and were used to grow the ciliates. The ciliates were then harvested by centrifugation at 200 × g for 5 min and washed three times by centrifugation at 150 × g for 5 min in 10 ml of phosphate-buffered saline (Sigma). The washed ciliates were counted using a hemocytometer.

DNA isolation, library preparation, and sequencing

The *M. avidus* ciliates were harvested by centrifugation at 200 × g for 5 min and washed more than three times by centrifugation at 150 × g for 5 min in Hanks' balanced salt solution (Sigma) or filtered seawater. The genomic DNA of *M. avidus* was extracted using a HiGene™ Genomic DNA Prep Kit (Biofact, Daejeon, Korea). Using a Covaris G-tube, fragments of 20 kb were generated by shearing the genomic DNA according to the manufacturer's protocol. The AMPureXP bead purification system was used to remove small fragments. A total of 5 µg of each sample was used as the input for the preparation of the library to be used in PacBio sequencing (Pacific Biosciences). The SMRTbell library was constructed using a SMRTbell™ Template Prep Kit 1.0 (PN 100-259-100). Using the BluePippin Size selection system, small fragments were removed from the large-insert library. After a sequencing primer was annealed to the SMRTbell template, DNA polymerase was bound to the complex (Sequel Binding Kit 2.0). Purification was performed using SMRTbell clean up columns (SMRTbell® Clean Up Columns v2 Kit-Mag; PN 01-303-600). The purification step was performed after polymerase binding to remove the unbound polymerases and the polymerase molecules that are bound to small DNA inserts. A MagBead Kit was used for the binding of the library complex with MagBeads before sequencing, since MagBead-bound complexes provide more reads per single-molecule real-time (SMRT) cell. This polymerase-SMRTbell-adaptor complex was then loaded into zero-mode waveguides. The SMRTbell library was sequenced using three SMRT cells (Pacific Biosciences, Sequel™ SMRT® Cell 1 M v2) using a sequencing kit (Sequel Sequencing Kit 2.1) and 1 × 600 min movies were captured for each SMRT cell using the PacBio sequencing platform (Pacific Biosciences).

Genomic DNA (gDNA) (200 ng) for Miseq was sheared using an S220 Ultra sonicator (Covaris) (peak incident power 175 W, duty factor 5%, 200 cycles per burst, treatment time 35 s). Library preparation was performed using an Illumina TruSeq Nano DNA sample prep kit (Illumina) according to the manufacturer's instructions. Briefly after

clean-up of fragmented gDNA using sample purification beads, the fragmented gDNA was end-repaired at 30 °C for 30 min, followed by size selection for 500 bp insert size using sample purification beads. A single 'A' nucleotide was added to the 3' ends of the blunt fragments using the a-tailing mix reagent by incubation at 37 °C for 30 min followed by incubation at 70 °C for 5 min. Indexing adapters were ligated to the ends of the DNA fragments using ligation mix 2 reagents at 30 °C for 10 min. After washing twice with sample purification beads, a PCR was performed to enrich the DNA fragments with adapter molecules on both ends. The thermocycler conditions were as follows: 95 °C for 3 min, 8 cycles of 98 °C for 20 s, 60 °C for 15 s, and 72 °C for 30 min, with a final extension at 72 °C for 5 min. Eventually, the quality and the band size of the libraries were assessed using an Agilent 2100 Bioanalyzer (Agilent). The libraries were quantified by qPCR using a CFX96 Real-Time System (Bio-Rad). After normalization, sequencing of the prepared library was performed using the MiSeq system (Illumina) with 300 bp paired-end reads.

Illumina Miseq sequencing, PacBio SMRT sequencing, and hybrid assembly

A total of 16.1 Gb of paired-end fastq files were produced using Illumina MiSeq paired-end sequencing, and Raw sequence data were deposited into Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra/>) with the accession no. PRJNA763762. Using these data, the genome size of coconut was estimated by *k*-mer distribution using the KmerGenie program (Chikhi and Medvedev 2014). Pre-assembled short-read next generation sequencing contigs were used to correct and derive a compact representation of the 7.8 Gb SMRT (Pacific Biosciences) long reads using a DBG2OLC hybrid assembler (Ye et al. 2016). The DBG2OLC assembler was used to assemble the raw PacBio SMRT sequence data, with the Illumina Miseq contig sequence assembly utilized as an anchor for error correction. The overlap and the consensus steps were executed with the following parameters: *k*-mer value: 19, adaptive *k*-mer matching threshold: 0.0001, fixed *k*-mer matching threshold: 2, minimum overlap score between a pair of long reads: 8, removal of chimeric reads: allow. The quality of the resulting assembly was assessed using a local Perl script (<https://github.com/aubombarely/GenoToolBox/blob/master/SeqTools/FastaSeqStats>). The assembled contigs were scanned for *M. avidus* mitochondrial sequence using local Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) with the *Tetrahymena thermophila* mtDNA sequence (NC_003029) as the search query. The complete *M. avidus* mitochondrial sequence with terminal repeat sequences at both ends was selected.

Annotation of the *M. avidus* mtDNA genome and phylogenetic analysis

Open reading frame (ORF) finding and gene prediction were performed using BLAST (BLASTn and BLASTx), GeneMarkS (Besemer et al. 2001), and Geneious (ver. R11). The large and small subunits of ribosomal RNA (rRNA) were identified using BLASTn with published ciliate rRNAs as queries and the transfer RNAs (tRNAs) were identified using the tRNAscan-SE search server (Lowe and Eddy 1997) (<http://lowelab.ucsc.edu/tRNAscan-SE/>). All protein-coding, rRNA, and tRNA genes, and genes in the novel mt-genomes were confirmed by multiple sequence alignment with published ciliate data using the T-Coffee package (Di Tommaso et al. 2011) (<http://www.tcoffee.org>). A graphical linear map of the complete mt-genome was produced using Circos v0.67 (Krzywinski et al. 2009). The whole mt-genome alignment of *M. avidus* was compared with that of other related ciliates (Table 1) using the MAUVE program (Mauve version 20,150,226 build 10) (Darling et al. 2010). To compare the *M. avidus* mt-genome with other ciliate mt-genomes, the respective amino acid sequences of the protein-encoding gene *rpl14* were aligned using ClustalX and neighbor-joining (NJ) analysis was conducted using MEGA 7.0 with 1000 bootstrap replicates (Kumar et al. 2016). In addition, the distance tree of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) genes was constructed using MEGA 7.0 with 1000 bootstrap replicates. The list of the taxa used in the *cox1* alignments is listed in Table 10. The additional sequences used for comparison and their GenBank accession numbers were as follows: *Philasterides dicentrarchi* *cox1_a* (accession number: MN531306.1), *P. dicentrarchi* *cox1_b* (GQ342957.1), *Pseudocohnilembus persalinus* *cox1* from freshwater-reared rainbow trout, *Oncorhynchus mykiss* (GU584095), *P. persalinus* *cox1* from Turbot (*Scophthalmus maximus*) (MH608212.2), *P. persalinus* *cox1* (GQ500579), *Uronema marinum* isolate ZZF20170302 *cox1* from Turbot (MG001901.1), and *Uronema heteromarinum* isolate FXP08082901 *cox1* (MG001901.1).

