Title

A Reevaluation of the Phylogenetic Position of Antonbruunia (Annelida: Phyllodocida) and New Species Description

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Publication Date

2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

A Reevaluation of the Phylogenetic Position of Antonbruunia (Annelida: Phyllodocida) and New Species Description

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

Sonja Huč

Committee in charge:

Professor Gregory Rouse, Chair
Professor Nicholas Holland
Professor Deirdre Lyons

2022

The Thesis of Sonja Huč is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

DEDICATION

Dedicated to my cat, Oskar, who spent countless hours in front of the computer screen with me, silently cheering me on (or hoping I would finally do something interesting) and giving support (waiting for food) when I most needed it.

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ACKNOWLEDGEMENTS

Thank you to my great-grandmother (Mama) Milena Trontelj, for being one of the first to spark my love of marine invertebrates. Thank you to my husband, Oton Pavlič for his incredible support throughout my MS degree. Thank you to my parents Helena Trontelj and Branko Huč for their support in my academic endeavors. Thank you to Pooja Balaji, Tiffany Wong, and Gabriella Berman for being next to me for my entire degree, Viktoria Forte Berlec, Michelle Berra, Tjaša Trajbarič for being with me in spirit from across the ocean, and the rest of my friends and family, who are equally as important parts of my support system, enabling me to complete this degree. Thank you to Marina McCowin and Avery Hiley for their continuous support as friends and colleagues in the lab, and for being available for every question I ever had. Thank you to Charlotte Seid for her support, friendship, and kind mentorship. Thank you also to Chrissy Tustison, Kaila Pearson, Cassandra Koga, Eesha Rangani, Kiirah Green, Paul Proctor, Rachel Kroesche, Hee-seung Hwang, María Emilia Bravo and others for making the lab feel like a family. Thank you to my advisor Greg Rouse for making this possible, to my advising committee member Nick Holland for all the stories, and to my advising committee member Dede Lyons for her encouragement.

This thesis in full is currently being prepared for submission for publication of the material. Huč, Sonja; Hiley, Avery S.; McCowin, Marina F.; Rouse, Greg W. The thesis author was the primary researcher and author of this material. Thank you also to the captains and crews of the research vessels that made sample collection possible throughout the years and Dr. Fredrik Pleijel and Dr. Greg Rouse for collecting annelid specimens by hand. Thank you to Dr. Charlotte Seid for her assistance with sample organization and draft review, and thank you to the entire Rouse Lab for their support.

ABSTRACT OF THE THESIS

A Reevaluation of the Phylogenetic Position of Antonbruunia (Annelida: Phyllodocida) and New Species Description

by

Sonja Huč

Master of Science in Marine Biology

University of California San Diego, 2022

Professor Gregory Rouse, Chair

The new species *Antonbruunia milenae* sp. nov. found off the coast of San Diego, CA, USA at a depth of 845 m inside the *Calyptogena pacifica* Dall, 1891 clam is described. It is used to reevaluate the phylogenetic position of *Antonbruunia* Hartman & Boss, 1966 with analyses using 20 Pilargidae Saint-Joseph, 1899 species and three Nephtyidae Grube, 1850 species as an outgroup. The three gene and sixteen gene concatenated maximum likelihood phylogenies show that *Antonbruunia* is a pilargid, making Antonbruunidae Fauchald, 1977 a junior synonym of Pilargidae. In addition, Hermundurinae subfam. nov. is erected, *Glyphohesione* Friedrich, 1970 is moved out of Pilarginae Saint-Joseph, 1899, and Synelminae Salazar-Vallejo 1987 is emended to include *Antonbruunia*, *Glyphohesione*, and *Otopsis* Ditlevsen, 1917.

INTRODUCTION

Annelids are one of the most diverse animal groups in existence, with over 20,000 described species (Capa & Hutchings, 2021; G. Rouse et al., 2022). Until the last few decades, morphology was the only way of creating phylogenetic trees and placing annelid taxa within the tree of life. Now that molecular phylogenetic methods are well established and readily available, our understanding of the placement of many taxa has been revised using DNA, for instance on lower (K. A. M. Pearson & Rouse, 2022; Watson et al., 2019) or higher (Summers & Rouse, 2014; Tilic et al., 2022) taxonomic levels. This is an ongoing process, partially because it is more difficult to obtain samples of some clades due to rarity or inaccessibility of their habitat, as is the case with the genus *Antonbruunia* Hartman & Boss, 1966. It is mostly found in the deep sea, and each species has been found only once (Hartman & Boss, 1965; Mackie et al., 2015; Quiroga & Sellanes, 2009). With the collection of a fourth species, *Antonbruunia milenae* sp. nov. described herein, the position of the genus in the annelid phylogeny can be thoroughly reevaluated.

Antonbruunia is a genus of inquiline phyllodocid annelid worms that live inside chemosynthetic bivalves from the families Lucinidae J. Fleming, 1828, Thyasiridae Dall, 1900 (1895), and Vesicomyidae Dall & Simpson, 1901 (Imparidentia) (Mackie et al., 2015). The first species to be described, *Antonbruunia viridis* Hartman & Boss 1965, was found inhabiting the mantle cavity of *Opalocina fosteri* Hartman & Boss 1965 (Bivalvia: Lucinidae) off the coast of Madagascar at a depth of 70 m. In each opened bivalve, there was almost always one smaller male and one larger female present. The specific epithet '*viridis'* was chosen because of the females' vibrant green coloration in life, while the generic name was chosen in honor of Danish oceanographer and marine biologist Anton Frederick Bruun (Roberts, 2013), because the

research vessel from which the worm was discovered was named after him (Hartman & Boss, 1965).

Since its first description, the taxonomic placement of *Antonbruunia* has been a source of discussion and uncertainty. Hartman & Boss (1965) speculated that their new genus was related to, but not within the family Pilargidae Saint-Joseph, 1899. This is because *Antonbruunia*, like the discovered pilargids up to that point, had only simple chaetae, paired enlarged cirri on the first segment, and a simple prostomium. Unlike pilargids, *Antonbruunia* was sexually dimorphic, with parapodia that appeared uniramous even though they were biramous and lacked eyes. Fauchald (1977) erected Antonbruunidae Fauchald, 1977 with the only differing characteristics from Pilargidae being sexual dimorphism and an inquiline lifestyle. However, some researchers have since argued that *Antonbruunia* should be within Pilargidae, stating that pilargids are a relatively morphologically diverse family (Glasby, 1993; S. I. Salazar-Vallejo, 1986). Salazar-Vallejo (1986) argued that the differences Fauchald listed for *Antonbruunia* are a result of their lifestyle, and that creating a new family was unjustified since similar morphological changes have occurred in other annelid groups. Miura & Laubier (1990) considered if *Antonbruunia* should be in Chrysopetalidae Ehlers 1864, though ultimately decided the genus was more related to Pilargidae based on morphology. Fitzhugh & Wolf (1990) erroneously (Glasby, 1993) decided Salazar-Vallejo mistook the type of chaetae present in *Antonbruunia viridis* and therefore acknowledged Antonbruunidae, since Salazar-Vallejo was also unclear in the interpretation of the prostomial appendages. Others ultimately concluded that there was not enough information available at the time to confidently place these worms in the tree of life based solely on morphology (Beesley et al., 2000; Pleijel & Dahlgren, 1998; Purschke et al., 2019; G. Rouse et al., 2022; G. Rouse & Pleijel, 2001).

A second species, *Antonbruunia gerdesi* Quiroga & Sellanes, 2009, was discovered inside *Calyptogena gallardoi* Sellanes & Krylova, 2005 (Bivalvia: Vesicomyidae) at a depth of 750 – 900 m off the coast of Chile (Quiroga & Sellanes, 2009). Within three of the four bivalves where the species was found there was only a single worm, while the fourth bivalve contained three. Mackie et al. (2015) found *Antonbruunia sociabilis* Mackie, Oliver & Nygren, 2015 inside *Thyasira scotiae* P. G. Oliver & Drewery, 2014 (Bivalvia: Thyasiridae) off the coast of Scotland at a depth of 1200 m. The name refers to the fact that multiple specimens were found within each dissected clam. They generated mitochondrial 16S rRNA (16S) and nuclear 18S rRNA (18S) sequences to assess the position of *Antonbruunia*.

However, neither of the two competing theories, that *Antonbruunia* is either closely related to Pilargidae but distant enough to warrant its own family, or that *Antonbruunia* lies within Pilargidae, could be properly assessed. Mackie et al. (2015) analyzed the *Antonbruunia sociabilis* sequences with representatives from fifteen other families of annelids and found that *Antonbruunia* is a sister taxon to Pilargidae, represented in their tree by the genera *Ancistrosyllis* McIntosh, 1878 and *Sigambra* Müller, 1858. However, this result was only seen in the Bayesian analysis when a concatenated data set of 16S and 18S sequences was used; in all other variations with maximum likelihood or single gene analyses, the position of these worms was inconclusive. They also lacked a sufficient diversity of Pilargidae to allow for the placement of *Antonbruunia* within Pilargidae.

In 2020, *Antonbruunia* specimens were found inside *Calyptogena pacifica* Dall, 1891 (Bivalvia: Vesicomyidae) from a whalefall off California at a depth of ~845 m. This find, along with previously collected yet unstudied pilargid museum specimens, presents an opportunity to investigate the phylogenetic position of *Antonbruunia* and other pilargid genera with greater

phylogenetic resolution than previously possible. This study combines newly obtained genetic and morphological pilargid data and publicly available sequences to reassess the phylogenetic position of *Antonbruunia* (species overview in Table 1) and describe the newly found species.

Table 1: Overview of *Antonbruunia* species.

Species	**Location**	**Depth (m)**	**Host**	**Worms per Host**	**Habitat**
A. viridis	Madagascar	70	*Lucina fosteri*	Usually 2 (male + female)	Hypoxic sediment
A. gerdesi	Chile	800	*Calyptogena gallardoi*	Usually 1	Gass hydrate environment
A. sociabilis	Scotland	1200	*Thyasira scotiae*	2-9	Sulfide seep
A. milenae sp. nov.	CA, USA	845	*Calyptogena pacifica*	2 (male + female)	Whale fall

METHODS

Sample Collection

Specimens of *Antonbruunia milenae* sp. nov. were recovered inside *Calyptogena pacifica* (Bivalvia: Vesicomyidae) clams collected using the ROV (remotely operated vehicle) *Doc Ricketts* piloted off the R/V (research vessel) *Western Flyer* in 2020 at the Rosebud whalefall, 845 m depth, off San Diego, California, USA. Thirteen pilargid and one nephtyid species were collected from various localities (Fig. 1, Table 2) between 1998 and 2020 either by hand (SIO-BIC: A4721, A4846, A2831, A5771, A13955) or by research vessels (R/V Western Flyer, R/V Atlantis, R/V Falkor, R/V Nereus, R/V Melville, R/V Burin) using dredges, the Soutar boxcore, the van Veen grab, the ROVs Doc Ricketts and SuBastian, or the HOV (human operated vehicle) Alvin. Live specimens were photographed, morphological voucher specimens were fixed in 5-10% formalin in sea water, rinsed and transferred to 50% ethanol, while specimens for genetic analysis (whole animals or samples of the posterior or midsection) were fixed directly in 95% ethanol. Specimens were deposited at the Benthic Invertebrate Collection at Scripps Institution of Oceanography (SIO-BIC), La Jolla, California, USA. Additionally, whole genomic DNA extractions of *Pilargis wolfi* Salazar-Vallejo & Harris, 2006 (USNM1499416) and *Hermundura fauveli* Berkeley & Berkeley, 1941 (USNM1643733) were borrowed from the National Museum of Natural History (USNM) and the physical voucher for the same specimen of *Hermundura fauveli* was borrowed from the Florida Museum of Natural History (UF). Sequences from the rest of the specimens were obtained from NCBI GenBank and the Barcode of Life Database (BOLD). Specimen details are summarized in Table 2.

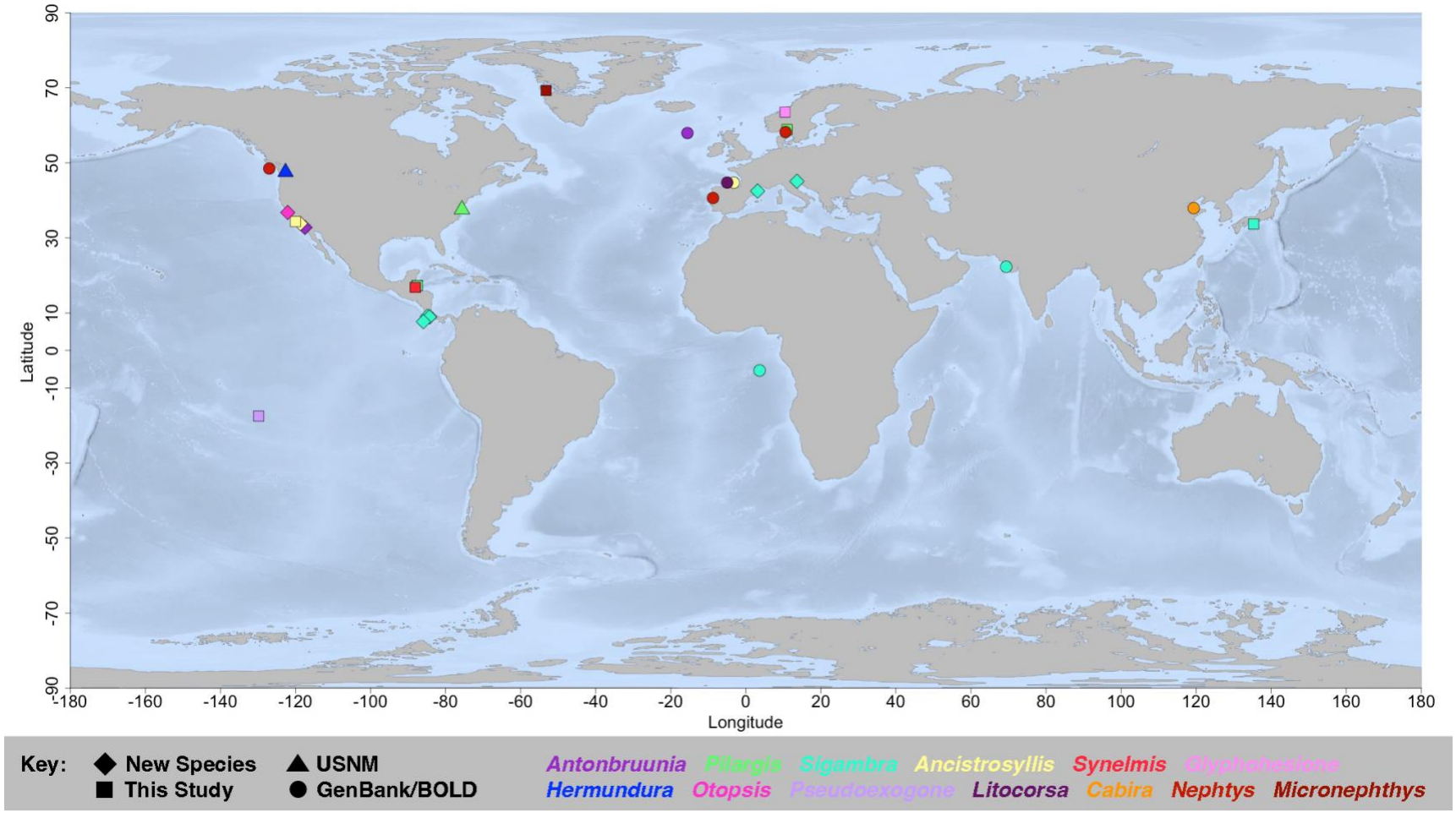

Figure 1: Collection locality map of annelids used in this study. Diamond shapes represent new species obtained in this study, square shapes represent other annelids obtained in this study, triangle shapes represent pilargids borrowed from USNM, and circle shapes represent the localities of annelids from GenBank and BOLD. Shapes are color coded by annelid genera.

Table 2: All used and obtained sequences in this study. Key: newly obtained sequences are set in bold, a single asterisk (*) next to a sequence SIO-BIC or USNM code or GenBank or BOLD accession number signifies the sequence was obtained through Sanger sequencing and not whole genome skimming.

