

ORIGINAL ARTICLE OPEN ACCESS

The Sea Cucumber-Infecting Parasite *Apostichocystis gudetama* gen. nov. sp. nov. Expands Marine-Host-Specific Clade of Apicomplexans

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Received: 26 February 2025 | **Revised:** 30 April 2025 | **Accepted:** 5 May 2025

Funding: This work was supported by University of Miami, Ministerio de Ciencia, Innovación y Universidades, PID2020-118836GA-I00; Departament de Recerca i Universitats de la Generalitat de Catalunya, 2021 SGR 00420.

Keywords: apicomplexa | aquaculture | coccidia | holobiont | holothuria | host-microbe interactions | metagenomics | parasitism | protococcidia | symbiosis

ABSTRACT

Unknown ellipsoid bodies, later classified as apicomplexan cysts, are prevalent in the ovaries of Japanese sea cucumbers (*Apostichopus japonicus*), where they can lead to lower fecundity in infected individuals and adverse effects on wild populations as well as aquaculture efforts for this endangered species. Apicomplexans are widespread and essential to marine environments, where they can affect the health and fitness of host populations. We performed genomic sequencing of recovered cysts to gain more ecological and evolutionary information on this parasite. We recovered this apicomplexan's complete nuclear ribosomal RNA (*rrn*) operon, the entire mitochondrial genome, and a partial apicoplast (relic chloroplast) genome. The *rrn* operon phylogeny revealed this parasite as being closely related to coccidian-like parasites of marine fish (ichthyocolid) and cnidarians (corallicolid), while organelle phylogenomics hint at a closer relation to the protococcidian *Eleutheroschizon*. Using this new phylogenetic context and previous morphological descriptions, we describe this parasite as *Apostichocystis gudetama* gen. nov. sp. nov. Mining available microbiomes reveal the presence of *Apostichocystis* spp. beyond its host range, alluding to other potential hosts or cryptic, closely related lineages. Its phylogenetic placement has important implications concerning the evolution of parasitism within Apicomplexa and the divergence of a marine-host-specific clade of coccidian-like parasites.

1 | Introduction

The Japanese sea cucumber (*Apostichopus japonicus*) is a commercially valuable species currently endangered according to the International Union for Conservation of Nature Red List of Threatened Species due to overharvesting (Robinson and Lovatelli 2015). Aquaculture of these sea cucumbers has expanded significantly in recent years, especially in Japan and China (Yu et al. 2014; Robinson and Lovatelli 2015; Yang

et al. 2015), where they are a popular seafood. To account for over-harvesting, some Japanese hatcheries restock wild *A. japonicus* populations by releasing juveniles to fishery grounds (Sakai 2015). Through these hatchery operations, technicians first noted unknown ellipsoidal bodies (UEBs), about ~250 µm in length, within the ovaries of female sea cucumbers (Figure 1). The individuals with UEBs-filled ovaries did not spawn with the normal temperature cue used for spawning induction of *A. japonicus* and have been shown to have lower fecundity than their

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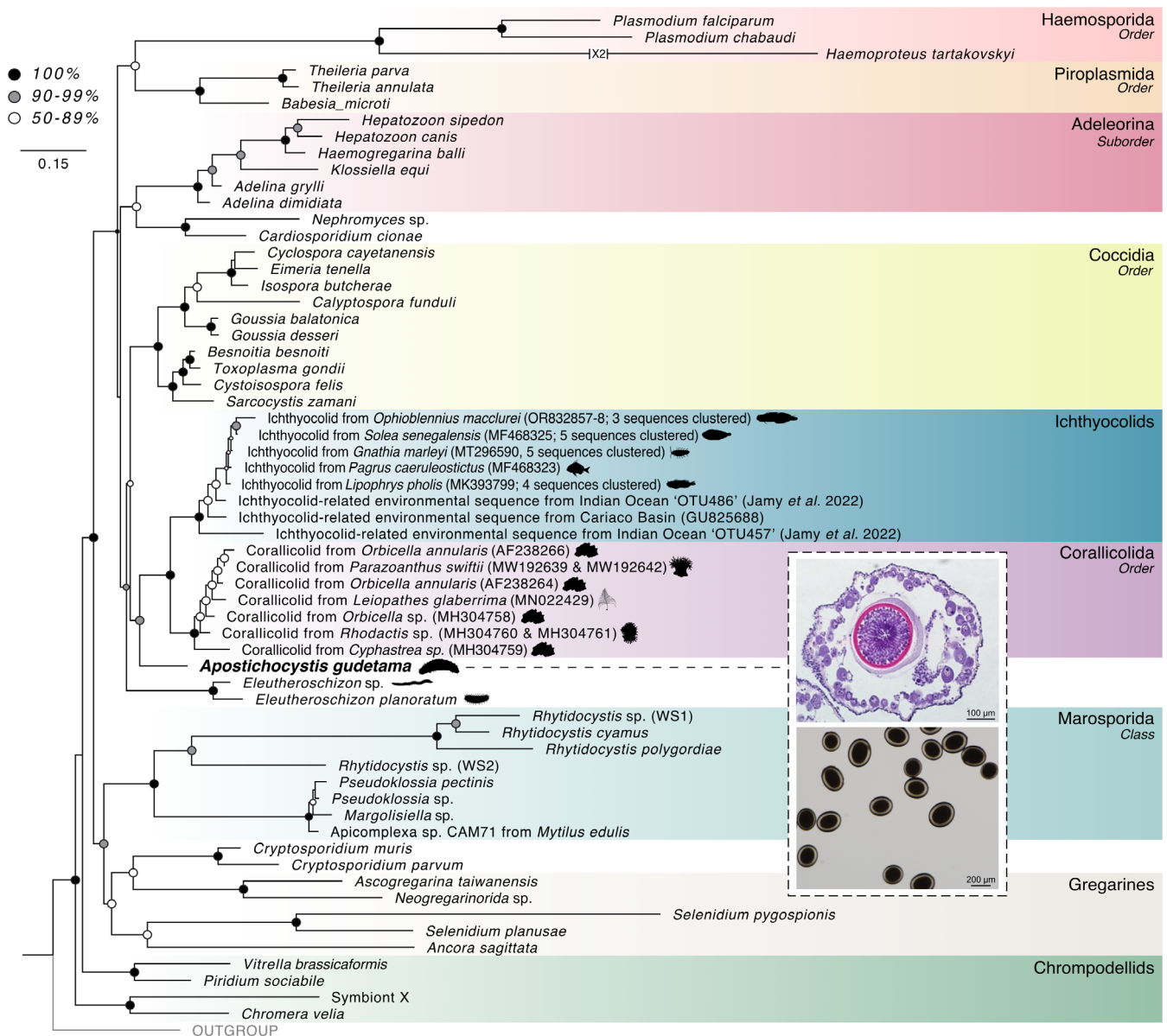