Results

Complete mt-genome organization and composition

The mt-genome of *M. avidus* was observed to be linear with two terminal repeat sequences (TRS; 30 bp) located at both ends and 38,695 bp in length with 47 genes, including 40 protein-coding genes (29 coding sequences, four large subunit ribosomal proteins [*rpl*], seven small subunit ribosomal

Table 1 Mitochondrial genome sequences of ciliates sequenced completely prior to the present study and used for sequence analyses

Species	Order	Family	Length (bp)	GenBank No.	Ref.
<i>Tetrahymena pyriformis</i>	Hymenostomatida	Tetrahymenidae	47,296	NC_000862.1	Burger et al. 2000
<i>Tetrahymena pigmentosa</i> strain UM1060	Hymenostomatida	Tetrahymenidae	46,990	DQ927305.1	Moradian et al. 2007
<i>Tetrahymena paravorax</i> strain RP	Hymenostomatida	Tetrahymenidae	47,496	DQ927304.1	Moradian et al. 2007
<i>Tetrahymena malaccensis</i> strain MP75	Hymenostomatida	Tetrahymenidae	47,691	DQ927303.1	Moradian et al. 2007
<i>Tetrahymena thermophila</i> strain SB210	Hymenostomatida	Tetrahymenidae	47,577	AF396436.1	Brunk et al. 2003
<i>Tetrahymena pigmentosa</i>	Hymenostomatida	Tetrahymenidae	46,990	NC_008339.1	Moradian et al. 2007
<i>Tetrahymena paravorax</i>	Hymenostomatida	Tetrahymenidae	47,496	NC_008338.1	Moradian et al. 2007
<i>Tetrahymena malaccensis</i>	Hymenostomatida	Tetrahymenidae	47,691	NC_008337.1	Moradian et al. 2007
<i>Ichthyophthirius multifiliis</i>	Hymenostomatida	Ichthyophthiriidae	51,686	NC_015981.1	Moradian et al. 2007
<i>Paramecium caudatum</i>	Peniculida	Parameciidae	43,660	NC_014262.1	Barth and Berendonk 2011
<i>Paramecium aurelia</i>	Peniculida	Parameciidae	40,469	NC_001324.2	Pritchard et al. 1990
<i>Uronema marinum</i>	Philasterida	Uronematidae	39,845	NC_039174.1	Li et al. 2018
<i>Miamiensis avidus</i>	Philasterida	Philasteridae	38,695	PRJNA763762	This study
<i>Oxytricha trifallax</i>	Sporadotrichida	Oxytrichidae	69,800	JN383843.1	Swart et al. 2012
<i>Laurentiella strenua</i>	Sporadotrichida	Oxytrichidae	66,721	KX529838.1	Smith and Keeling 2013
<i>Moneuplotes minuta</i>	Hypotrichia	Euplotidae	41,978	GQ903130	de Graaf et al. 2009
<i>Nyctotherus ovalis</i>	Metopida	Metopidae	41,666	GU057832	de Graaf et al. 2011

Bold Species indicate the mitogenome sequences of ciliates sequenced completely used for sequence analyses

proteins [*rps*]), two ribosomal RNA (rRNA) genes, and five transfer RNA (tRNA) genes (Fig. 1). The mt-genome organization of *M. avidus*, including a list of gene order, gene length, alternative start codons, and intergenic spacer regions, is given in Table 2. The nucleotide composition of the entire *M. avidus* mtDNA sequence was found to be 40.8% thymine, 39.1% adenine, 10.4% guanine, and 9.7% cytosine, while the complete mtDNA sequence had a high AT content of 79.87% (Table 3). Clusters of orthologous protein-coding genes (COGs) located in the *M. avidus* mt-genome included genes encoding 13 energy pathway proteins, including ATP synthase subunit 9 (*atp9*), two subunits of cytochrome c oxidase (*cox1* and *cox2*), apocytochrome b (*cob*), and nine NADH dehydrogenase subunits (*nad1a*, *nad1b*, *nad2*, *nad3*, *nad4*, *nad5*, *nad7*, *nad9*, and *nad10*), genes for translation, ribosomal structure, and biogenesis, including six ribosomal proteins (*rpl2*, *rps12*, *rps13*, *rpl14*, *rpl16*, and *rps19*), and genes encoding proteins involved in

defense mechanisms, such as putative mitochondrial protein *orf386* (*orf386*) (Table 4).