Species	Locality	Depth (m)	COI	16S rRNA	18S rRNA	Mitogenome	Reference
Antonbruunia milenae sp. nov.	San Diego, CA, USA (32.7769, -117.4881)	845	**SIO-BIC: A12278*, A12279*, A12280*, A12281*, A12283***	**SIO-BIC A12279***	**SIO-BIC A12279***	**SIO-BIC A12279**	This study
Antonbruunia sociabilis	Scotland (57.9500, -15.5500)	1200	-	KP992845*	KP992843	-	(Mackie et al., 2015)
Ancistrosyllis breviceps	Santa Barbara, CA, USA (34.3700, -120.0300)	400	**SIO-BIC A6261***	**SIO-BIC A6261***	**SIO-BIC A6261***	-	This study
Ancistrosyllis groenlandica	N Spain, unknown	-	KT307624*	-	DQ790075*	-	(Aylagas et al., 2016; Struck et al., 2007)
Ancistrosyllis tiffanyae sp. nov.	Santa Monica, CA, USA (33.8385, -118.6904)	711	**SIO-BIC A12326***	**SIO-BIC A12326**	**SIO-BIC A12326**	**SIO-BIC A12326**	This study
Cabira pilargiformis	Bohai Sea, China (37.948, 119.361)	16	HZPLY558-13*	-	-	-	Zhou
Glyphohesione klatti	N Trondheim, Norway (63.5086, 10.4419)	35	**SIO-BIC A6066***	**SIO-BIC A6066**	**SIO-BIC A6066**	**SIO-BIC A6066**	This study
Hermundura fauveli	Bremerton, WA, USA (47.5471, -122.6620)	-	BBPS179-19*	**USNM1643733***	**USNM1643733***	**USNM1643733**	Paulay, This study
Litocorsa stremma	N Spain	-	KT307655*	-	-	-	(Aylagas et al., 2016)
Otopsis fredi sp. nov.	Monterey, CA, USA (36.7721, -122.0831)	2892-2937	**SIO-BIC: A15574*, A15575*, A15576*, A15577*, A15578*, A15579***	**SIO-BIC A15574**	**SIO-BIC A15574**	**SIO-BIC A15574**	This study

Table 2: All used and obtained sequences in this study, continued.

Species	Locality	Depth (m)	COI	16S rRNA	18S rRNA	Mitogenome	Reference
Pilargis verrucosa	Koster, Sweden	-	**SIO-BIC A13955***	**SIO-BIC A13955***	**SIO-BIC A13955***	**SIO-BIC A13955**	This study
Pilargis wolfi	Chesapeake Bay, VA, USA (37.5848, -75.6486)	-	**USNM1499416***	**USNM1499416***	**USNM1499416***	**USNM1499416**	This study
Pseudoexogone cf. *backstromi*	S Pacific (-17.4904, -129.8267)	18-20	**SIO-BIC A2813***	**SIO-BIC A2813***	**SIO-BIC A2813***	-	This study
Sigambra cortesi sp. nov.	W Costa Rica (8.9308, -84.3072)	967-1005	**SIO-BIC: A1516***	**SIO-BIC A1516**	**SIO-BIC A1516**	**SIO-BIC A1516**	This study
	W Costa Rica (9.1174, -84.8396)	1795	**A9597***				
	W Costa Rica (8.9309, -84.3133)	999	**A9843***				
Sigambra gabriellae sp. nov.	W Costa Rica (7.6802, -85.9118)	528.2	**SIO-BIC A10175***	**SIO-BIC A10175**	**SIO-BIC A10175**	**SIO-BIC A10175**	This study
Sigambra hanaokai	Tanabe Bay, Japan (33.6997, 135.3268)	36	**SIO-BIC A5771***	**SIO-BIC A5771***	**SIO-BIC A5771***	-	This study
Sigambra magnuncus	W Africa	-	GQ426642*	GQ426621*	GQ426593*	-	(Böggemann, 2009)
Sigambra otoni sp. nov.	Rovinj, Croatia (45.0915, 13.6244)	25	**SIO-BIC A15568*, A15569*, A15570*, A15571*, A15572*, A15573***	**SIO-BIC A15568***	**SIO-BIC A15568***	-	This study
	Banyuls-sur-Mer, France (42.4958, 3.1400)	24	**A5926***	**A5926***	**A5926***	-	

Table 2: All used and obtained sequences in this study, continued.

Species	Locality	Depth (m)	COI	16S rRNA	18S rRNA	Mitogenome	Reference
Sigambra parva	NW India (22.3000, 69.3900)	-	KY775644*	-	-	-	(Vijapure et al., 2019)
Synelmis amoureuxi	Carrie Bow Cay, Belize (16.8032, -88.0778)	10	**SIO-BIC A4721***	**SIO-BIC A4721**	**SIO-BIC A4721**	**SIO-BIC A4721**	This study
Micronephthys minuta	Disko Island, Greenland (69.2901, -53.2143)	190	**SIO-BIC A15537***	**SIO-BIC A15537***	**SIO-BIC A15537***	**SIO-BIC A15537**	This study
Nephtys hombergii	Ria Aveiro, Portugal, Bohuslän, Sweden	-	GU179410*	GU179354*	GU179377*	-	(Ravara et al., 2010)
Nephtys sp.	NE Pacific	-	KY972420	-	-	KY972376, KY972406, KY972433, KY972447, KY972462, KY972476, KY972490, KY972504, KY972532	(Bernardino et al., 2017)

DNA extraction, amplification, and sequencing

DNA was extracted using the Zymo Research Quick-DNA™ Miniprep (most specimens) or Microprep (SIO-BIC: A2813, A4846, A6066, A6261, A9843, A13955) Plus Kit following the manufacturer-supplied protocol. Regions of the mitochondrial genes cytochrome oxidase subunit I (COI) and 16S rRNA (16S) and the nuclear gene 18S rRNA (18S) were amplified on Eppendorf thermal cyclers using the primers in Table 3. PCR amplification was done with a mixture of 12.5 µl of Apex 2.0x Taq Red DNA Polymerase Master Mix (Genesee Scientific) for most amplifications or Conquest™ PCR 2x Master Mix-1 (Lamda Biotech) (for amplifications of: COI: SIO-BIC: A12280, A12283, A13955, and A15568 and USNM1499416; 16S using primer pair AnnF/16Sb: SIO-BIC A13955, USNM1499416, and USNM1643733; 16S using primer pair 16SarL/16SbrH: SIO-BIC: A5771, A5926, A6261; 18S: SIO-BIC A13955, USNM1499416, and USNM1643733; only primer pair 18S-3F/18S-bi for 18S: SIO-BIC A5926; only primer pair 18S-1F/18S-5R for 18S: SIO-BIC A4846, A5771, and A5926), 8.5 µl ddH_2O, 1 µl of each appropriate forward and reverse primer (Table 3) and 2 µl of eluted DNA. The primer pair AnnF/16Sb was used in PCR reactions for 16S for samples SIO-BIC: A13955, A15537, and A15568, and USNM1499416 and USNM1643733, while the primer pair 16SarL/16SbrH was used for all other 16S PCR amplifications. The ExoSAP-IT protocol (USB, Affymetrix, Ohio, USA) was then used to purify the PCR products, which were then Sanger sequenced by Eurofins Genomics (Louisville, KY). The "De Novo Assembly" option with default settings was used to assemble consensus sequences in Geneious Prime v. 2022.2.2 (www.geneious.com, Kearse et al., 2012).

Table 3: Forward (F) and reverse (R) primers used for each gene and references.

Gene	Primer Name	Primer Sequence	Reference
COI	polyLCO (F)	(5'-GAYTATWTTCAACAAATCATAAAGATATTGG-3')	(Carr et al., 2011)
COI	polyHCO (R)	(5'-TAMACTTCWGGGTGACCAAARAA TCA-3')	(Carr et al., 2011)
16S	16S-AnnF (F)	(5'-GCGGTATCCTGACCGTRCWAAGGTA -3')	(Sjölin et al., 2005)
16S	16Sb (R)	(5'-CTCCGGTTTGAACTCAGATCA -3')	(Palumbi, 1996)
16S	16SarL (F)	(5'-CGCCGTTTATCAA AAACAT-3')	(Palumbi et al., 1991)
16S	16SbrH (R)	(5'-CCGGTCTGAACTCAGATCACGT-3')	(Palumbi et al., 1991)
18S	18S-1F (F)	(5'-TACCTGGTTGATCCTGCCAGTAG-3')	(Giribet et al., 1996)
18S	18S-5R (R)	(5'-CTTGGCAAATGCTTTCGC-3')	(Giribet et al., 1996)
18S	18S-3F (F)	(5'-GTTCGATTCCGGAGAGGGA-3')	(Giribet et al., 1996)
18S	18S-bi (R)	(5'- GAGTCTCGTTCGTTATCGGA-3')	(Whiting et al., 1997)
18S	18S-a2.0 (F)	(5'- ATGGTTGCAAAGCTGAAAC-3')	(Whiting et al., 1997)
18S	18S-9R (R)	(5'- GATCCTTCCGCAGGTTCACCTAC-3')	(Giribet et al., 1996)

Up to 684 base pairs were amplified for COI with the reaction protocol of the initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 40 s, annealing at 42°C for 45 s, and elongation at 72°C for 50 s, followed by the final extension at 72°C for 5 minutes. Up to 530 base pairs were amplified for 16S using the primer pair 16SarL/16SbrH with the reaction protocol of the initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 40 s, annealing at 50°C for 40 s, and elongation at 72°C for 50 s, followed by the final extension at 72°C for 5 minutes. Up to 442 base pairs were amplified for 16S using the primer pair AnnF/16Sb with the reaction protocol of the initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s, and elongation at 70°C for 45 s, followed by the final extension at 72°C for 7 minutes. Up to 1,792 base pairs were amplified for 18S with the reaction protocol for primer pairs 18S-1F/18S-5R and 18S-a2.0/18S-9R being

an initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 90 s, followed by the final extension at 72°C for 8 minutes, and for primer pair 18S-3F/18S-bi the reaction protocol is an initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 90 s, followed by the final extension at 72°C for 8 minutes.

The DNA extractions from specimens SIO-BIC: A1516, A4721, A6066, A10175, A13955, A12279, A12326, A15537, and A15574, USNM1499416, and USNM1643733 were sent to Novogene (en.novogene.com) for library preparation and whole genome sequencing using 150 base pair (bp) paired-end reads on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA); Table 4 shows the number of generated paired-end 150 bp long raw reads per sample. Before this, sequences SIO-BIC A13955, USNM1499416, and USNM1643733 were amplified to reach sufficient DNA concentration using the Illustra Ready-To-Go GenomiPhi V3 DNA Amplification Kit (Cytiva, Marlborough, MA, USA) according to the manufacturer protocol, except in the amount of DNA added for sample USNM1643733, because there was only 4.65 ng of total DNA available instead of the recommended minimum of 10 ng. The sequence reads, which were trimmed and cleaned with Trimmomatic v. 0.39 (Bolger et al., 2014), were then assembled using MitoFinder v. 1.4 (Allio et al., 2020), with the organism genetic code that was used to translate the 13 protein-coding genes being The Invertebrate Mitochondrial Code (NCBI; transl_table = 5). The reference file used for MitoFinder contained the complete records for all RefSeq annelid mitogenomes that were publicly available on NCBI GenBank. The integrated MitoFinder pipeline with the MEGAHIT v. 1.2.9 (D. Li et al., 2016) and Arwen v.1.2.3 (Laslett & Canbäck, 2008) parameters was used to annotate the assembled mitochondrial genomes. Sample USNM1643733 was also assembled and annotated using

NOVOPlasty (Dierckxsens et al., 2017) and MitoZ (Meng et al., 2019). The MITOS Web Server (Bernt et al., 2013) was used to check annotations, and Geneious Prime v. 2022.2.2 (www.geneious.com, Kearse et al., 2012) was used to manually modify the annotated mitogenome assemblies if needed, extract the finalized nucleotide genes, and translate the protein-coding genes into amino acids, also using The Invertebrate Mitochondrial genetic code. The paired-end reads were also used to obtain mapped 18S sequences for samples SIO-BIC: A1516, A4721, A6066, A10175, A12326, and A15574, using MITObim (Hahn et al., 2013), Minimap2 v. 2.22 (H. Li, 2018, 2021), SamTools 1.13 (Danecek et al., 2021) to interleave and map the reads to a reference file of publicly available Phyllodocida Dales, 1962 18S sequences from NCBI GenBank. Mapped reads were visualized with Geneious Prime v. 2022.2.2 (www.geneious.com, Kearse et al., 2012) and a consensus 18S sequence was chosen for each specimen.

Table 4: Number, total length, and individual length of Novogene genome skimming raw reads for the specified species and the number of reads retained after using Trimmomatic.

Species	Total Number of Raw Reads (Paired-End)	Total Length of Raw Reads (Paired-End)	Individual Length of Raw Reads	Number of Reads Post-Trimmomatic (Paired-End)
Antonbruunia milenae sp. nov.	3,702,263	555,339,450	150	3662081 (98.91%)
Ancistrosyllis tiffanyae sp. nov.	4,226,103	633,915,450	150	4160625 (98.45%)
Otopsis fredi sp. nov.	10,035,386	1,505,307,900	150	9912137 (98.77%)
Sigambra cortesi sp. nov.	8,748,561	1,312,284,150	150	8609906 (98.42%)
Sigambra gabriellae sp. nov.	9,106,118	1,505,307,900	150	8996276 (98.79%)
Glyphohesione klatti	9,850,116	1,477,517,400	150	9682419 (98.30%)
Synelmis amoureuxi	10,165,381	1,524,807,150	150	10023655 (98.61%)
Pilargis verrucosa	8,696,621	1,304,493,150	150	8479066 (97.50%)
Pilargis wolfi	7,213,725	1,082,058,750	150	7062447 (97.90%)
Hermundura fauveli	7,387,747	1,108,162,050	150	7253809 (98.19%)
Micronephthys minuta	6,284,087	942,613,050	150	6159575 (98.02%)

Morphological analyses

Parapodia and isolated chaetae of preserved specimens were permanently mounted on slides with Aquamount ® (Thermo Fisher Scientific) and observed on a Leica DMR HC with compound light microscope with differential interference contrast. A Leica MZ12.5 stereomicroscope was used for observing whole or larger sections of the preserved specimens. A Canon Rebel T6i camera was used for taking light micrographs. Additionally, a Zeiss EVO10 was used for scanning electron microscopy (SEM). SEM samples that were prepared for SEM were first dehydrated in an ethanol series, transferred to hexamethyldisilazane (HMDS) and then air dried in a fume hood overnight. Larger pieces were first rehydrated in distilled water, soaked in 4% osmium tetroxide for 1.5 hours, and then rinsed with more distilled water for three days and finally once again dehydrated in an ethanol series. Once dry, samples were mounted on double-sided adhesive carbon tab and aluminum tape covered aluminum stubs, then sputter coated with gold-palladium (Au-Pd) using the Quorum SC7620 Mini Sputter Coater.

Phylogenetic analyses

Nephtyidae Grube, 1850 were chosen as the outgroup for Pilargidae and *Antonbruunia* based on (Tilic et al., 2022), who showed them as sister to Pilargidae. All obtained single gene sequence datasets were aligned in the program Mesquite v3.61 (Maddison, 2019) using MAFFT v.7.453 (Katoh, 2002) under the default G-INSI-I method with gaps included for all the nucleotide single gene datasets (see Table 5) used and MUSCLE v.3.8.31 (Edgar, 2010) with the default setting and gaps included for the all the amino acid single gene datasets used (see Table 5). The concatenated COI, 16S and 18S phylogeny single gene datasets used Sanger sequences whenever possible, while datasets used in the concatenated 13 protein-coding, 12S rRNA, and 16S mitochondrial and 18S nuclear gene phylogeny used data obtained through genome skimming whenever possible. RAxML GUI 2.0 v.2.0.10 (Edler et al., 2020) was used to concatenate the single gene datasets for each phylogeny. The "raxml-ng-ARM64" setting was used for the maximum likelihood + transfer bootstrap expectation + consensus analysis of the three-gene phylogeny with 20 runs, 500 reps, no predetermined outgroup, and a seed of 552840. The substitution model, proportion of invariant sites, and rate heterogeneity were chosen with the integrated "RUN MODELTEST" function (see Table 5). The 16-gene (see Table 5) maximum likelihood + thorough bootstrap + consensus phylogeny was analyzed using the "raxml-ng" setting with 20 runs, 500 reps, no predetermined outgroup, and a seed of 802774. The substitution model, proportion of invariant sites, and rate heterogeneity were chosen with the integrated "modeltest-ng" option (Table 5). FigTree v1.4.4 (Andrew Rambaut, 2009) was used for displaying the tree. PopART (Leigh & Bryant, 2015) was used to create and visualize the *Antonbruunia milenae* sp. nov. haplotype network.

Table 5: Data type (either nucleotide (NT) or amino acid (AA)) and RAxML modeltest results of single gene datasets, grouped by concatenated 16 gene and concatenated three gene phylogenies.