Codon usage

Seven alternative start codons were found to be used in the *M. avidus* mt-genome, in which 37 of the 40 protein-coding genes have been shown to use TAA as a termination codon, and three genes (*vejR*, *rps13*, and *rps3_a*) were shown to use TAG as a termination codon. On the whole, 27.5% of the genes were found to have an ATG start codon; 27.5%, ATT (*rpl2*, *rps12*, *rpl6/orf176*, *orf73*, *rpl16*, *nad4L/orf119*, *nad9*, *cob*, *nad5*, *nad1_a*, and *rps13*); 20%, TTA (*rps3_b/orf149*, *rps19*, *atp9*, *nad2_a/orf371*, *orf159*, *rpl14*, *orf143*, and *rps3_a/orf320*); 12.5%, ATA (*orf437*, *rps14/orf107*, *rps7/orf229*, *cox2*, and *nad6/orf246*); 5%, ATC (*nad4* and *vejR/orf571*); 5%, TTG (*orf195* and *orf492*); and only 2.5%,

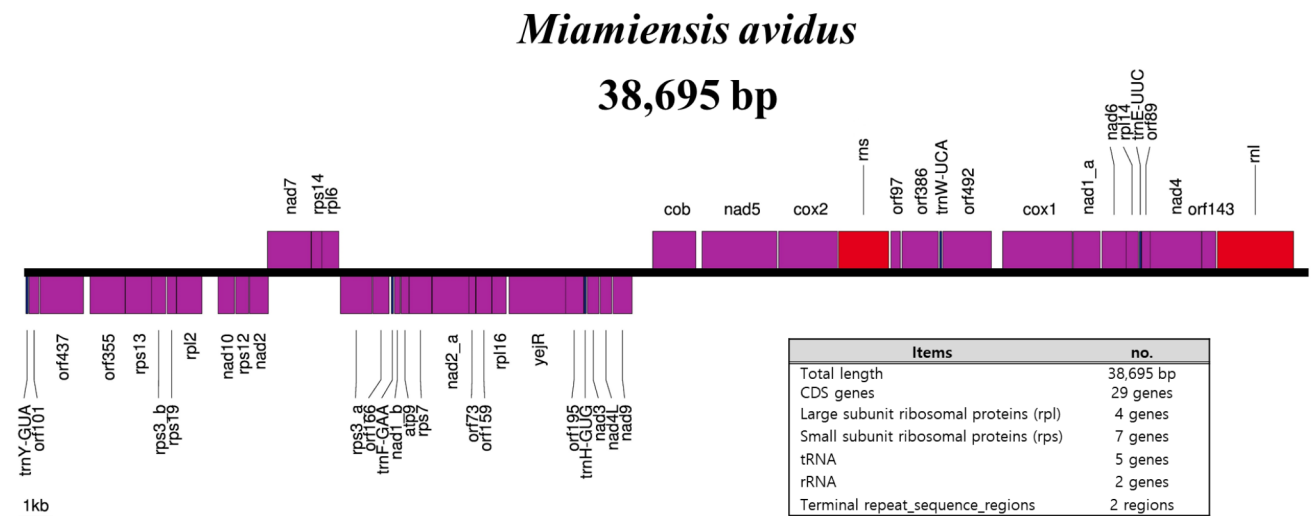
Table 2 Mitochondrial genome organization of *M. avidus*

Name	Type	Position		Size		Direction	Intergenic nucleotides	Codon	
		Start	Finish	DNA length	AA length			Start/Stop	Anticodon
repeat region	Repeat region	1	30	30		forward			
<i>trnY(gua)</i>	tRNA	45	125	81		reverse	14		GUA
<i>orf101</i>	CDS	143	448	306	101	reverse	17	ATG/TAA	
<i>orf437</i>	CDS	471	1784	1314	437	reverse	22	ATA/TAA	
<i>orf355</i>	CDS	1976	3043	1068	355	reverse	191	ATG/TAA	
<i>rps13</i>	CDS	3044	3841	798	265	reverse	0	ATT/TAG	
<i>rps3_b/orf149</i>	CDS	3822	4271	450	149	reverse	-20	TTA/TAA	
<i>rps19</i>	CDS	4297	4584	288	95	reverse	25	TTA/TAA	
<i>rpl2</i>	CDS	4588	5358	771	256	reverse	3	ATT/TAA	
<i>nad10</i>	CDS	5833	6339	507	168	reverse	474	ATG/TAA	
<i>rps12</i>	CDS	6363	6770	408	135	reverse	23	ATT/TAA	
<i>nad2_b</i>	CDS	6781	7359	579	192	reverse	10	GTG/TAA	
<i>nad7</i>	CDS	7326	8645	1320	439	forward	-34	ATG/TAA	
<i>rps14/orf107</i>	CDS	8649	8972	324	107	forward	3	ATA/TAA	
<i>rpl6/orf176</i>	CDS	8957	9487	531	176	forward	-16	ATT/TAA	
<i>rps3_a/orf320</i>	CDS	9524	10,486	963	320	reverse	36	TTA/TAG	
<i>orf166</i>	CDS	10,496	10,996	501	166	reverse	9	ATG/TAA	
<i>trnF(gaa)</i>	tRNA	11,060	11,131	72		reverse	63		GAA
<i>nad1_b</i>	CDS	11,152	11,331	180	59	reverse	20	ATG/TAA	
<i>atp9</i>	CDS	11,349	11,597	249	82	reverse	17	TTA/TAA	
<i>rps7/orf229</i>	CDS	11,594	12,283	690	229	reverse	-4	ATA/TAA	
<i>nad2_a/orf371</i>	CDS	12,290	13,405	1116	371	reverse	6	TTA/TAA	
<i>orf73</i>	CDS	13,383	13,604	222	73	reverse	-23	ATT/TAA	
<i>orf159</i>	CDS	13,611	14,090	480	159	reverse	6	TTA/TAA	
<i>rpl16</i>	CDS	14,091	14,528	438	145	reverse	0	ATT/TAA	
<i>yejR/orf571</i>	CDS	14,604	16,319	1716	571	reverse	75	ATC/TAG	
<i>orf195</i>	CDS	16,274	16,861	588	195	reverse	-46	TTG/TAA	
<i>trnH(gug)</i>	tRNA	16,867	16,939	73		reverse	5		GUG
<i>nad3</i>	CDS	16,964	17,326	363	120	reverse	24	ATG/TAA	
<i>nad4L/orf119</i>	CDS	17,349	17,708	360	119	reverse	22	ATT/TAA	
<i>nad9</i>	CDS	17,734	18,321	588	195	reverse	25	ATT/TAA	
<i>cob</i>	CDS	18,936	20,243	1308	435	forward	614	ATT/TAA	
<i>nad5</i>	CDS	20,421	22,685	2265	754	forward	177	ATT/TAA	
<i>cox2</i>	CDS	22,729	24,516	1788	595	forward	43	ATA/TAA	
<i>rns</i>	rRNA	24,535	26,053	1519		forward	18		
<i>orf97</i>	CDS	26,112	26,405	294	97	forward	58	ATG/TAA	
<i>orf386</i>	CDS	26,446	27,549	1104	367	forward	40	ATG/TAA	
<i>trnW(uca)</i>	tRNA	27,578	27,648	71		forward	28		UCA
<i>orf492</i>	CDS	27,672	29,150	1479	492	forward	23	TTG/TAA	
<i>cox1</i>	CDS	29,480	31,588	2109	702	forward	329	ATG/TAA	
<i>nad1_a</i>	CDS	31,603	32,427	825	274	forward	14	ATT/TAA	
<i>nad6/orf246</i>	CDS	32,471	33,211	741	246	forward	43	ATA/TAA	
<i>rpl14</i>	CDS	33,189	33,578	390	129	forward	-23	TTA/TAA	
<i>trnE(uuc)</i>	tRNA	33,595	33,665	71		forward	16		UUC
<i>orf89</i>	CDS	33,667	33,936	270	89	forward	1	ATG/TAA	
<i>nad4</i>	CDS	33,855	35,483	1629	542	forward	-82	ATC/TAA	
<i>orf143</i>	CDS	35,480	35,911	432	143	forward	-4	TTA/TAA	
<i>rnl</i>	rRNA	35,947	38,251	2305		forward	35		
repeat region	Repeat region	38,666	38,695	30		reverse	414		

a GTG start codon (*nad2_b*) in the *M. avidus* mt-genome (Table 2).

Table 3 Nucleotide composition of mitochondrial genome of *M. avidus*

Nucleotide	Length (bp)	A (%)	C (%)	T (%)	G (%)	A+T (%)	G+C (%)
Entire seq.	38,695	15,126 (39.09)	3,743 (9.67)	15,778 (40.78)	4,048 (10.46)	30,904 (79.87)	7,791 (20.13)
Protein-coding seq.	31,752	11,988 (37.76)	2,898 (9.13)	13,410 (42.23)	3,456 (10.88)	25,398 (79.99)	6,354 (20.01)
rRNA genes seq.	3,824	1,467 (38.36)	359 (9.39)	1,409 (36.85)	589 (15.40)	2,876 (75.21)	948 (24.79)
tRNA gene seq.	368	98 (26.63)	55 (14.95)	127 (34.51)	88 (23.91)	225 (61.14)	143 (38.86)

**Fig. 1** Visual representation of the linear mitochondrial genome of *Miamiensis avidus*. Protein-coding genes (40) are purple, tRNAs (5) are dark blue, rRNAs (2) are red, and Terminal Repeat Sequences

(TRS; 30 bp) at both ends are not shown. Gene composition in the whole mitochondrial genome of the *M. avidus* is indicated by a table below the mtDNA map

Comparative analysis of the complete mitogenomes of ciliates

The genome structure and organization of the mt-genomes (mitogenomes), the mitochondrially encoded gene content, and the TRS in the mitochondrial genome of *M. avidus* determined in the present study were compared to that of the mt-genomes of other ciliates reported previously (Table 1). As shown in Table 5, with the exception of a few gene losses, ciliate mitochondrial genomes share largely the same complement of known protein-coding genes. The content of the identified protein-coding genes was almost identical in *M. avidus* and *U. marinum* mtDNAs, both organisms belonging to the order Philasterida. Genes for the small and large subunit ribosomal RNAs (*rnl_b_1* and *rns_b*, respectively) were found in *M. avidus* mtDNA in contrast to the split large and small subunit ribosomal RNA genes and the duplicated large subunit rRNA genes in the *Tetrahymena* genus (Table 6). The *rnl* and *rns* genes in *M. avidus* were found to be not duplicated, similar to those in *T. pyriformis* and *I. multifiliis*.

The *M. avidus* mitogenome was also found to contain 12 *M. avidus* and ciliate-specific ORFs, called *Ymf* genes,

for which function a cannot be assigned, due to lack of sufficient sequence similarity to strongly indicate homology (Table 8). Three of these ORFs (*orf386*, *orf437*, and *orf492*) were also observed in *Tetrahymena*, *Ichthyophthirius*, and *Paramecium* mtDNAs (*Ymf67*, *Ymf66*, and *Ymf68* in *I. multifiliis*). The linear mitogenomes of ciliates have between 18 and 35 bp repeat sequences and have symmetrical ends forming TRS (Table 9). The terminal repeat regions in *M. avidus* and *U. marinum* were found to be 30 and 32 nt long, respectively. In six ciliate species, the terminal repeats at both ends of the mitochondrial DNA were observed to be completely different.

In general, *Miamiensis*, *Uronema*, *Tetrahymena*, and *Ichthyophthirius* mtDNAs had almost the same preference for relative synonymous codons, with only a slight difference (Fig. 2). The most frequent amino acids in the *M. avidus* mitogenome were leucine (Leu), phenylalanine (Phe), and isoleucine (Ile), with percentages of occurrence of 13.4%, 11.37%, and 10.51%, respectively. The least frequent amino acids were cysteine (Cys), histidine (His), and proline (Pro), with percentages of occurrence of 0.95%, 1.47%, and 1.72%, respectively.

Table 4 Clusters of orthologous protein-coding genes (COGs) located in *M. avidus* mt-genome