Mitochondrial Genome Phylogeny Dataset					
Gene	**Data Type**	**Substitution model**	**Stationary frequencies**	**Proportion of invariant sites**	**Rate heterogeneity**
ATP6	AA	MTZOA	+F (empirical)	-	+GAMMA (mean)
ATP8	AA	MTART	-	-	+GAMMA (mean)
COI	AA	MTZOA	-	+I (ML estimate)	+GAMMA (mean)
COII	AA	MTZOA	-	-	+GAMMA (mean)
COIII	AA	MTZOA	-	+I (ML estimate)	+GAMMA (mean)
CYTB	AA	MTZOA	-	+I (ML estimate)	+GAMMA (mean)
ND1	AA	MTART	-	+I (ML estimate)	+GAMMA (mean)
ND2	AA	MTMAM	+F (empirical)	-	+GAMMA (mean)
ND3	AA	MTART	-	+I (ML estimate)	+GAMMA (mean)
ND4	AA	MTZOA	+F (empirical)	-	+GAMMA (mean)
ND4L	AA	MTMAM	-	-	+GAMMA (mean)
ND5	AA	MTART	+F (empirical)	+I (ML estimate)	+GAMMA (mean)
ND6	AA	MTART	-	+I (ML estimate)	+GAMMA (mean)
12S	NT	TIM2	-	-	+GAMMA (mean)
16S	NT	TN93	-	+I (ML estimate)	+GAMMA (mean)
18S	NT	TN93	-	+I (ML estimate)	+GAMMA (mean)
Concatenated COI, 16S rRNA, and 18S rRNA Phylogeny Dataset					
Gene	**Data Type**	**Substitution model**	**Stationary frequencies**	**Proportion of invariant sites**	**Rate heterogeneity**
COI	NT	GTR	-	+I (ML estimate)	+GAMMA (mean)
16S	NT	TIM3	-	+I (ML estimate)	+GAMMA (mean)
18S	NT	TIM3	-	+I (ML estimate)	+GAMMA (mean)

RESULTS

Phylogenies

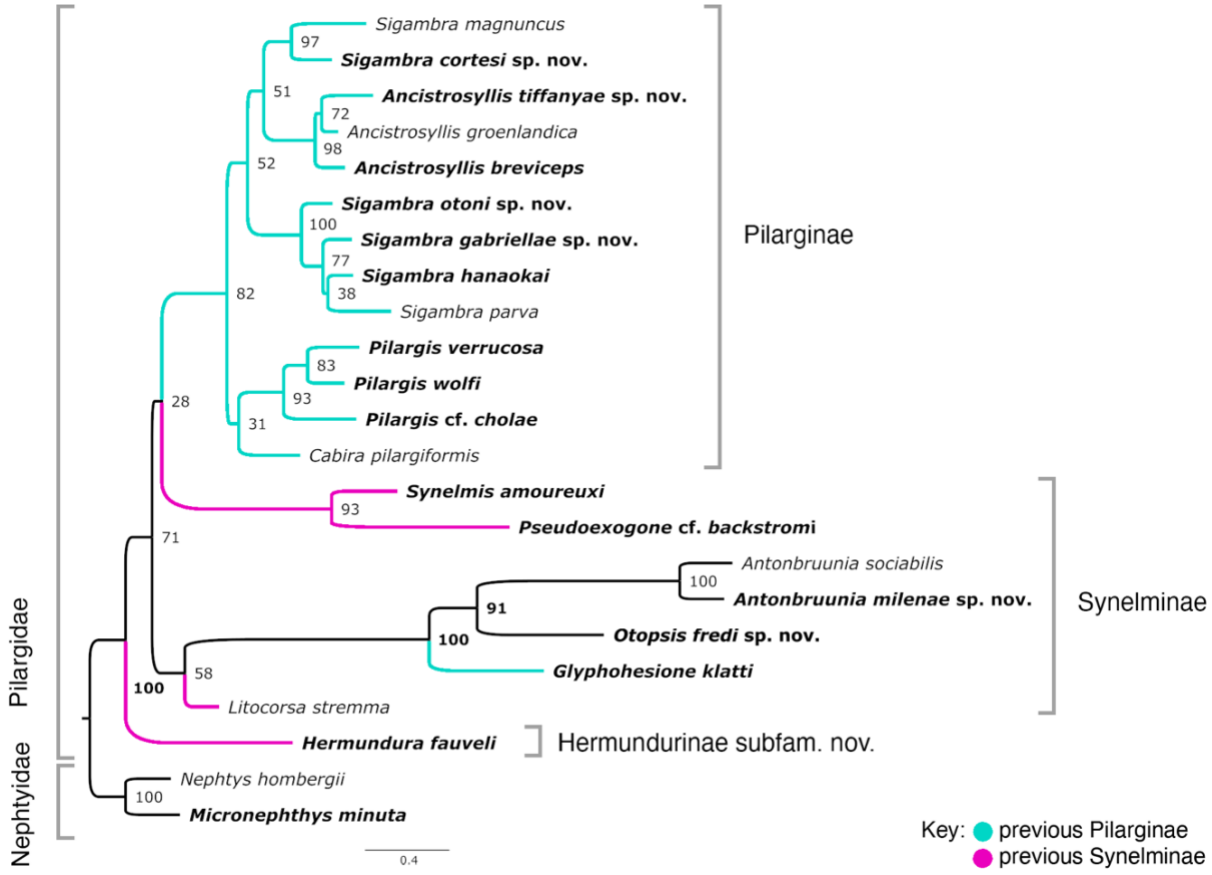

Figure 2: Concatenated mitochondrial COI, 16S rRNA, and nuclear 18S rRNA gene Maximum Likelihood (ML) tree of Pilargidae with two nephtyids as an outgroup using nucleotide sequences. ML thorough bootstrap values are represented by numbers at the nodes. Species names set in bold indicate new sequences obtained in this study. Grey brackets group species together by family and new subfamily, while fuchsia branches indicate species belonging to Synelminae, teal branches indicate species belonging to Pilarginae, and black pilargid branches indicate unknown subfamily division until this study.

The concatenated mitochondrial COI, 16S rRNA, and nuclear 18S rRNA maximum likelihood tree rooted by nephtyids (Fig. 2) shows four major pilargid clades. The first clade, *Hermundura fauveli*, has a high support value of 100. The second clade branches off with a support value of 71 and contains *Litocorsa stremma* Pearson, 1970 as the sister taxa to *Glyphohesione* Friedrich, 1970, *Otopsis* Ditlevsen, 1917, and *Antonbruunia*. In that clade, *Antonbruunia* is sister to *Otopsis fredi* sp. nov. with high support of 91, and *Glyphohesione klatti*

Friedrich, 1950 is sister to them both with a high support value of 100. The third clade branching off has a very low support value of 28, though *Synelmis amoureuxi* Salazar-Vallejo, 2003 and *Pseudoexogone* cf. *backstromi* Augener, 1922 are sister clades with a high support value of 93. The fourth clade is split into two groups with a good support value of 82. On one side, *Pilargis* Saint-Joseph, 1899 forms a well-supported clade (support values 93 and 83), sister to *Cabira pilargiformis* Uschakov & Wu, 1962 with a low support value of 31. On the other side, there are two clades of well supported *Sigambra* with the well supported *Ancistrosyllis* clade in between, but the support values on the nodes showing the relationship between these three clades are low, at 51 and 52.

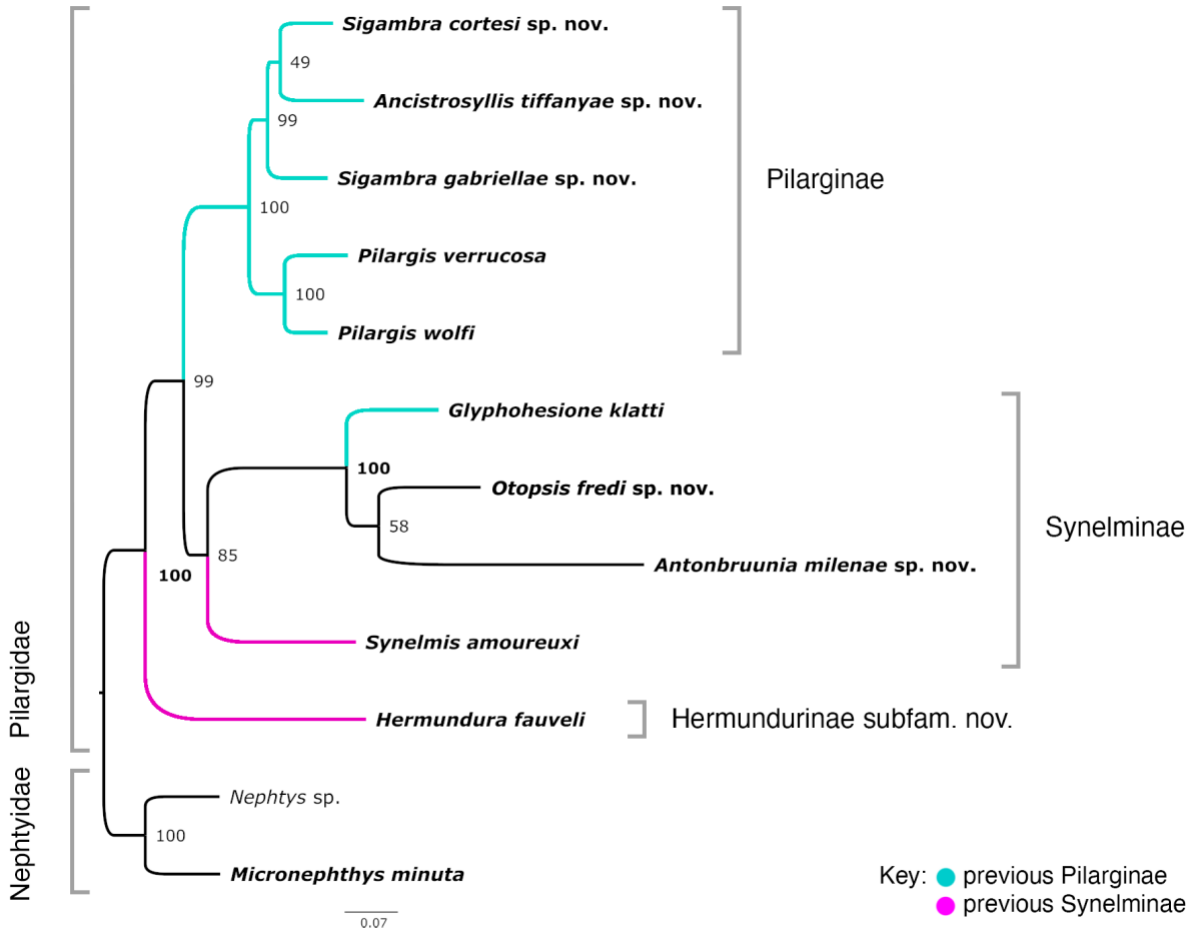

Figure 3: Concatenated Maximum Likelihood (ML) tree of Pilargidae with two nephtyids as an outgroup using the thirteen protein-coding mitochondrial genes, two non-protein-coding mitochondrial genes 12S rRNA and 16S rRNA, and the nuclear 18S rRNA gene. The protein-coding genes are input as amino acid sequences, while the non-protein-coding genes are input as nucleotide sequences. ML thorough bootstrap values are represented by numbers at the nodes. Species names set in bold indicate new sequences obtained in this study. Grey brackets group species together by family and new subfamily, while fuchsia branches indicate species belonging to Synelminae, teal branches indicate species belonging to Pilarginae, and black pilargid branches indicate unknown subfamily division until this study.

The mitochondrial genome and nuclear 18S rRNA maximum likelihood tree (Fig. 3) shows three major pilargid clades rooted by the nephtyids. First, *Hermundura fauveli* branches off with a high bootstrap support value of 100. Then, the tree splits into two clades with a high support value of 99. In the first, *Antonbruunia milenae* sp. nov. is sister to *Otopsis fredi* sp. nov. with a low support value of 58, but they are sister to *Glyphohesione klatti* with a high support value of 100. The clade they form is sister to *Synelmis amoureuxi* with a good support value of

85. The second major clade is split into the well supported *Pilargis* clade and the *Sigambra* and *Ancistrosyllis* clade. There, *Ancistrosyllis tiffanyae* sp. nov. forms a clade with *Sigambra cortesi* sp. nov. with a low support value of 49, and they are sister to *Sigambra gabriellae* sp. nov. with a high support value of 99.

Haplotype Network

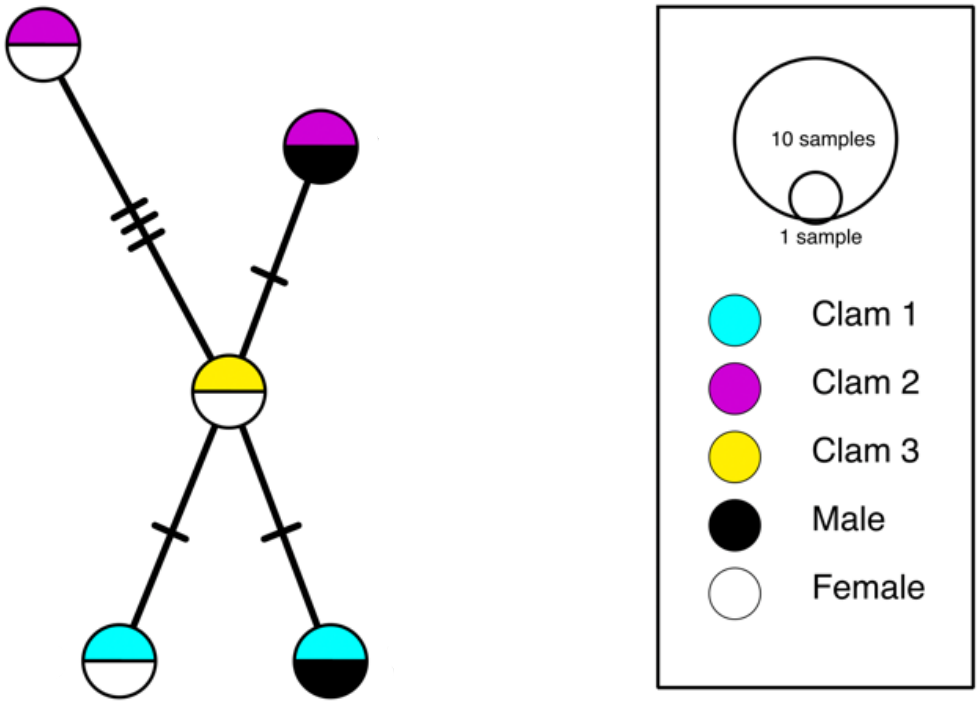

Figure 4: Antonbruunia milenae sp. nov. COI haplotype network. Each circle represents one individual; the bottom half of the circles is white for females or black for males, while the top part of the circles is turquoise for specimens found in clam 1 (SIO-BIC M18180), purple for specimens found in clam 2 (SIO-BIC M18183), or yellow for specimens found in clam 3 (SIO-BIC M18184). Each mark on the line connecting two circles represents a difference of one base pair between two haplotypes.

The COI haplotype network of *Antonbruunia milenae* sp. nov. (Fig. 4) shows that each of the five sequenced specimens has its own haplotype. Sequences are from two to five base pairs different from each other. Of the sequenced annelids, those found in the same clam did not have the same haplotype.

Mitogenomes

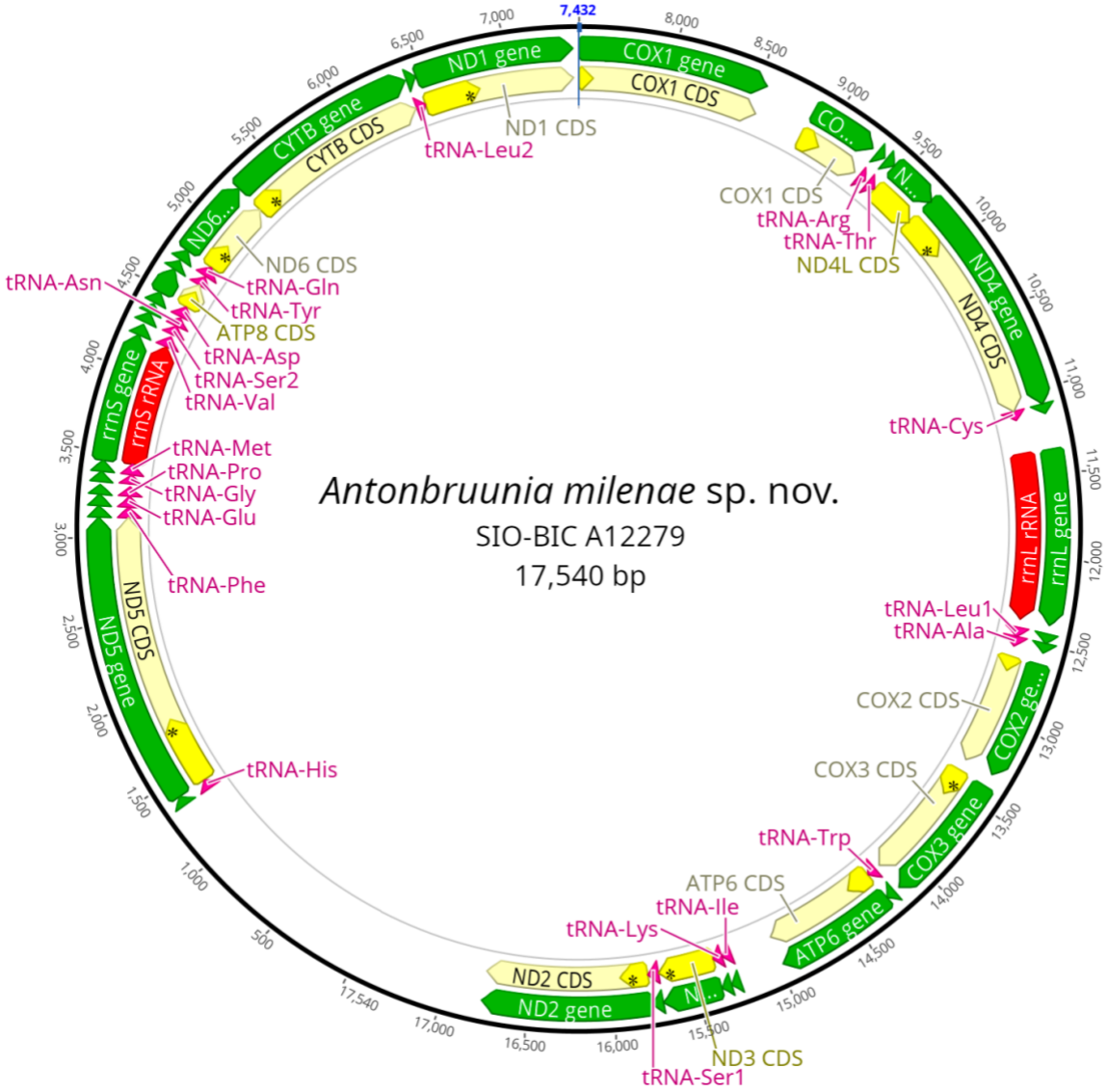

Figure 5: Circularized mitogenome of *Antonbruunia milenae* sp. nov. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