Start	End	Score	Strand	Functional category (COGclassification)	COG hit name	gene name	Annotation
3044	3841	30.43478	-	J = Translation, ribosomal structure and biogenesis	DR2125	<i>rps13</i>	ribosomal protein S13
4297	4584	43.75	-	J = Translation, ribosomal structure and biogenesis	SPAC1751.02c	<i>rps19</i>	ribosomal protein S19
4588	5358	30.45455	-	J = Translation, ribosomal structure and biogenesis	BMEI0760	<i>rpl2</i>	ribosomal protein L2
5833	6339	56.33803	-	C = Energy production and conversion	XF0306	<i>NAD10</i>	NADH dehydrogenase subunit 10
6363	6770	51	-	J = Translation, ribosomal structure and biogenesis	TM1505	<i>rps12</i>	ribosomal protein S12
6781	7359	27.86885	-	C = Energy production and conversion	AGc2364	<i>nad2</i>	NADH dehydrogenase subunit 2
7326	8645	46.88995	+	C = Energy production and conversion	BMEI1155	<i>nad7</i>	NADH dehydrogenase subunit 7
11,152	11,331	39.21569	-	C = Energy production and conversion	RSc2055	<i>nad1</i>	NADH dehydrogenase subunit 1
11,349	11,597	34.69388	-	C = Energy production and conversion	MPN603	<i>atp9</i>	ATP synthase F0 subunit 9
14,091	14,528	34.69388	-	J = Translation, ribosomal structure and biogenesis	Cj1700c	<i>rpl16</i>	ribosomal protein L16
16,964	17,326	33.33333	-	C = Energy production and conversion	mll1372	<i>nad3</i>	NADH dehydrogenase subunit 3
17,734	18,321	42.37288	-	C = Energy production and conversion	mll1369	<i>nad9</i>	NADH dehydrogenase subunit 9
18,936	20,243	23.36957	+	C = Energy production and conversion	CC0473	<i>cob</i>	apocytochrome b
20,421	22,685	34.65909	+	CP = Cytoplasm	RSc2051	<i>nad5</i>	NADH dehydrogenase subunit 5
22,729	24,516	44.77612	+	C = Energy production and conversion	BMEI1466	<i>cox2</i>	cytochrome c oxidase subunit 2
26,389	27,549	22.35294	+	V = Defense mechanisms	UU510	<i>orf386</i>	putative mitochondrial protein orf386
29,408	31,588	37.35955	+	C = Energy production and conversion	CC3406	<i>cox1</i>	cytochrome c oxidase subunit 1
31,603	32,427	29.32331	+	C = Energy production and conversion	RC1230	<i>nad1</i>	NADH dehydrogenase subunit 1
33,189	33,578	38.20225	+	J = Translation, ribosomal structure and biogenesis	TM1490	<i>rpl14</i>	ribosomal protein L14
33,855	35,483	29.2	+	C = Energy production and conversion	BMEI1146	<i>nad4</i>	NADH dehydrogenase subunit 4

Functional genes annotated by COGs of the mitochondrial genomes of 14 related ciliates (Huang et al. 2021) is in **bold**

Mitochondrial genome organization and phylogenetic relationships

To assess the extent and comparison of ciliate mitochondrial genome arrangements more closely, mt-genomes were aligned using the MAUVE program. The mt-genomes of *Miamiensis* and *Uronema* were found to be largely collinear. Those of *Tetrahymena* and *Ichthyophthirius* were largely collinear as well. In the large subunit ribosomal protein (*rpl14*)-based phylogeny, class Oligohymenophorea was monophyletic with the orders Philasterida, Hymenostomatida, and Peniculida clustered together, supported by significant statistical values (Fig. 3). Considering the order Philasterida to which *M. avidus* and *U. marinum* belong as a benchmark, the order Hymenostomatida, which is most closely related to Philasterida, revealed only slight differences in the order of common genes (Table 5).

Two major clusters were observed in the phylogenetic tree of the *cox1* gene in the *M. avidus* isolates (Fig. 4; Table 10). Cluster I included 11 strains (GJ01, Mie0301, WDB-0708, SJF-06 A, YS2, WD4, JJ4, JJ3, SJF-03 A, WS1, and Nakajima) and cluster II contained 8 strains (Iyo1, xiapul,

SJF-03B, YK1, YK2, JF05To, RF05To, and SK05Kyo). While the highest bootstrap values were observed in two major clusters of *cox1* gene (100%) including *M. avidus* SCUTICA2 strain, the low bootstrap values were observed in *Philasterides dicentrarchi* strains (33–52%).

Discussion

The complete mt-genome of *M. avidus* was observed to be remarkably compact when compared to other ciliate mitogenomes in similar taxonomic positions (Burger et al. 2000; Brunk et al. 2003; de Graaf et al. 2009; Harikrishnan et al. 2010; Gao et al. 2018; Power et al. 2019; Retalack et al. 2019) and had the shortest genome length and the least coding sequence (CDS) of genes. The most remarkable observation in the order Philasterida and Hymenostomatida mt-genomes is the very low GC content observed in certain species, for instance, in the five *Tetrahymena* species (18.5–21.3%), *U. marinum* (19.0%), and *I. multifiliis* (16.7%), while species such as *N. ovalis* and *P. Aurelia* had relatively high GC contents of 41.5% and 41.2%,

Table 5 Protein-coding genes in Ciliate mitochondrial genomes

Genes	<i>Miamiensis avidus</i>	<i>Uronema marinum</i>	<i>Tetrahymena pyriformis</i>	<i>Ichthyophthirius multifiliis</i>	<i>Paramecium aurelia</i>	<i>Oxytricha trifallax</i>	<i>Moneuplotes minuta</i>	<i>Nyctotherus ovalis</i>
<i>nad1 a</i>	*	*	*	*	*	*	*	* (no split determined)
<i>nad1 b</i>	*	*	*	*	*	*	*	
<i>nad2 a</i>	<i>ymf65 like (orf371)</i>	<i>ymf65</i>	<i>ymf65</i>	<i>ymf65</i>	<i>ymf65_a + b</i>	*	*	*
<i>nad2 b</i>	<i>nad2</i>	<i>nad2</i>	<i>nad2</i>	<i>nad2</i>	<i>nad2</i>	*	*	*
<i>nad3</i>	*	*	*	*	*	*	*	*
<i>nad4</i>	*	*	*	*	*	*	*	*
<i>nad4L</i>	* (<i>orf119</i>)	*	*	<i>ymf58</i>	*	*	*	*
<i>nad5</i>	*	*	*	*	*	*	*	*
<i>nad6</i>	* (<i>orf246</i>)	*	*	*	*	*	*	<i>ord236</i>
<i>nad7</i>	*	*	*	*	*	*	*	*
<i>nad9</i>	*	*	*	*	*	*	*	*
<i>nad10</i>	*	*	*	*	*	*	*	*
<i>cob</i>	*	*	*	*	*	*	*	*
<i>cox1</i>	*	*	*	*	*	*	*	*
<i>cox2</i>	*	*	*	*	*	*	*	*
<i>atp9</i>	*	*	*	*	*	*	*	*
<i>ccmF/yejR</i>	<i>yejR like (orf571)</i>	*	*	*	*	*	*	
<i>rps2</i>						*		<i>orf262</i>
<i>rps3 a</i>	<i>ymf64 like (orf320)</i>	<i>ymf64</i>	<i>ymf64</i>		<i>ymf64</i>	*	*	
<i>rps3 b</i>	<i>rps3 like (orf149)</i>	<i>rps3</i>	<i>rps3</i>	<i>rps3</i>	<i>rps3</i>	*	<i>orf190</i>	
<i>rps4</i>			<i>ymf76</i>		<i>ymf81 + 85</i>	*	*	
<i>rps7</i>	<i>ymf63 like (orf229)</i>	<i>ymf63</i>	<i>ymf63</i>		<i>ymf63</i>	*	<i>orf170</i>	
<i>rps8</i>			<i>ymf74</i>		<i>ymf84</i>	*	<i>orf125</i>	*
<i>rps10</i>			<i>ymf59</i>		<i>ymf59</i>	*	<i>orf111</i>	
<i>rps12</i>	*	*	*	*	*	*	*	*
<i>rps13</i>	*	*	*	*	*	*	<i>orf102</i>	
<i>rps14</i>	* (<i>orf14</i>)	*	*	*	*	*	<i>orf49+</i>	*
<i>rps19</i>	*	*	*	*	*	*	<i>orf155</i>	<i>orf199</i>
<i>rpl2</i>	*	*	*	*	*	*	*	*
<i>rpl6</i>	* (<i>orf176</i>)	*	*	<i>Ymf60</i>		*	*	*
<i>rpl14</i>	*	*	*	*	*	*	*	*
<i>rpl16</i>	*	*	*	*	*	*	*	*

Table 6 Ciliate mitochondrially encoded rRNAs

Genes	<i>Miamiensis avidus</i>	<i>Uronema marinum</i>	<i>Tetrahymena pyriformis</i> ^a	<i>Ichthyophthirius multifiliis</i>	<i>Paramecium aurelia</i> ^a	<i>Oxytricha trifallax</i> ^a	<i>Moneuplotes minuta</i> ^a	<i>Nyctotherus ovalis</i>
<i>rnl_a_1</i>			*	*	*	*	*	
<i>rnl_a_2</i>			*	*				
<i>rnl_b_1</i>	*	*	*	*	*	*	*	
<i>rnl_b_2</i>			*	*				
<i>rns a</i>			*	*	*	*	*	
<i>rns b</i>	*	*	*	*	*	*	*	

Estimates based on an experimental reassessment (van Hoek et al. 2000; Swart et al. 2012) and our sequence alignments

respectively (Johri et al. 2019). The low GC content of *Paramecium* mitogenomes is marked by a highly biased codon usage, with most synonymous positions exhibiting a strong bias for A or T nucleotides (Barth and Berendonk 2011). Only TAA was found to terminate protein-coding genes, whereas TAG never occurred in *P. caudatum* and

Tetrahymena. In the *M. avidus* mitogenome, 37 of the 40 protein-coding genes were shown to use TAA as the termination codon, and three genes (*yejR*, *rps13*, and *rps3_a*) were shown to use TAG as the termination codon.

COGs located in the *M. avidus* mitogenome showed that most of the functional genes were related to secondary

Table 7 Ciliate mitochondrially encoded tRNAs

tRNA	<i>Miamiensis avidus</i>	<i>Uronema marinum</i>	<i>Tetrahymena pyriformis</i>	<i>Ichthyophthirius multifiliis</i>	<i>Paramecium aurelia</i>	<i>Oxytricha trifallax</i>	<i>Moneuplotes minuta</i>	<i>Nyctotherus ovalis</i>
<i>trnC</i>						*		
<i>trnE</i>	*	*	*	*		*	*	
<i>trnF</i>	*	*	*	*	*	*	*	*
<i>trnH</i>	*	*	*			*	*	
<i>trnK</i>						*		
<i>trnL</i>			*			*		
<i>trnM i</i>		*	*		*	*		
<i>trnM ii</i>						*	*	
<i>trnQ</i>						*	*	
<i>trnW</i>	*	*	*	*	*	*	*	*
<i>trnY</i>	*	*	*	*	*	*	*	*

Table 8 *M. avidus*-specific ORFs in mitochondrial genome

CDS	<i>Miamiensis avidus</i>	<i>Uronema marinum</i>	<i>Tetrahymena pyriformis</i>	<i>Ichthyophthirius multifiliis</i>	<i>Paramecium aurelia</i>	<i>Oxytricha trifallax</i>	<i>Moneuplotes minuta</i>	<i>Nyctotherus ovalis</i>
<i>orf73</i>	*							
<i>orf89</i>	*	* (<i>ymf70</i>)	* (<i>orf89</i>)	* (<i>Ymf70</i>)				
<i>orf97</i>	*	* (<i>ymf56</i>)	* (<i>orf97</i>)					
<i>orf101</i>	*	* (<i>ymf57</i>)	* (<i>orf100</i>)	* (<i>Ymf57</i>)				
<i>orf143</i>	*							
<i>orf159</i>	*	* (<i>orf149</i>)	* (<i>ymf59</i>)	* (<i>ymf59</i>)				
<i>orf166</i>	*	* (<i>ymf75</i>)	* (<i>orf188</i>)	* (<i>ymf75</i>)				
<i>orf195</i>	*	* (<i>orf202</i>)						
<i>orf355</i>	*	* (<i>orf346</i>)	* (<i>orf421</i>)					
<i>orf386</i>	*	* (<i>ymf67</i>)	* (<i>orf443</i>)	* (<i>Ymf67</i>)	* (<i>orf13</i>)			
<i>orf437</i>	*	* (<i>ymf66</i>)	* (<i>orf448</i>)	* (<i>Ymf66</i>)	* (<i>orf17</i>)			
<i>orf492</i>	*	* (<i>ymf68</i>)	* (<i>orf593</i>)	* (<i>Ymf68</i>)	* (<i>orf14</i>)			