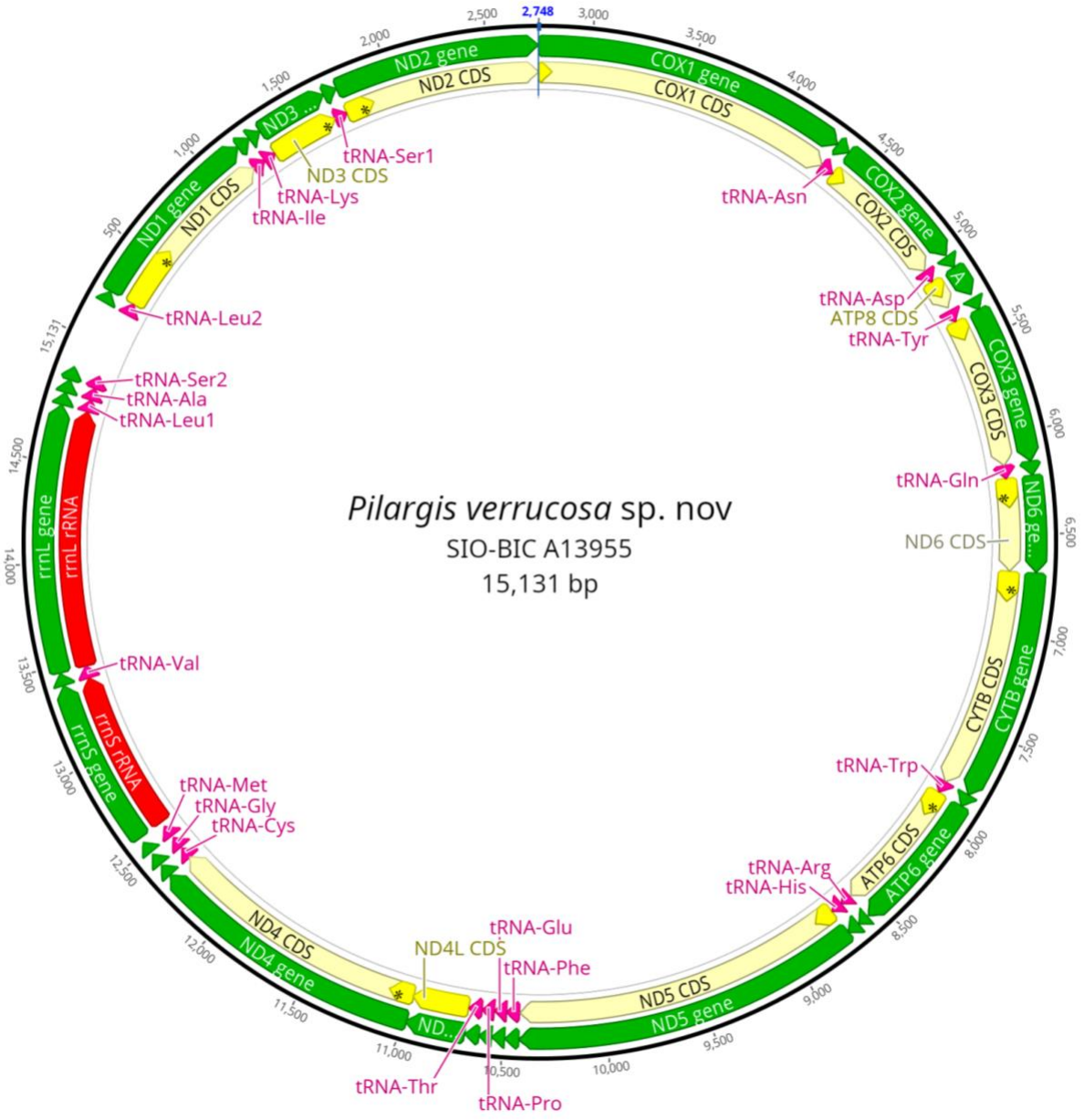

Figure 6: Circularized mitogenome of *Pilargis verrucosa*. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

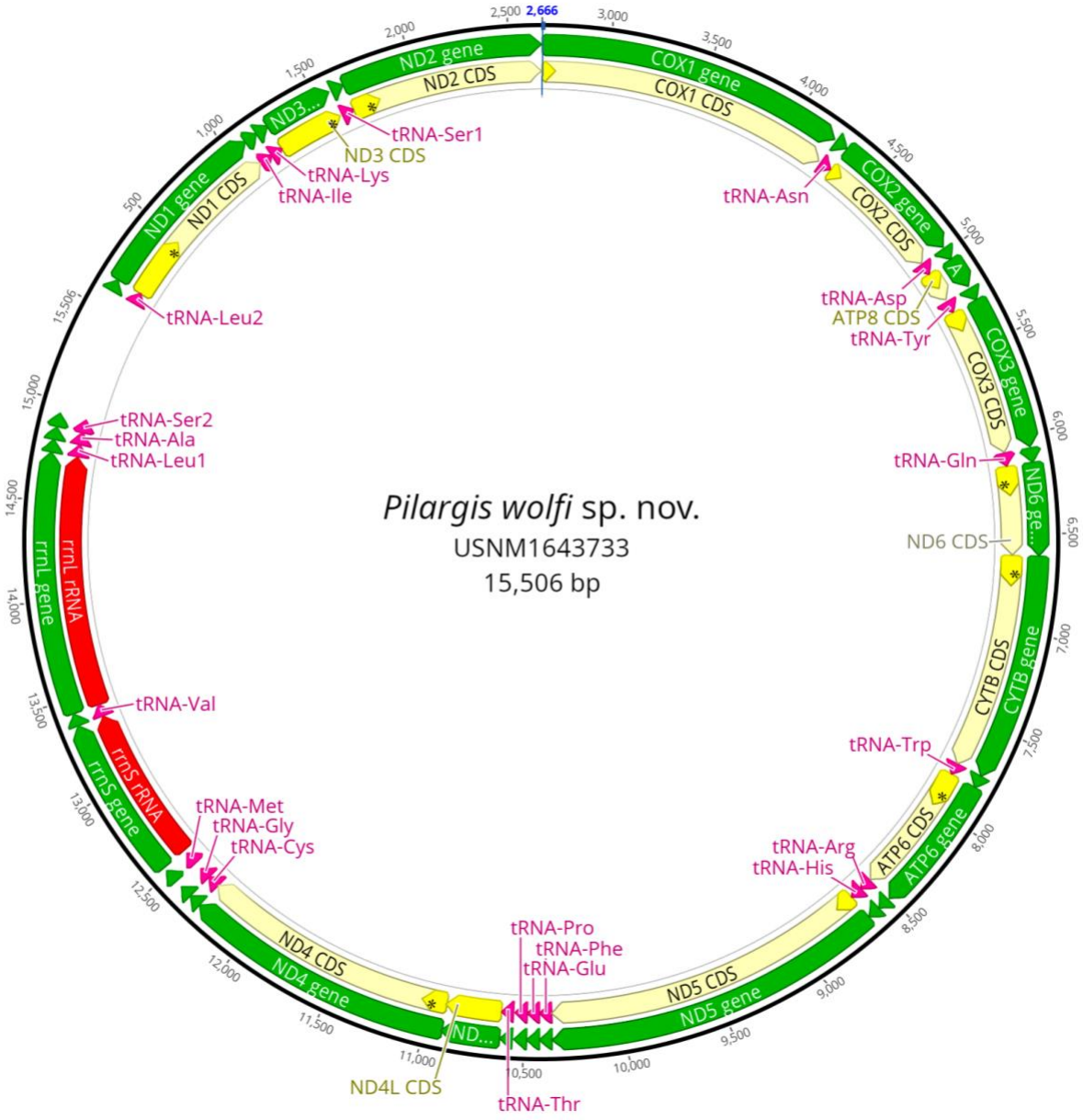

Figure 7: Circularized mitogenome of *Pilargis wolfi*. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

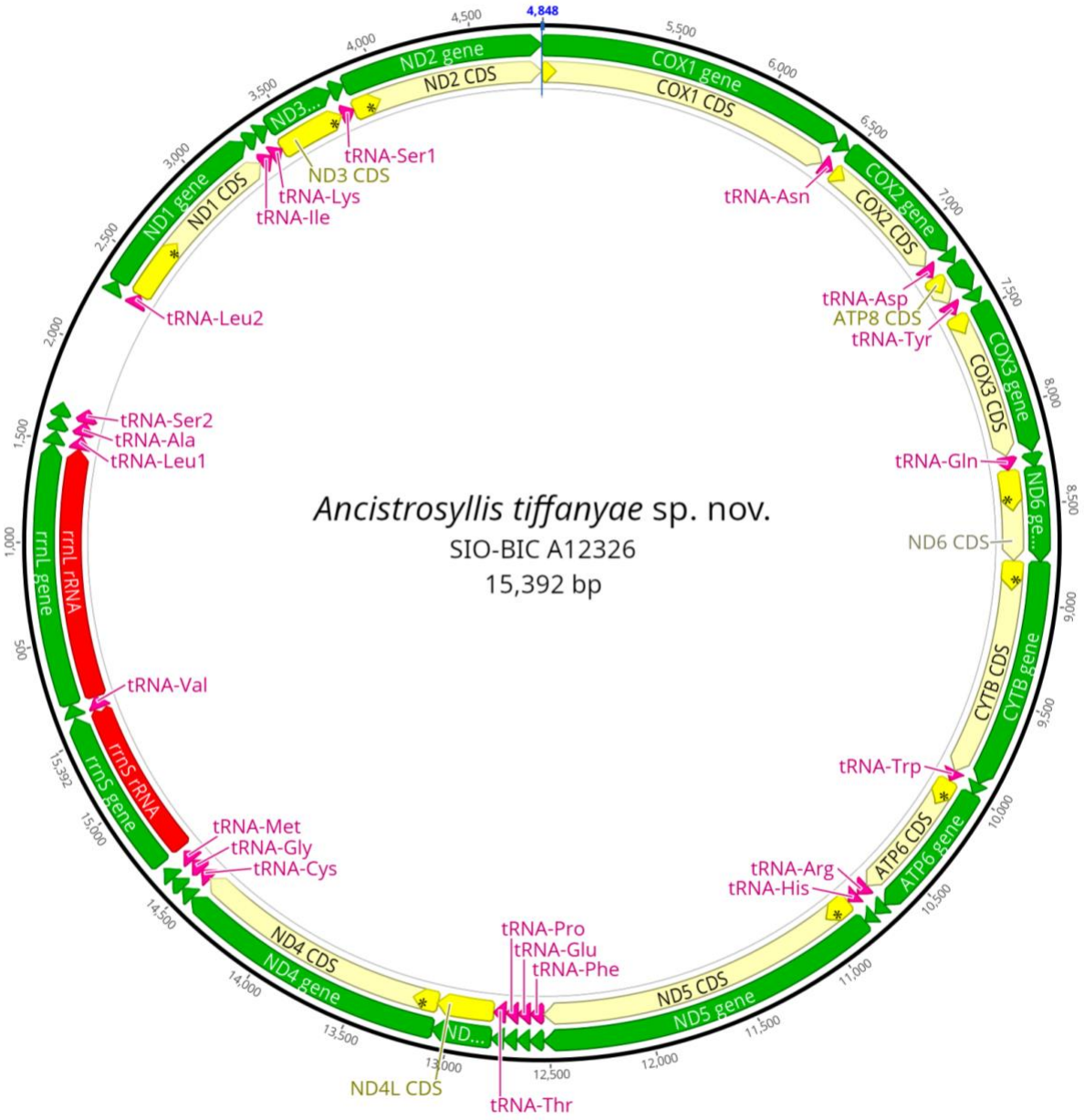

Figure 8: Circularized mitogenome of *Ancistrosyllis tiffanyae* sp. nov. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

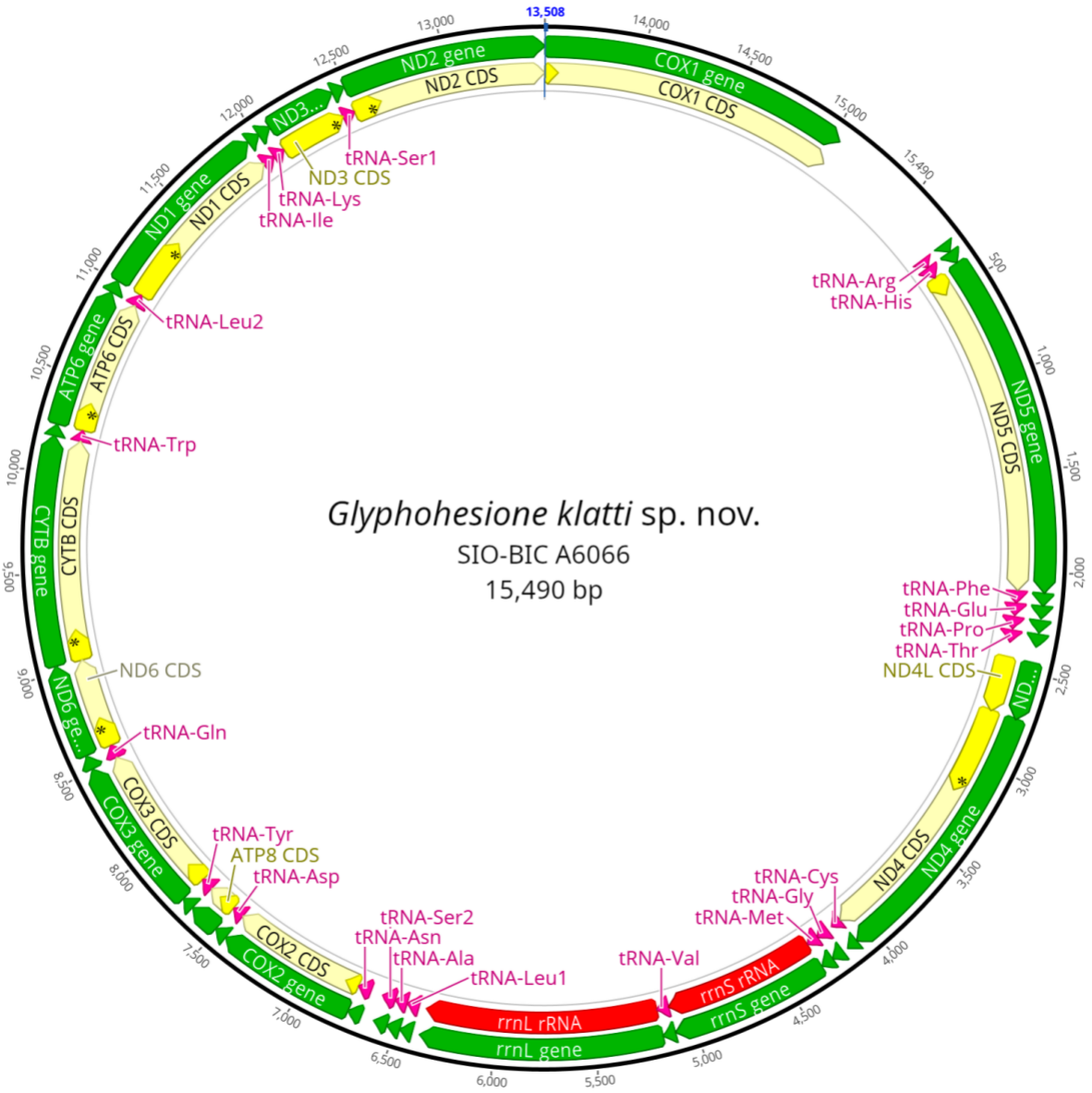

Figure 9: Circularized mitogenome of *Glyphohesione klatti*. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