Numbers of *M. avidus* CDS indicate number of amino acid residues in each ORF

Table 9 Comparison of Terminal Repeat Sequences (TRS) with other ciliates

Species	Sequences (5' \diamond 3')	Lengths	GC contents (%; TRS / mtDNA)	Identity (%)
<i>Miamiensis avidus</i>	ATTATACATCTTACTTTTAAGTTAGTCAAT	30	20.00 / 20.13	ID
<i>Uronema marinum</i>	TATAGTTCACTACTTGTTACCTATGTATCATA	32	28.13 / 19.00	47.0
<i>Tetrahymena pyriformis</i>	AGTAGATATGAACTAAAGAGACACGAGGGT	31	38.71 / 21.32	45.1
<i>Paramecium aurelia</i>	AAATATTAATATATTTATATTTTTTATTTTAAT	34	0.00 / 41.24	58.3
<i>Oxytricha trifallax</i>	CGACTCCTCTATCCTCATCCTAGACTCCGCTTACT	35	51.42 / 23.83	37.1
<i>Moneuplotes minuta</i>	ATAGTATATAATGTATAC	18	16.67 / 35.26	26.6

metabolite biosynthesis, transport, and catabolism, as has been observed in the mitochondrial genomes of 14 related ciliates (Huang et al. 2021). The *M. avidus* mitogenome shares a number of structural features with the existing ciliate mt-genome showing molecular affinity and similarities in encoded gene content with other members of the order,

such as Philasterida, Hymenostomatida, and Peniculida. Indeed, *M. avidus* had extensive gene loss, especially for ribosomal proteins, compared to species within Hymenostomatida, with only two ribosomal genes in its mitochondrial genome and only five tRNAs.

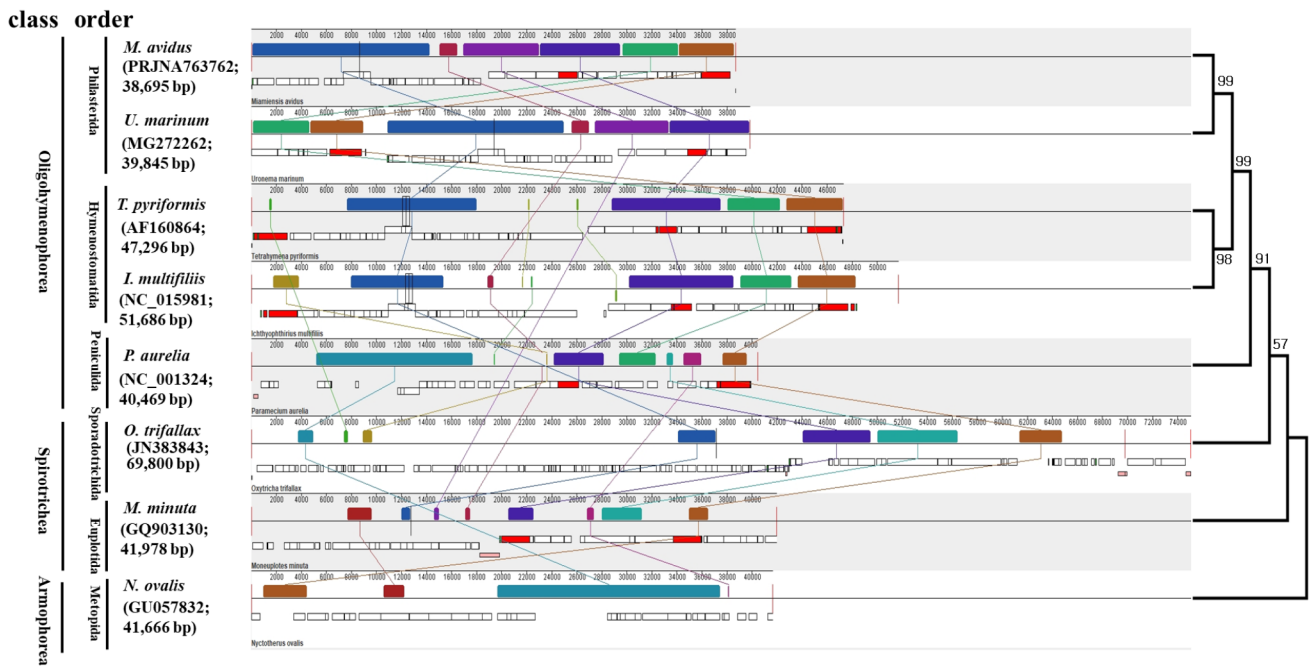


Fig. 3 Whole mitochondrial genome alignment of other ciliate genomes by the MAUVE program (Mauve version 20,150,226 build 10). Color blocks of the same color represent homologous regions

between different mitogenomes. GenBank accession numbers are provided (in parentheses) for all reference sequences