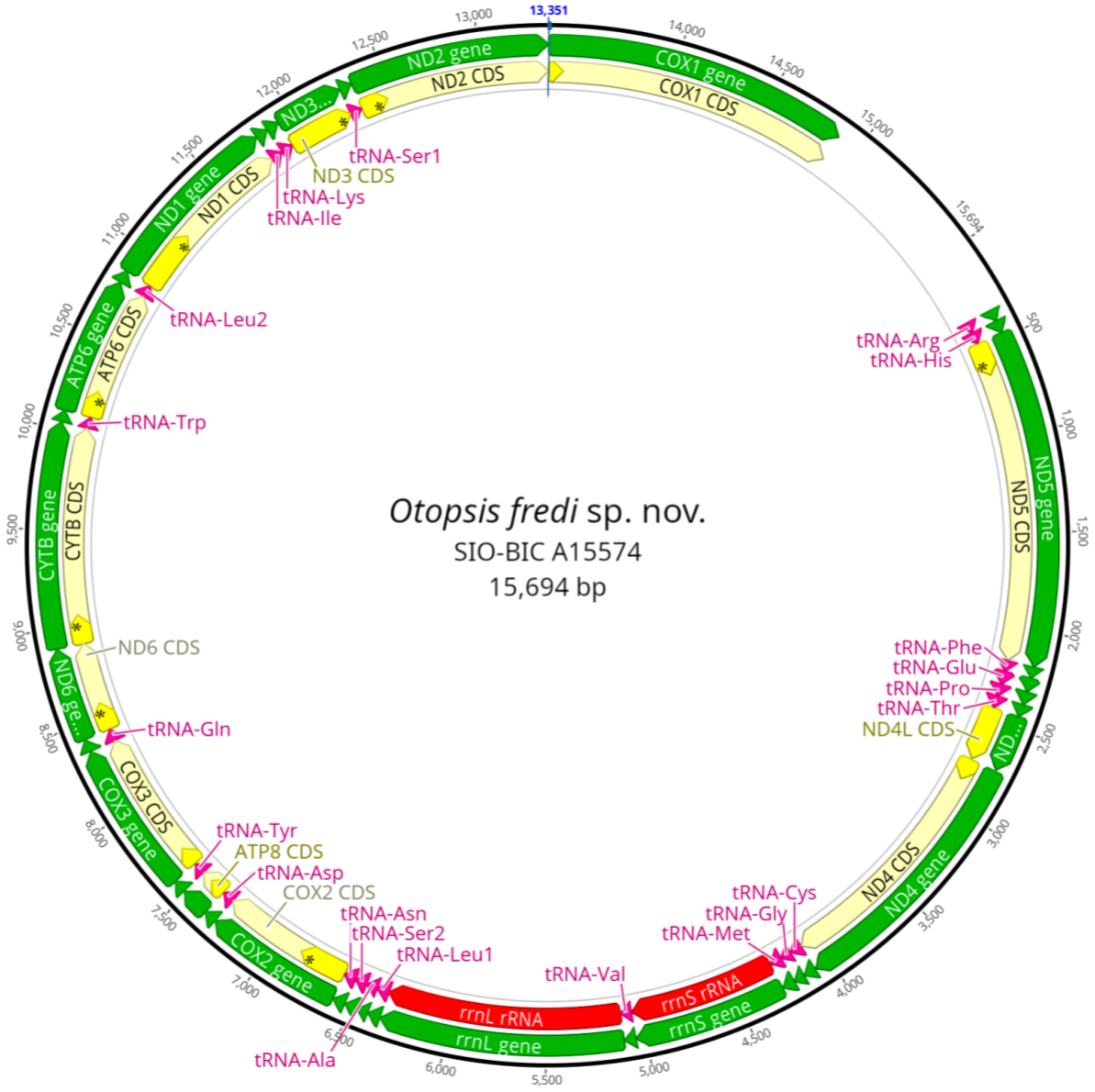

Figure 10: Circularized mitogenome of *Otopsis fredi* sp. nov. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

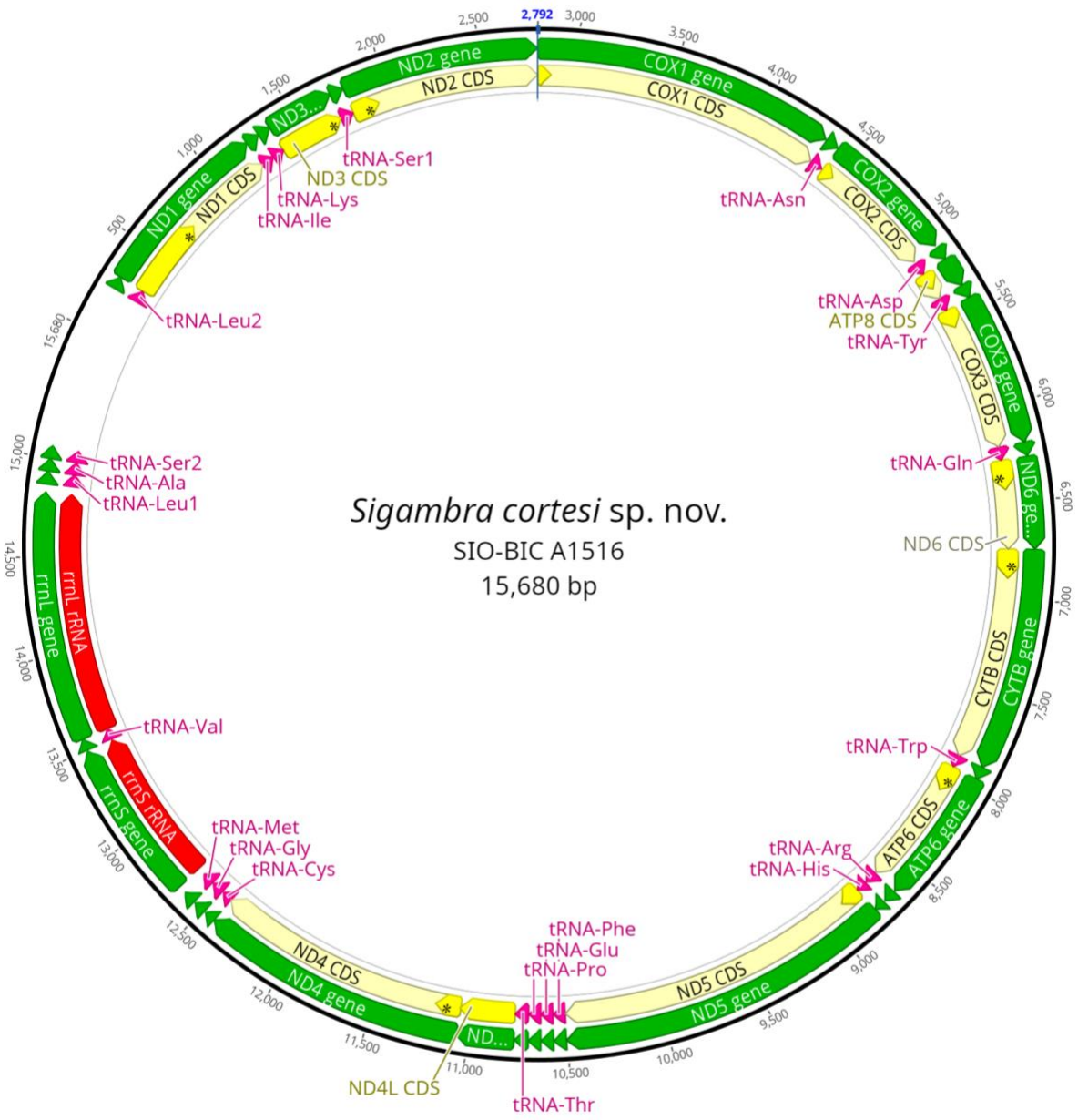

Figure 11: Circularized mitogenome of *Sigambra cortesi* sp. nov. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene

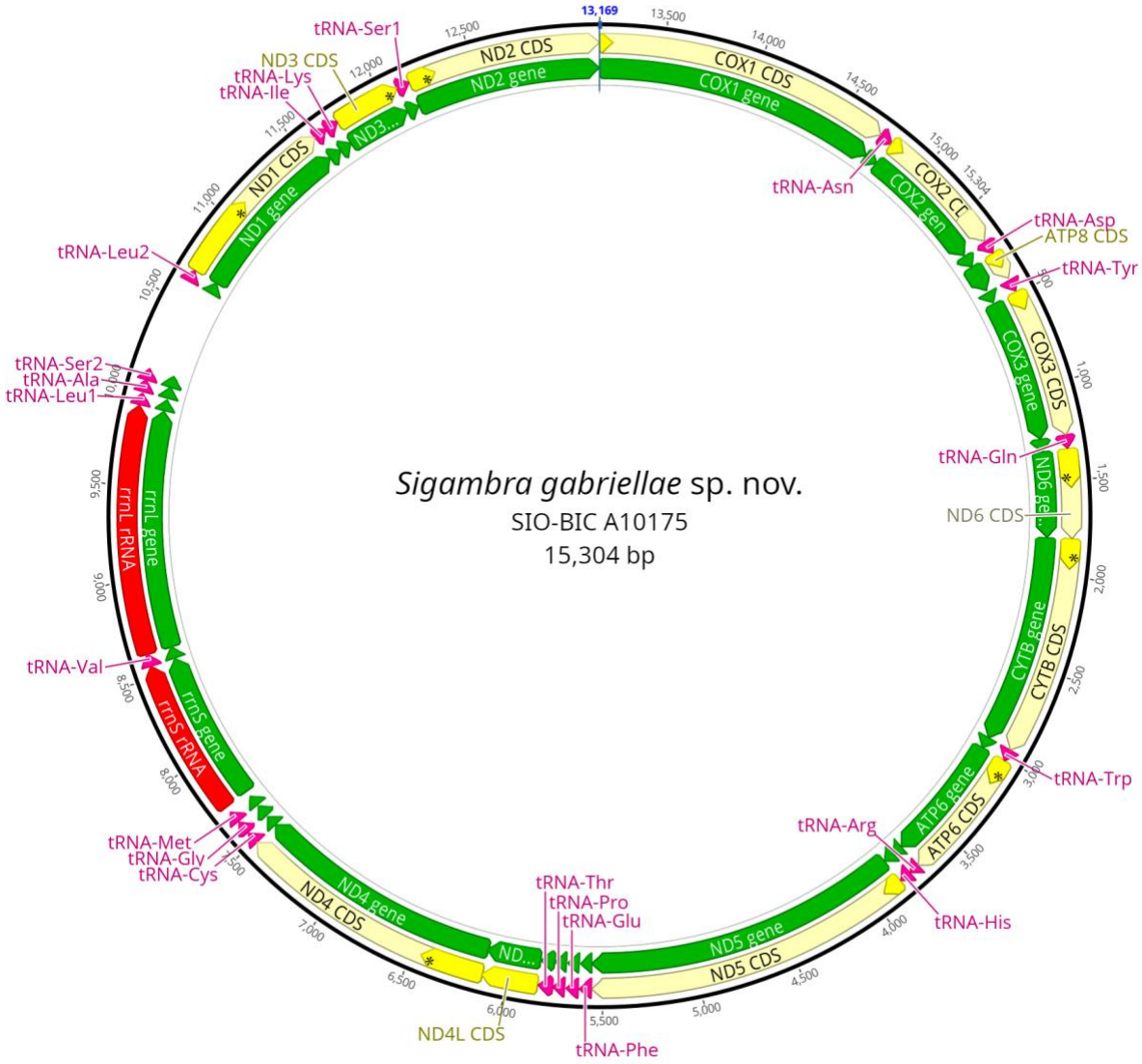

Figure 12: Circularized mitogenome of *Sigambra gabriellae* sp. nov. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

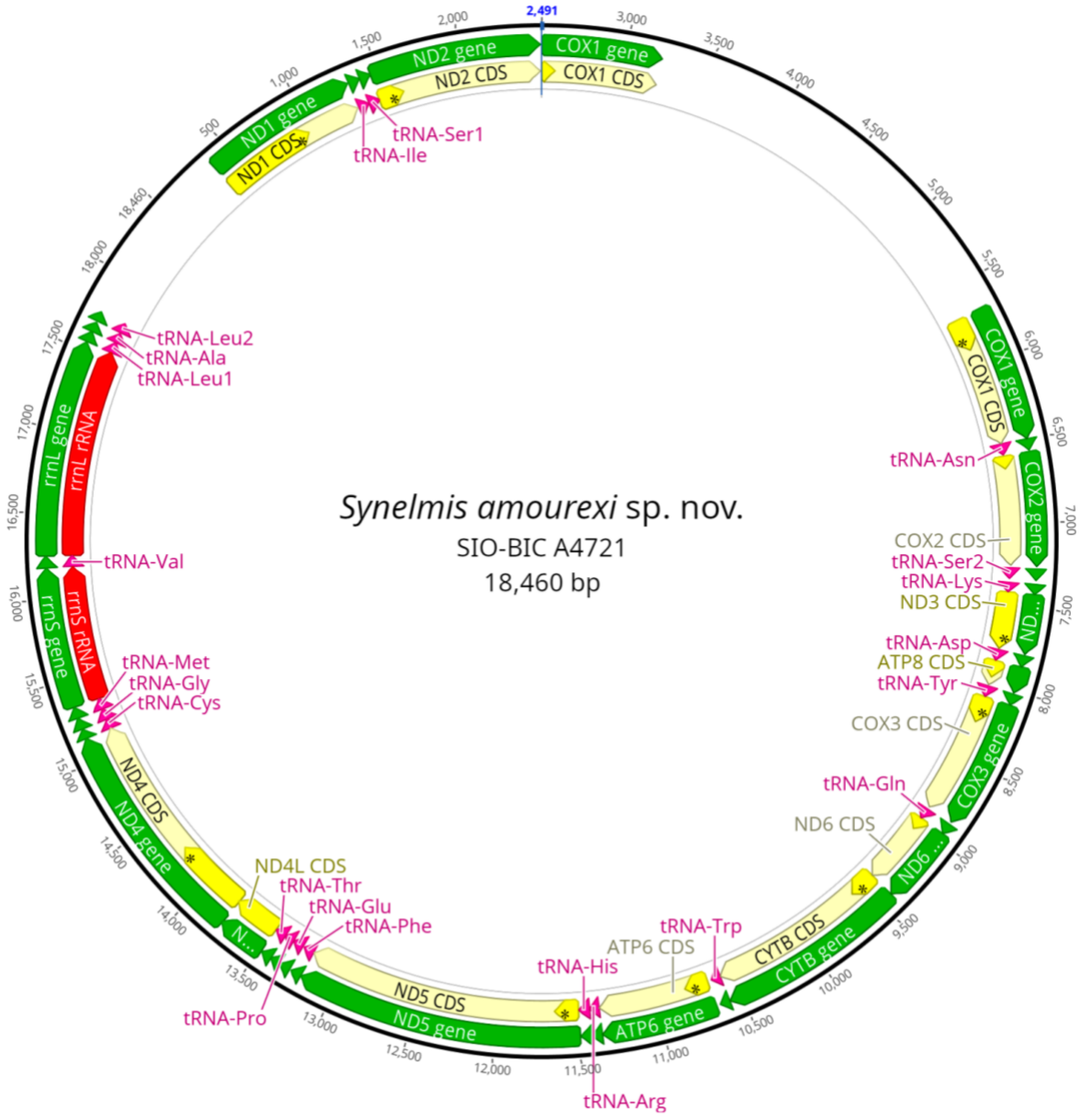

Figure 13: Circularized mitogenome of *Synelmis amoureuxi*. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

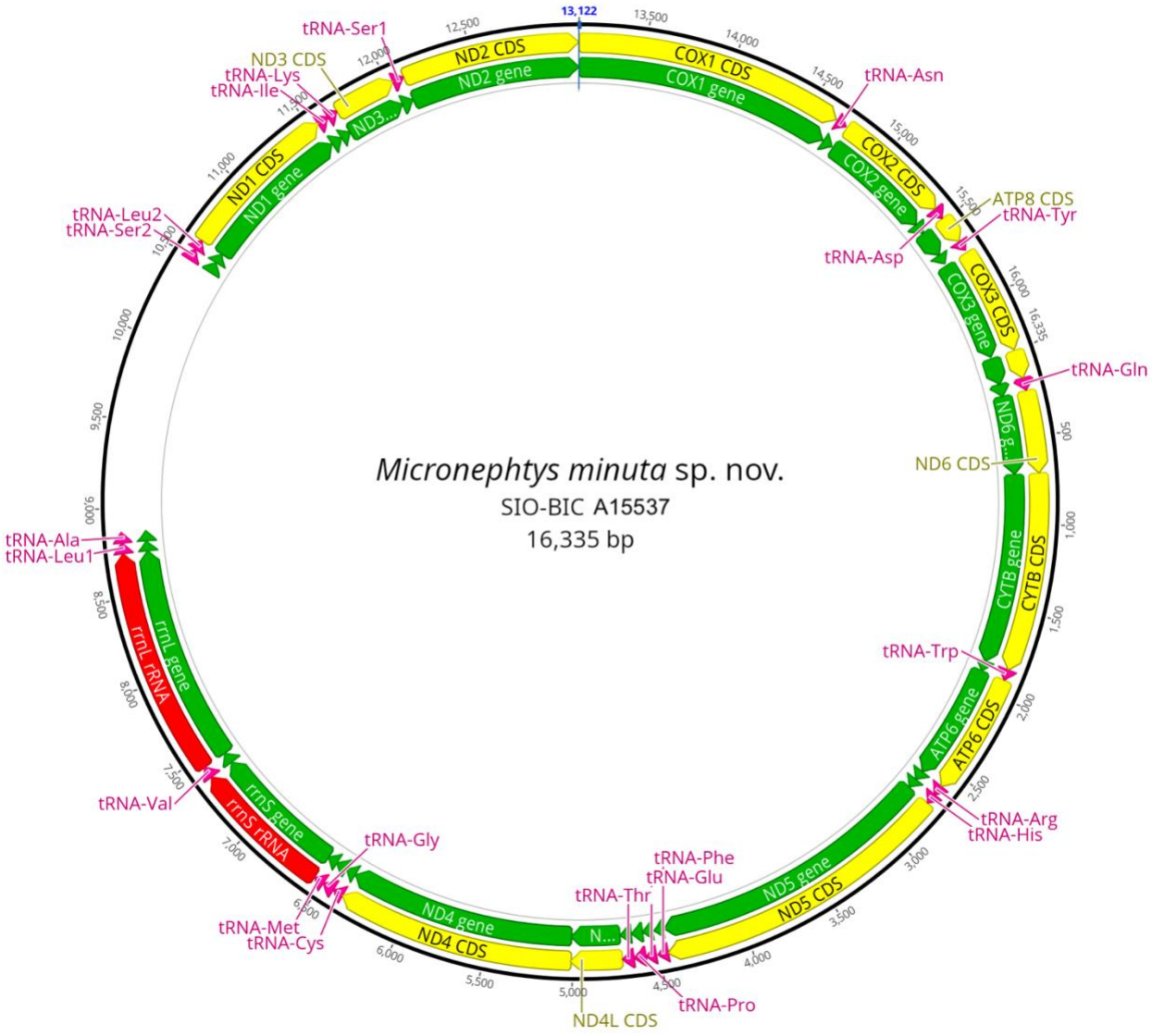

Figure 14: Circularized mitogenome of Micronephthys minuta. The black circle is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

The complete circularized mitochondrial genomes for pilargids *Antonbruunia milenae* sp. nov. (17,540 bp) (Fig. 5), *Pilargis verrucosa* Saint-Joseph, 1899 (15,131 bp) (Fig. 6), *Pilargis wolfi* (15,506 bp) (Fig. 7), of *Ancistrosyllis tiffanyae* sp. nov. (15,392 bp) (Fig. 8), *Glyphohesione klatti* (15,490 bp) (Fig. 9), *Otopsis fredi* sp. nov. (15,694 bp) (Fig. 10), *Sigambra cortesi* sp. nov. (15,680 bp) (Fig. 11), *Sigambra gabriellae* sp. nov. (15,304) (Fig. 12), and *Synelmis amoureuxi* (18,460 bp) (Fig. 13) have a length of between 15,131 bp and 18,460 bp. *Antonbruunia milenae* sp. nov. and *Synelmis amoureuxi* have an intron in their COI gene, 305 bp and 2,459 bp long, respectively. The outgroup complete circularized mitogenome, *Micronephthys minuta* Théel, 1879 (Fig. 14), is 16,335 bp long. Unlike the pilargid mitogenomes, its COI gene starts with the start codon GTG instead of ATG or ATA. Figure 15 shows the incomplete mitogenome of *Hermundura fauveli*, which is missing the tRNA-Ala gene. All the complete mitogenomes fall into one of four distinct mitogenome gene order groups, shown in Figure 16. Group I is comprised of *Ancistrosyllis tiffanyae* sp. nov., *Sigambra cortesi* sp. nov, *Sigambra gabriellae* sp. nov, *Pilargis verrucosa*, *Pilargis wolfi*, and the outgroup *Micronephthys minuta*, Group II is *Synelmis amoureuxi*, Group III is comprised of *Glyphohesione klatti* and *Otopsis fredi* sp. nov., and Group IV is *Antonbruunia milenae* sp. nov.

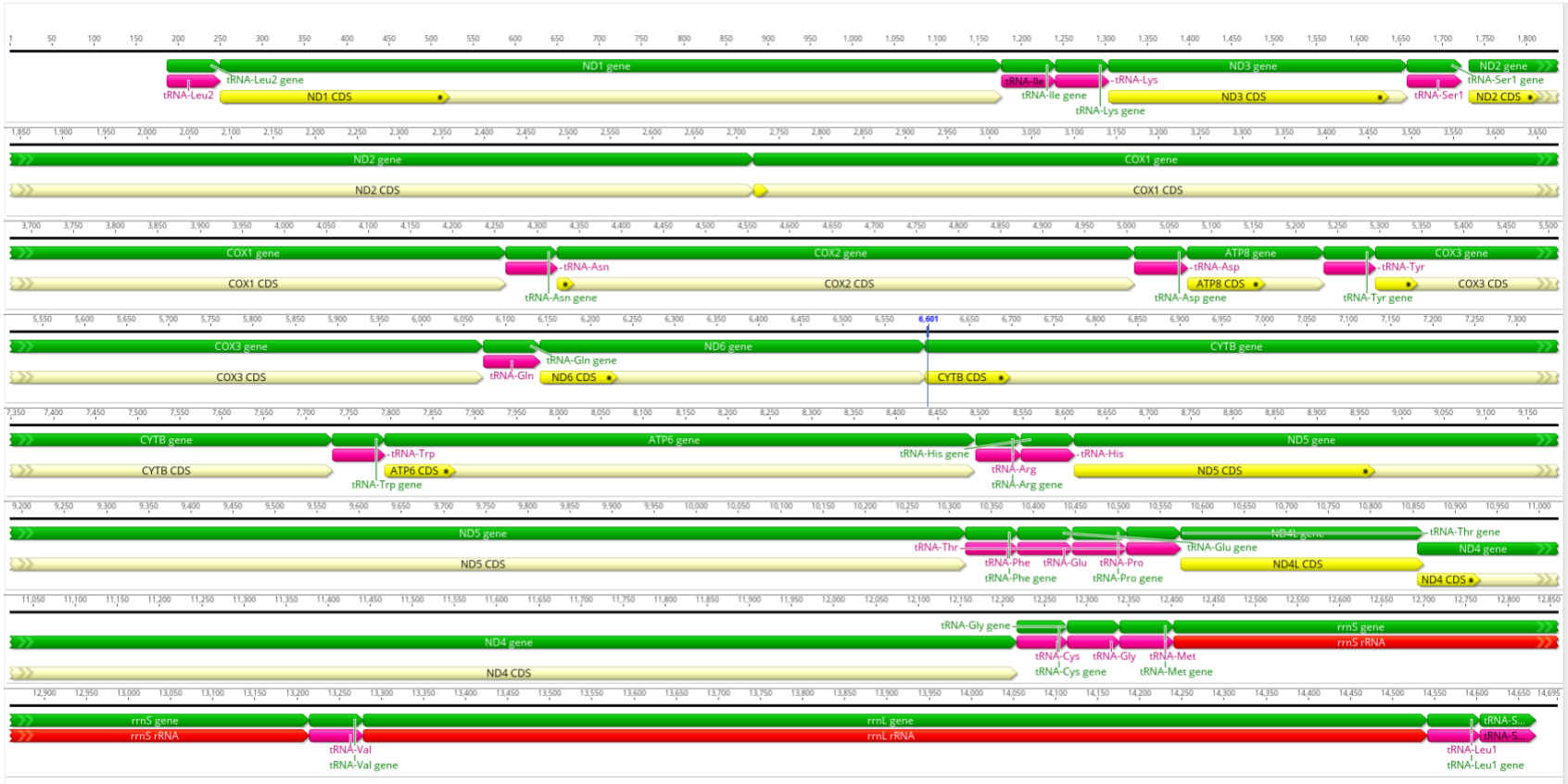

Figure 15: Incomplete mitogenome of *Hermundura fauveli*. The black line is the nucleotide sequence, with numbers marking nucleotides. Green arrows are genes, while the yellow arrows show which genes are protein-coding, red arrows show which genes are non-protein-coding, and pink arrows show which genes are tRNAs. The direction of the arrows is the direction of the gene.

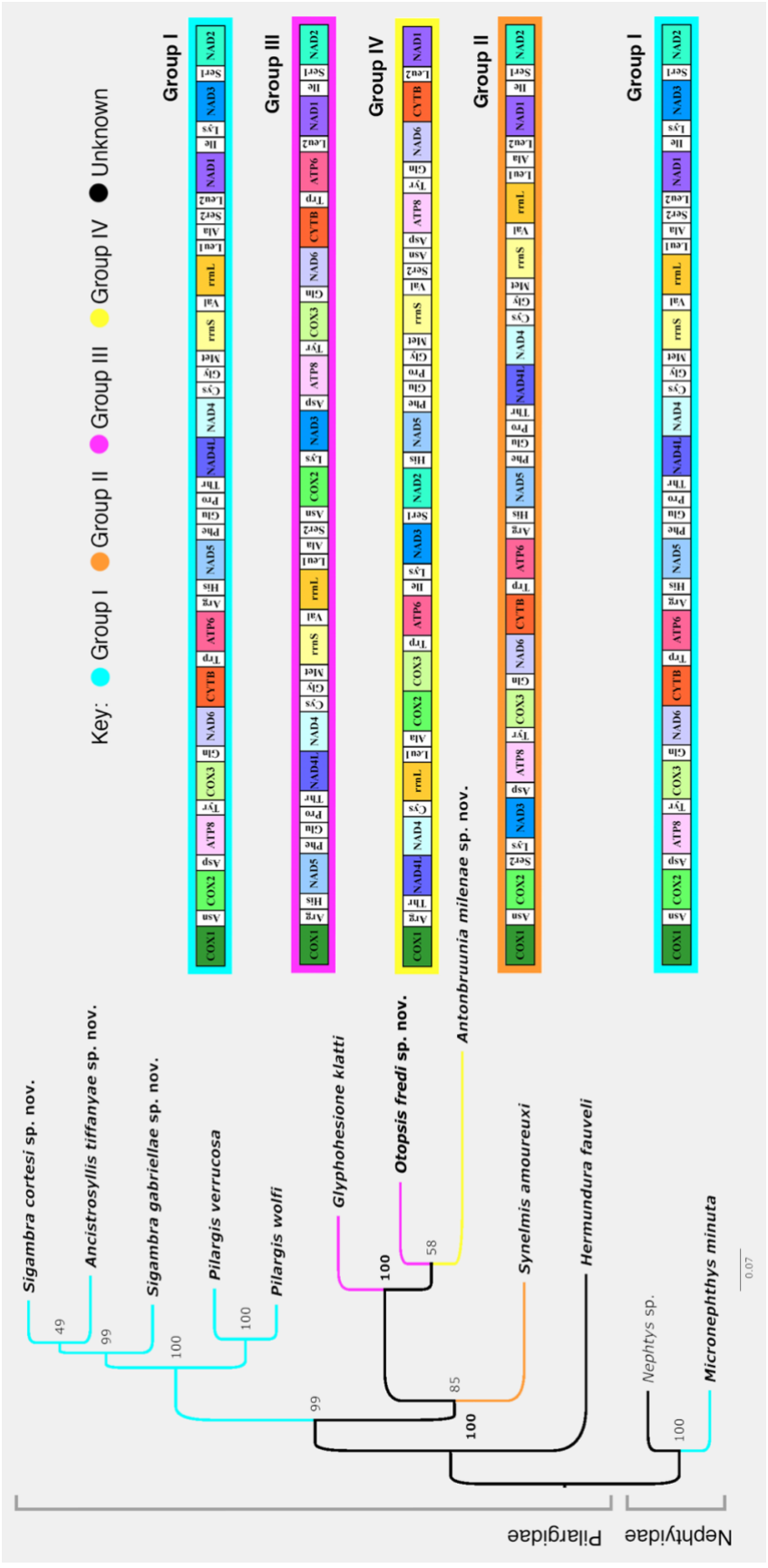

Figure 16: Sixteen gene phylogeny and gene orders. Tree branches are color coded for mitogenome gene order group when known.

Taxonomy

Pilargidae Saint-Joseph, 1899

Hermudnurinae subfam. nov.

(Emerson & Fauchald, 1971; Fauvel, 1932; Glasby & Hocknull, 2010; Monro, 1936; Müller, 1858; S. I. Salazar-Vallejo, 1990)

Diagnosis.

Body long, subcylindrical, anteriorly inflated. Reduced prostomium and palps. Antennae absent. First segment lacks parapodia and cirri. Parapodia with reduced notopodia, emergent straight notospines, capillary chaetae present, no notoaciculae. Neuropodia with neuroaciculae, capillary neurochaetae. Pygidium enlarged, at least two pygidial cirri.

Type genus. *Hermundura* Müller, 1858

Included genera: *Hermundura* Müller, 1858.

Synelminae Salazar-Vallejo 1987 (emended)

(Augener, 1922; Chamberlin, 1919; Ditlevsen, 1917; Ehlers, 1920; Fauchald, 1977; Friedrich, 1950; Licher, 1994; T. H. Pearson, 1970; S. Salazar-Vallejo et al., 2007; S. I. Salazar-Vallejo, 1986, 2003)

Diagnosis (emended).

Body cylindrical or dorsoventrally flattened, integument smooth. Prostomium with two or no fused or biarticulate palps; no or three antennae. First segment with two pairs of cirri. Notopodia with or without emerging straight or curved spines. Lacking jaws.

Type genus. *Synelmis* Chamberlin, 1919

Included genera: *Antonbruunia* Hartman & Boss, 1966, *Glyphohesione* Friedrich, 1970, *Litocorsa* Pearson, 1970, *Otopsis* Ditlevsen, 1917, *Pseudoexogone* Augener, 1922, Synelmis Chamberlin, 1919.

Remarks.

Synelminae is emended based on the phylogenetic tree in this study to newly include *Glyphohesione* (previously in Pilarginae Saint-Joseph, 1899), *Antonbruunia* (previously in Antonbruunidae), and *Otopsis* (previously unassigned subfamily level) by allowing for dorsoventrally flattened body shape. *Hermundura* (now in Hermundurinae subfam. nov.) is excluded by requiring the presence of cirri on the first segment.

***Antonbruunia* Hartman & Boss, 1965**

***Antonbruunia milenae* sp. nov.**

Figures 17-21

Material examined.

Holotype: SIO-BIC A12281* (prepared for SEM), female of pair found in *Calyptogena pacifica* clam SIO-BIC M18183, Rosebud whalefall, San Diego, CA, USA, 32.7769° N 117.4881° W, ~845 m depth, February 9, 2020, ROV Doc Ricketts, R/V Western Flyer.

Paratypes: SIO-BIC: A12280* (prepared for SEM) male of pair found in clam M18183, A12278* male and A12279* female of pair found in clam M18180, A12282 male and A12283* female of pair found in clam M18184, Rosebud whalefall, San Diego, CA, USA, 32.7769° N 117.4881° W, ~845 m depth, February 9, 2020, ROV Doc Ricketts, R/V Western Flyer. For accession number details, see table 2. * indicates sequenced specimens.

Description.

Holotype length ~18.5 mm, width up to ~1 mm including parapodia, ~80 segments. Segment width rapidly increases in anterior tenth (~10 segments), remains evenly wide ~40 segments, gradually decreases in posterior fourth (Fig. 17A, H). Body vermiform, arched dorsally, ventrally flattened (Fig. 17A, H), with ventral groove (Fig. 18A, F). Translucent yellowish white body color in life, dark greenish dorsal median longitudinal stripe and one horizontal medio-dorsal stripe per segment, except on first four bright, opaque white segments. Green coloration width decreases, fades to partial or complete absence in posterior eighth (Fig. 17A, H). Color entirely yellowish white after preservation (formalin/ethanol).

Prostomium shape trapezoidal, lacking eyes, with pair of conical 0.1 mm palps on anterior margin, pair of tapered conical 0.2 mm anterior dorso-lateral antennae, single posterior dorsal 0.2 mm median antenna similar to paired antennae, but obviously narrower base (Fig. 18A, B). Nuchal organs not clearly observed, may be present at posterior margin of prostomium, as seen in male paratype SIO-BIC A12280 (Fig. 19A). Peristomium present as ventral transverse slit-like mouth, no visible margins (Fig. 18A). Jawless pharynx. First segment achaetous, two pairs of tapered conical cirri; dorsal cirrus 130 μm, base almost 2x width of 70 μm ventral cirrus (Fig. 18A, B). First segment dorsal cirri not obviously longer than following parapodial dorsal cirri, ventral cirrus longer, much more conical than parapodial ventral cirri (Fig. 18A, B).

Subbiramous parapodia appear uniramous (Fig. 20A, C). Neuropodia with conical, distally tapered dorsal cirri with single embedded distally pointed notoacicula; much smaller, bluntly rounded ventral cirri, with base ~two thirds width of dorsal cirri (Fig. 18A, E, F, 20A, C). Ventral cirri much shorter, blunter on anterior parapodia (Fig. 18A, B, 19A), lengthening, tapering towards posterior (Fig. 18E, F, 19C). Parapodia with multiple thickly arranged chaetae in two bundles (Fig. 18D, 20C, E), with embedded, distally pointed neuroaciculae (Fig. 20C, D). Anterior, posterior parapodia at least 27 chaetae, largest middle parapodia up to 135 chaetae. Chaetae simple, slightly curved, distally thickened, bifid with angled straight distal tooth, inwardly curved proximal tooth (Fig. 18C, 19D, 20B). Pygidium with two ~0.1 mm pygidial cirri, inflated in distal fourth (Fig. 17A, 18F). Anus dorsal (Fig. 18F, 19B).