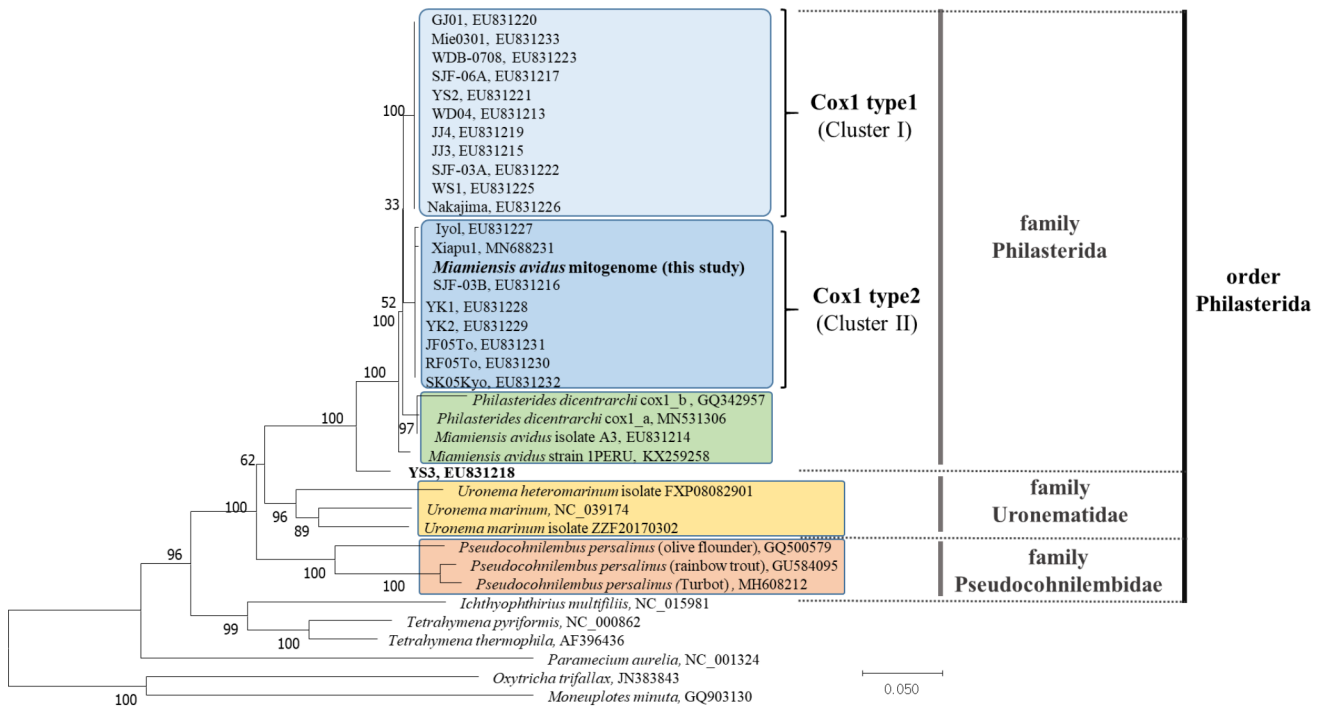


Fig. 4 Phylogenetic tree of *Miamiensis avidus* strains based on the Cox 1 gene. Abbreviation: Cox 1, Cytochrome c oxidase I

RNA (SSU and LSU) and *cox1* genes, *cox1* was similar to SSU and better than LSU in discriminating between *M. avidus* and related scuticociliates, which has been used

previously in investigations of a wide range of human and nonhuman infectious diseases. These *cox1* types have not been found to reflect geographical origins and host species

Table 10 *Miamiensis avidus* isolates used in this study

<i>M. avidus</i> strain	Host	Sam-pling year	Geo-graphic origin	Genbank accession No.	Cox 1 Type
WS1	<i>Parali-chthys olivaceus</i>	2003	Korea, Wando	EU831225	1
SJF-03 A	<i>P. olivaceus</i>	2003	Korea, Wando	EU831222	1
SJF-03B	<i>P. olivaceus</i>	2003	Korea, Wando	EU831216	2
YK1	<i>P. olivaceus</i>	2003	Korea, Youngk-wang	EU831228	2
YK2	<i>P. olivaceus</i>	2003	Korea, Youngk-wang	EU831229	2
Mie0301	<i>P. olivaceus</i>	2003	Japan, Owase	EU831233	1
JJ3	<i>P. olivaceus</i>	2004	Korea, Jeju	EU831215	1
JJ4	<i>P. olivaceus</i>	2004	Korea, Jeju	EU831219	1
WD04	<i>P. olivaceus</i>	2004	Korea, Wando	EU831213	1
YS2	<i>P. olivaceus</i>	2005	Korea, Yosu	EU831221	1
JF05To	<i>P. olivaceus</i>	2005	Japan, Tottori	EU831231	2
RF05To	<i>Pleu-ronichthys cornutus</i>	2005	Japan, Tottori	EU831230	2
SK05Kyo	<i>P. olivaceus</i>	2005	Japan, Kyoto	EU831232	2
A3	<i>P. olivaceus</i>	2006	Korea, Jeju	EU831214	-
SJF-06 A	<i>P. olivaceus</i>	2006	Korea, Goheung	EU831217	1
YS3	<i>P. olivaceus</i>	2006	Korea, Yosu	EU831218	-
Iyo1	<i>P. olivaceus</i>	2006	Japan, Iyo	EU831227	2
Nakajima	<i>P. olivaceus</i>	2006	Japan, Matsuyama	EU831226	1
WDB-0708	<i>P. olivaceus</i>	2007	Korea, Wando	EU831223	1
GJ01	<i>P. olivaceus</i>	2007	Korea, Geoje	EU831220	1
1PERU	<i>Parali-chthys adspersus</i>	2014	Peru	KX259258	-
xiapu1	<i>Larimich-thys crocea</i>	2018	China	MN688231	2
SCUTICA2(this study)	<i>P. olivaceus</i>	2008	Korea, Pohang	PRJNA7637622	

(Jung et al. 2011). In the present study, we performed a comparative and phylogenetic analysis of *cox1* genes from the mitochondrial genomes of *M. avidus* and the serotypes of

M. avidus strains. The mitochondrial *cox1* gene of *M. avidus* which belongs to the order Philasterida of the subclass Scuticociliatida showed promise as a valuable genetic marker for species detection. Based on previous studies, intraspecific variations in *cox1* are not attributed to the infectivity and virulence demonstrated in some strains (Nakajima, WS1, YK1, Iyo1, and Mie 0301) of *M. avidus* that are highly pathogenic to olive flounder (Song et al. 2009). However, the *cox1* cluster I and II types in the serotypes A and B of the *M. avidus* strains showed cross-immobilization/agglutination activities with the anti-sera against serotypes A and B, respectively. The present study can be utilized in epidemiological studies by informing detection, taxonomic research, strain identification, geographical spread, and disease control.

Miamiensis avidus is a dangerous parasitic pathogen that causes scuticociliatosis in fish and high mortality rates in mariculture worldwide. Methods to identify this species from among closely related species were limited. Mitochondrial DNA sequences can be valuable genetic markers for species detection and diagnostics, which are increasingly used in molecular epidemiology and surveillance tool. This is the first report in which the mitogenome of *M. avidus* was thoroughly compared to seven related ciliate mitogenomes by analyzing the nucleotide composition, codon usage, genome organization, protein-coding genes, and the terminal repeat sequences. The results of this study could facilitate better understanding of scuticociliate infection, aid in the development of control measures against scuticociliatosis, and provide insights into the molecular epidemiology of scuticociliates.

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Declarations

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