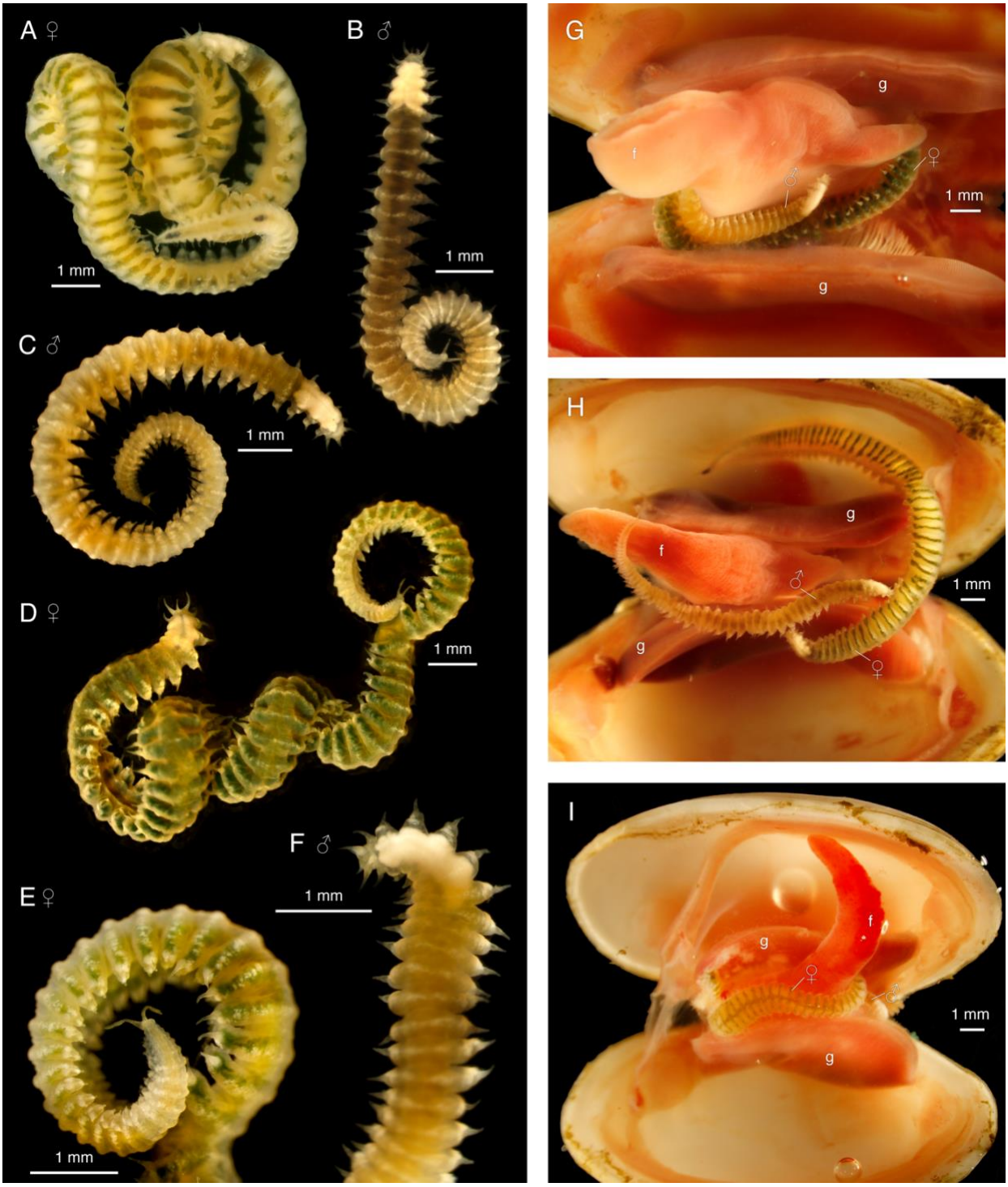

Figure 17: *Antonbruunia milenae* sp. nov. live photos. **A** female holotype SIO-BIC A12281 **B** male paratype SIO-BIC A12282 **C** male paratype SIO-BIC A12280 **D** female paratype SIO-BIC A12279 **E** pygidial cirri of female paratype SIO-BIC A12279 **F** anterior of male paratype SIO-BIC A12278 **G** paratypes SIO-BIC: A12278 and A12279 in clam M18180 **H** holotype SIO-BIC A12281 and paratype SIO-BIC: A12280 in clam M18183 **I** paratypes SIO-BIC: A12282 and A12283 in clam M18184. Abbreviations: f, foot; g, gills.

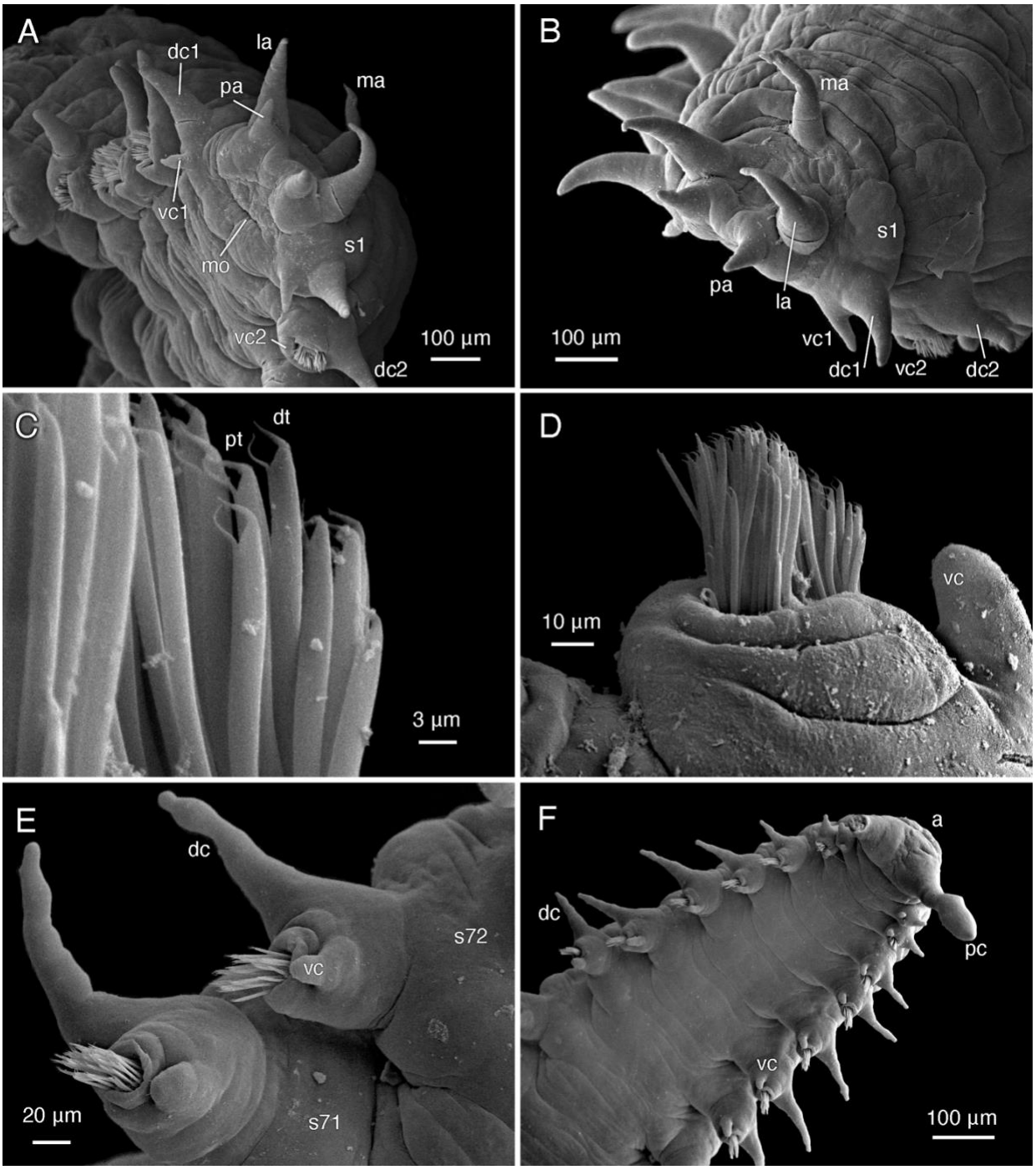

Figure 18: SEM images of *Antonbruunia milenae* sp. nov. holotype SIO-BIC A12281. **A** anteroventral view of anterior **B** laterodorsal view of anterior **C** detail of chaetae from anterior neuropodium **D** anterior neuropodium **E** posterior neuropodia **F** ventral view of posterior. Abbreviations: a, anus; dc, dorsal cirrus; dc1, dorsal cirrus of first segment; dc2, dorsal cirrus of second segment; dt, distal tooth of chaeta; la, lateral antenna; ma, median antenna; mo, mouth; np71, neuropodium of segment 71; np72, neuropodium of segment 72; pa, palp; pc, pygidial cirrus; pt, proximal tooth of chaeta; s1, segment 1; s71, segment 71; s72, segment 72; vc, ventral cirrus; vc1, ventral cirrus of first segment; vc2, ventral cirrus of second segment.

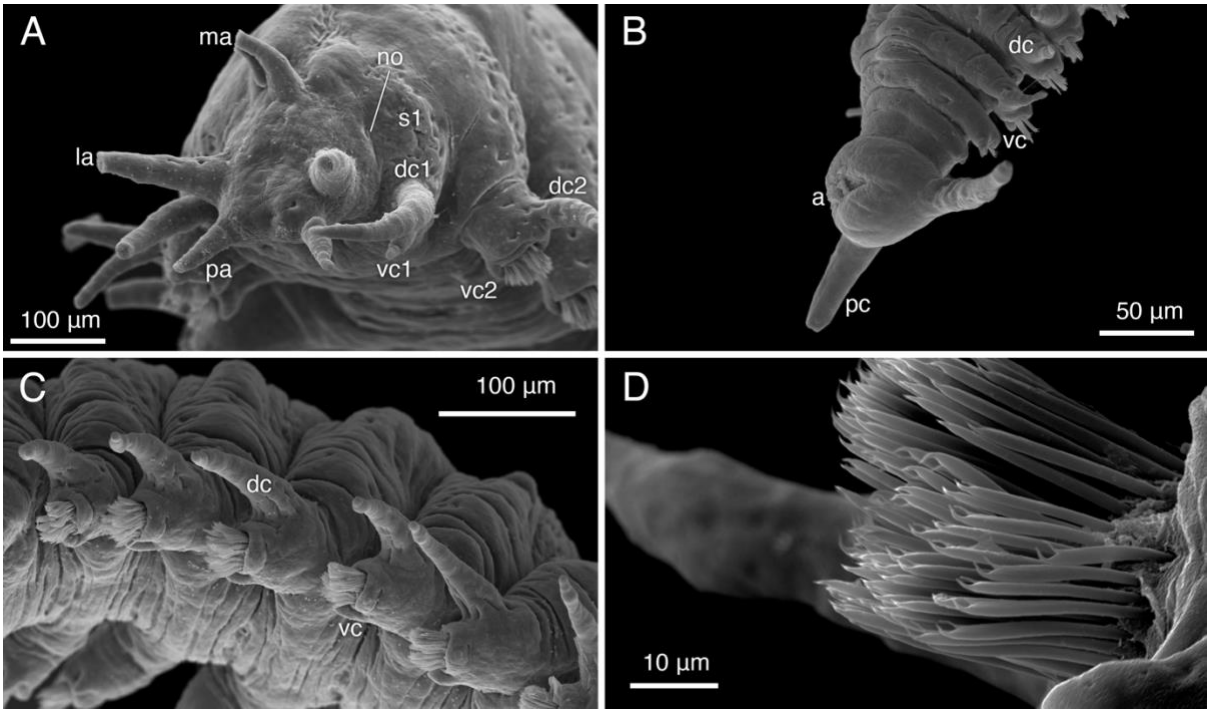

Figure 19: SEM images of *Antonbruunia milenae* sp. nov. paratype SIO-BIC A12280. **A** anterolateral view of anterior **B** dorsolateral view of pygidium **C** lateral view of midsection **D** chaetae closeup. Abbreviations: a, anus; dc, dorsal cirrus; dc1, dorsal cirrus of first segment; dc2, dorsal cirrus of second segment; la, lateral antenna; ma, median antenna; no, nuchal organ; pa, palp; pc, pygidial cirrus; vc, ventral cirrus; vc1, ventral cirrus of first segment; vc2, ventral cirrus of second segment.

Variation.

Sexual dimorphism present. Males smaller, ~50% body length of females (Fig. 17); female paratype SIO-BIC A12279 ~18.5 mm long like holotype, ~85 segments; three male paratypes ~8.5-10.5 mm, ~42-57 segments. Color variation between sexes: all males uniformly different from females; in life, dull yellowish white (some with slight dark green tinge in anterior two thirds) with opaque bright white patchy horizontal mediodorsal line on most segments (Fig. 17B, C, F). Color variation within females: in life, paratype SIO-BIC A12279 anteriorly instead of stripes more general green coloration than holotype, paratype SIO-BIC A12283 (Fig. 17D, G). Male dorsal cirri of first segment obviously longer than parapodial dorsal cirri (Fig. 19A), unlike females. Pygidial cirri of males (Fig. 19B) and paratype SIO-BIC A12283 conical and uniformly

tapering, no inflation. Paratype SIO-BIC A12279 inflation in distal fifth (Fig. 17E). Male pygidial cirri length ~ 50 μm, female up to 0.2 mm (Fig. 17E, 19B). Putative nuchal organs found in male paratype SIO-BIC A12280 between posterior prostomium and first segment (Fig. 19A).

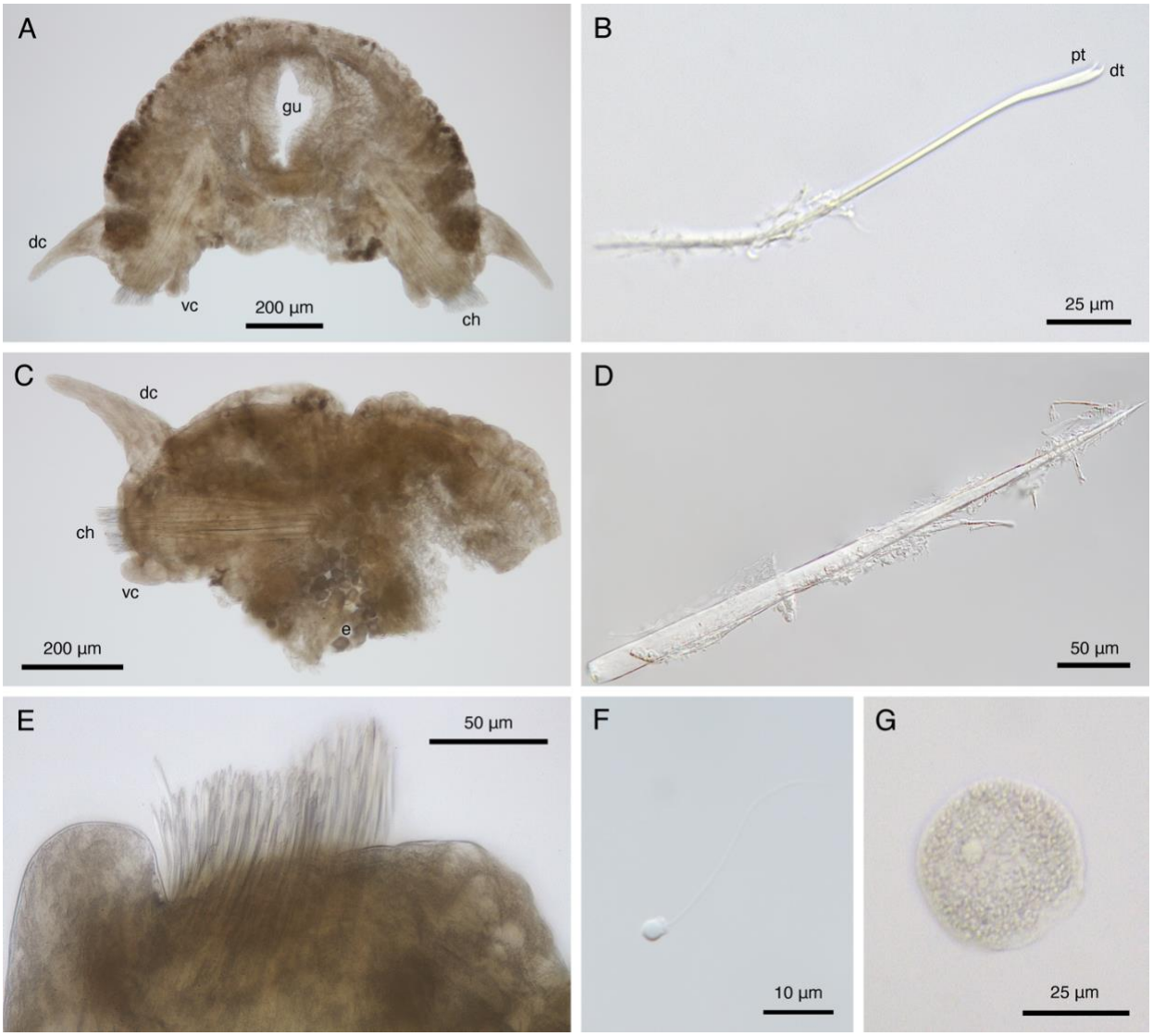

Figure 20: *Antonbruunia milenae* sp. nov. DIC microscopy images. **A** midanterior region cross section of male paratype SIO-BIC A12278 **B** single chaeta of female paratype SIO-BIC A12283 **C** anterior parapodium of holotype SIO-BIC A12281 **D** acicula of female paratype SIO-BIC A12283 **E** detail of anterior neuropodium of holotype SIO-BIC A12281 **F** sperm of male paratype SIO-BIC A12280 **G** egg of holotype SIO-BIC A12281. Abbreviations: ch, chaetae; dc, dorsal cirrus; dt, distal tooth of chaeta; e, egg; gu, gut; pt, proximal tooth of chaeta; vc, ventral cirrus.

Habitat.

Antonbruunia milenae sp. nov. is found within the host chemosynthetic vesicomyid clam *Calyptogena pacifica*. Inside each collected clam, two individuals were found, wrapped around the foot: one smaller male and one larger female (Fig. 17G, H, I). The clams were collected from the blackened sulfuric sediments surrounding the jaw bones of the Rosebud whalefall (Fig. 21).

Figure 21: Rosebud whalefall in February of 2020 with *Calyptogena pacifica* clams in a cluster by whale jawbone, in anoxic black sediments. Closeup of clam. Scale: red lasers are 29 cm apart.

Reproduction.

A male and female pair of non-closely related individuals lives in each clam; females have eggs 40 μm wide in their preserved state (Fig. 20G) and males have sperm with 5 μm head and midpiece regions and 40 μm long tails in unpreserved states (Fig. 20F).

Remarks.

Antonbruunia milenae sp. nov. is morphologically most similar to *A. sociabilis*. According to Mackie et al. (2015), *A. sociabilis* has only 32-52 segments, while *A. milenae* sp. nov. females have ~80-85 segments, males ~42-57. *Antonbruunia sociabilis* has no ventral groove. Chaetae number is lower in *A. sociabilis*; parapodia of the largest segments have 40-45 chaetae and posterior segment parapodia have less than 5-6 chaetae, while *A. milenae* has 27-135 chaetae. *Antonbruunia sociabilis'* bifid chaetae have a less curved proximal and more curved

distal tooth than *A. milenae* sp. nov. Additionally, *A. sociabilis* differs in the collection location of the North Atlantic Ocean E of Scotland, depth of 1187-1200 m, and host bivalve *Thyasira scotiae*. Genetically they clearly different.

Antonbruunia viridis is also very similar to *A. milenae* sp. nov., but there is no DNA to compare the two species. However, according to Hartman & Boss (1965), the female holotype of *A. viridis* is 14 mm long, has only 52 segments and 26-30 chaetae per parapodium compared to *A. milenae* sp. nov. with ~18.5 mm length, ~80-85 segments, and up to 135 chaetae. The shape of the chaetae teeth also seems to differ, but that is only visible in the SEM photos of *A. milenae* sp. nov. and not with DMR light microscopy, and SEM photos of *A. viridis* are not available to check in greater detail. *Antonbruunia milenae* sp. nov. dorsal parapodial cirri have a wider base, ventral parapodial cirri are more blunt and shorter relative to the dorsal cirri than in *A. viridis*, especially anteriorly. Additionally, the *A. viridis* collection location of the Indian Ocean off the SW coast of Madagascar, depth of 70-80 m, and host clam species *Opalocina fosteri* sets the species apart.

Antonbruunia milenae sp. nov. differs the most from *A. gerdesi*. According to Quiroga & Sellanes (2009), *A. gerdesi* males are 5.86 mm long with 33 segments, females 16.4 mm long with 53 segments, which is shorter than *A. milenae* sp. nov. However, *A. gerdesi* is about twice as wide as *A. milenae* sp. nov. *Antonbruunia gerdesi* lacks a ventral groove, has wider and shorter antennae, palps in proportion to the head, bifid chaetae teeth that are straight and similar in length instead of the proximal tooth being curved, and clearly different pygidial cirri (Quiroga & Sellanes, (2009) refer to them as anal cirri) that are proportionately shorter and thicker than those in *A. milenae* sp. nov. While both species are found in the Pacific Ocean at similar depths,

A. gerdesi is found much further south off Concepción, Chile, and inside the host clam species *Calyptogena gallardoi*.

In the emended description of the genus *Antonbruunia* by Mackie et al. (2015), the cirri on the first segment are referred to as tentacular cirri.

Parts of the holotype and paratypes were destroyed for the purpose of DNA collection, which is why lengths and segment counts are approximate for most specimens and unavailable for some, since they were inferred from photos of the live specimens when available.

Etymology.

Antonbruunia milenae sp. nov. is named after the lead author's maternal great-grandmother (Mama) Milena Trontelj for being the first to spark the lead author's love of marine invertebrates.

DISCUSSION

This is the broadest phylogenetic study of Pilargidae to date. Having access to genetic samples of *Antonbruunia milenae* sp. nov. and to all accepted pilargid genera except for *Sigatargis* Misra, 1999 has allowed for a thorough reevaluation of the phylogenetic position of *Antonbruunia*. In both the three-gene nucleotide and sixteen-gene amino acid and nucleotide phylogenies (Fig. 2, 3), *Antonbruunia* is nested within Pilargidae, being sister to *Otopsis fredi* sp. nov. and forming a very well supported clade (bootstrap support value of 100) with *Glyphohesione klatti*. There is also very strong support (bootstrap support value of 100) for *Hermundura fauveli* being the sister taxon to the clade including the rest of the pilargids and *Antonbruunia*. Therefore, Antonbruunidae is regarded as a junior synonym of Pilargidae.

The relationships between pilargid genera are not congruent in the two phylogenies when it comes to the position of *Synelmis amoureuxi*. In the three-gene phylogeny, *Synelmis amoureuxi* and well supported sister taxon *Pseudoexogone* cf. *backstromi* form one of four major pilargid clades, but with very poor support (bootstrap support value 28). In the sixteen-gene phylogeny, *Synelmis amoureuxi* is the fairly well supported (bootstrap support value of 85) sister taxon to the clade formed by *Glyphohesione klatti*, *Otopsis fredi* sp. nov., and *Antonbruunia milenae* sp. nov. Because of the greater phylogenetic resolution in that phylogeny due to a larger number of analyzed genes, and the much greater bootstrap support value, it is regarded as being a better representation of the actual evolutionary relationships between the genera, though additional statistical testing is needed. Additionally, albeit poorly supported (bootstrap value of 58), the synelminid *Litocorsa stremma* is a sister taxon to the rest of the clade containing *Antonbruunia*. With this evidence, the genus *Antonbruunia* is considered to belong to the subfamily Synelminae, along with the genus *Otopsis*, which had previously not been assigned a

subfamily affiliation. Additionally, *Glyphohesione* is transferred from Pilarginae to Synelminae to reflect its position in the phylogenies. Consequently, Pilarginae now includes only *Pilargis, Sigambra*, *Ancistrosyllis*, and *Cabira* Webster, 1879. The diagnosis of Pilarginae (S. I. Salazar-Vallejo, 1986) did not require emendment because all morphological traits still describe the remaining genera.

The position of *Hermundura fauveli* (the identification of which was confirmed using the physical voucher specimen borrowed from the Florida Museum of Natural History, which corresponds to the DNA extraction borrowed from the USNM), which branches off as the first major pilargid clade, is strongly supported with a bootstrap support value of 100 in both phylogenies. Because it is outside of both the Synelminae and Pilarginae clades, and because of its unique morphology within pilargids concerning the lack of enlarged cirri on the first segment (Emerson & Fauchald, 1971; Fauvel, 1932; Glasby & Hocknull, 2010; Monro, 1936; Müller, 1858), *Hermundura* is removed from Synelminae, and Hermundurinae subfam. nov. is erected, with *Hermundura* as the only included and type genus. Hartman (1947) had hypothesized that *Loandalia* Monro, 1936 and *Talehsapia* Fauvel, 1932, now known as *Hermundura* (Glasby & Hocknull, 2010), could potentially be their own family because of their morphological differences compared to the other pilargids. Besides the lack of enlarged cirri on the first segment, she also cited the lack of antennas on the prostomium, however that trait is also present in *Litocorsa* (Fauchald, 1977; T. H. Pearson, 1970) and is no longer unique to *Hermundura*. While not a new family, the phylogenies confirmed her suspicion of *Hermundura* being in some way separate to other pilargids.

Because of the exclusion and inclusion of genera into Synelminae, the original diagnosis (S. I. Salazar-Vallejo, 1986) required emendment. To include *Antonbruunia, Glyphohesione*, and

Otopsis, the body shape needs to allow for dorsoventral flattening and must not require iridescence. Because *Hermundura* was excluded, the first segment must have two pairs of cirri (referred to in the original diagnosis as the peristomium with or without two pairs of tentacular cirri). It was reiterated that notopodial spines may (*Synelmis*, *Glyphohesione*, *Pseudoexogone*, *Litocorsa*) or may not (*Antonbruunia*, *Otopsis*) be present. The spines may be straight or curved, as in *Pseudoexogone* (S. Salazar-Vallejo et al., 2007), which was described after the original diagnosis of Synelminae. Additionally, it was specified that Synelminae lack jaws because the original description included Fauvel's (1932) incorrect evaluation of *Hermundura*'s denticulate band-resembling pharyngeal structures (Glasby & Hocknull, 2010; S. I. Salazar-Vallejo et al., 2001).

The fourth (Fig. 2) or third (Fig. 3) major pilargid clade containing Pilarginae is congruent in topology across both phylogenies. *Pilargis* is a well-supported monophyletic clade. In Figure 2, *Ancistrosyllis* is also a well-supported monophyletic clade, however it is nested in between two well-supported *Sigambra* clades making *Sigambra* paraphyletic, although the nodes between the three generic clades have low support with bootstrap support values 51 and 52. In Figure 3, each *Sigambra* clade is represented by only one species, as is the *Ancistrosyllis* clade; the first clade, *Sigambra gabriellae* sp. nov. has high support (bootstrap support value of 99), while the position of *Ancistrosyllis tiffanyae* sp. nov. and *Sigambra cortesi* sp. nov. being sister taxa has low support (boostrap support value of 49). There is not currently enough evidence to either split Sigambra into two genera or consider *Ancistrosyllis* as a junior synonym of *Sigambra*. Phylogenetically, genetic samples of more species from both genera would be needed, particularly the type species *Sigambra grubii* Müller, 1859 and *Ancistrosyllis groenlandica* McIntosh, 1878. Though COI and 18S of the latter were used in this study, obtaining at least

mitogenomes is likely needed for further clarification of the phylogenetic relationships within Pilarginae. Morphologically, Hartman (1947) had already debated whether *Ancistrosyllis* was actually *Sigambra*. According to S. I. Salazar-Vallejo et al. (2019), the first difference between the two genera is the relative size of the antennae, dorsal cirri, and first segment cirri (regarded by him as tentacular cirri); *Ancistrosyllis* having shorter appendages and *Sigambra* longer. The second difference is the former having stronger body papillation and the latter having a mostly smooth integument. Because pilargids in general are rather morphologically diverse (G. Rouse et al., 2022), if it will be shown in the future the two genera are the same, it is not unlikely that a single genus has the morphological variation between *Sigambra* and *Ancistrosyllis*. For instance, *Pilargis* has varying papillation between species and varying dorsal cirri (S. I. Salazar-Vallejo & Harris, 2006), while *Antonbruunia* has variation in relative antennae length between species and varying relative first segment cirri size even within a single species due to sexual dimorphism. Should the two *Sigambra* clades (Fig. 2, 3) be split into two genera, the main morphological difference that appears to be present between the clades based on species in this study is the number of pharyngeal papillae being either eight or more than eight (usually 13-14) (Nishi et al., 2007). Therefore, the option of *Ancistrosyllis* being a junior synonym of *Sigambra* seems more likely, but further study is needed.

In addition to creating phylogenies, this study also presents the first ten pilargid mitochondrial genomes and the third nephtyid mitogenome (Bernardino et al., 2017a; Vallès et al., 2008). *Anotnbruunia milenae* sp. nov. and *Synelmis amoureuxi*, which have an intron in their COI gene, had mitogenome sizes of 17,540 bp and 18,460 bp respectively, while the rest of the pilargids had mitogenome sizes of 15,131 bp to 15,694 bp. Both species also had unique mitogenome gene orders (Fig. 16); *Pilargis*, *Sigambra*, and *Ancistrosyllis tiffanyae* sp. nov. have

the Group I gene order, *Glyphohesione klatti* and *Otopsis fredi* have the Group III gene order, while *Synelmis amoureuxi* is the only species in this study with the Group II gene order, and *Antonbruunia milenae* sp. nov. the only species with the Group IV gene order. Further statistical and phylogenetic analysis is needed to infer what kind of changes occurred between mitogenome orders and what the ancestral state gene order was. However, it is clear that *Antonbruunia milenae* sp. nov. has the most different gene order compared to the other represented genera; this could possibly be a result of genetic changes necessary for its inquiline lifestyle and may also be the cause of the long branch (Fig. 2, 3). The complete mitogenome of *Hermundura fauveli* was not obtained; despite multiple assembly attempts with different programs, the gene for tRNA-Ala was still missing. However, the rest of the genes were present and in order with no spaces in between large enough for a tRNA to be, so it is likely that tRNA-Ala is either before the first gene tRNA-Leu2 or after the last gene tRNA-Ser2. If that turns out to be true with further research, then the gene order for *Hermundura fauveli* would be the same as Group I. Additionally, *Micronephthys minuta* also has the gene order of Group I, which could mean that the Group I gene order is the ancestral state for at least pilargids.

The newly described species, *Antonbruunia milenae* sp. nov. is the fourth described within the genus. Based on the haplotype network (Fig. 4), it appears the male and female individuals in a pair in a single clam are not closely related, since they each have their own individual haplotype. It is interesting that there is an isolated heterosexual pair of worms in each clam, as this is not a common occurrence among commensal polychaetes (Britayev & Zamishliak, 1996); besides in *Antonbruunia viridis* and *A. milenae* sp. nov., this is observed in the sea star associated *Bathynoe cascadiensis* Ruff, 1991 (Ruff, 1991) and the deep-sea sponge dwelling Harmothoe hyalonemae Martin, Rossel & Uriz, 1992 (Martin et al., 1992). It is likely

that antagonistic intraspecific interactions are to blame for the exclusivity of the pair (Dimock, 1974), for instance either the annelids killing or repelling additional individuals from their host. Nothing else is known about the reproductive habits of the species, but based on their deep-sea inquiline lifestyle and restricted locality, it is unlikely that the larvae are planktrotrophic, which the small egg size (McHugh & Fong, 2002; Pernet & Jaeckle, 2004), also observed in *Antonbruunia sociabilis* (Mackie et al., 2015), indicates (G. Rouse et al., 2022). San Diego is the only known locality of *Antonbruunia* milenae sp. nov., but it's host species *Calyptogena pacifica* is found across the Pacific Ocean (Dall, 1891; Suzuki, 1941). If it should be observed that the new species of worm is present within it's host species throughout the clam's entire distribution, then perhaps the larvae of *A. milenae* sp. nov. are planktotrophic, like in other deep-sea annelids with a wide distribution such as some scale worms (Taboada et al., 2020). However, it might be exactly the inquiline, potentially parasitic lifestyle that accounts for the small egg size. While non-planktotrophic eggs are usually larger (G. Rouse et al., 2022), a decrease in egg size has been seen in some non-planktotrophic annelid clades, such as in the parasitic order Myzostomida von Graff, 1877 (Eeckhaut et al., 2003; G. W. Rouse & Grygier, 2005).

During this study, thirteen other pilargid species were morphologically examined. Five of them, yet to be described, were shown to be new species; *Ancistrosyllis tiffanyae* sp. nov., *Otopsis fredi* sp. nov., *Sigambra cortesi* sp. nov., *Sigambra gabirellae* sp. nov, and *Sigambra otoni* sp. nov. *Otopsis fredi* sp. nov. will be the fourth described species of the genus, and it is the first time DNA was extracted from this taxon. This study also provides the first DNA sequences of the genera *Pseudoexogone* and *Synelmis*, and a range extension of ~250 km for *Pilargis cholae* Salazar-Vallejo & Harris, 2006 (S. I. Salazar-Vallejo & Harris, 2006).

In conclusion, the present investigation has confirmed the phylogenetic placement of *Antonbruunia* within Pilargidae, facilitated by the collection and description of the new species *Antonbruunia milenae*, sp. nov. While Hermundurinae subfam. nov. was erected and Synelminae emended, more work must be done to fully elucidate the intergeneric relationships within Pilargidae. The twelfth pilargid genus *Sigatargis* Misra, 1999 cannot yet be placed within a subfamily without any genetic samples, this study was unable to obtain mitochondrial genomes of the genera *Cabira*, *Litocorsa*, and *Pseudoexogone*, and additional morphological and phylogenetic samples are needed to resolve the paraphyly of *Sigambra*.

ACKNOWLEDGEMENTS

This thesis in full is currently being prepared for submission for publication of the material. Huč, Sonja; Hiley, Avery S.; McCowin, Marina F.; Rouse, Greg W. The thesis author was the primary researcher and author of this material. Thank you also to the captains and crews of the research vessels that made sample collection possible throughout the years and Dr. Fredrik Pleijel and Dr. Greg Rouse for collecting annelid specimens by hand. Thank you to Dr. Charlotte Seid for her assistance with sample organization and draft review and thank you to the entire Rouse Lab for their support.

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