FIGURE 1 | This maximum likelihood tree was constructed using the 18S and 28S subunits of the rRNA operon using 5017 nucleic acid sites with RAxML General Time Reversible model with a GAMMA+P-Invar model of rate heterogeneity ('-m GTRGAMMAIX') and 1000 bootstrap replicates. Silhouettes of the primary hosts for select coccidians and protococcidians are also shown. (See also Supporting Information S1A). Upper image inset—hematoxylin and eosin-stained cysts. Lower image inset—stereoscopic micrograph of cysts isolated from the ovaries of the Japanese sea cucumber, *Apostichopus japonicus*.

non-infected counterparts (Unuma et al. 2020). These UEBs were later characterized as coccidian (Apicomplexa; Coccidia) cysts by sequencing of the small subunit ribosomal RNA (18S rRNA gene) (Unuma et al. 2020). Importantly, this parasite has been detected in more than half of the females during the reproductive season (Unuma et al. 2020), which infer a potential ecological trade-off and co-evolution between the host and parasite.

This sea cucumber-infecting parasite belongs to the phylum Apicomplexa, which encompasses many obligate associates of animal hosts. Dozens of apicomplexan-related lineages (ARLs) (Jan Janouškovec et al. 2012; Janouškovec et al. 2019) have been identified from environmental sequencing data and, along with more genomic work on core apicomplexans, has expanded our knowledge on the origin of parasitism (Mathur et al. 2019), organelle

evolution (McFadden and Yeh 2017), and the breadth of known microeukaryotic diversity (del Campo et al. 2019). The corallicolids (previously known as ARL-V) and ichthyocolids (previously known as ARL-VI), widespread symbionts of anthozoans and marine teleosts, respectively, are two recently characterized lineages of apicomplexan parasites from marine hosts that have enlightened our understanding of parasite evolution (Jacko-Reynolds et al. 2025; Bonacolta et al. 2024). New phylogenomic data suggest these parasites represent a more recently divergent lineage in the context of apicomplexan evolution (i.e., late-branching), possibly with the Protococcidiorida clade (Jacko-Reynolds et al. 2025). The Protococcidiorida is a poorly described group of apicomplexans, basal to Coccidia, which includes marine representatives such as the polychaeta-infecting *Eleutheroschizon dubosqi* (Valigurová et al. 2015). The 18S rRNA gene alone does not resolve the lower

taxonomic classification of the coccidian-like parasite of Japanese sea cucumber. Therefore, more work is needed to place this parasite within an evolutionary context confidently, correlate it to another known ARL, and to investigate its host and geographic distribution further.

To investigate the evolutionary history of this enigmatic parasite, deep genomic sequencing was conducted on isolated cysts recovered from the ovaries of *A. japonicus*. This approach recovered enough genetic material from this apicomplexan parasite to retrieve the complete nuclear ribosomal RNA (*rrn*) operon, entire mitochondrial genome, and partial apicoplast (non-photosynthetic chloroplast) genome. This new genetic information was utilized to learn more about this parasite's evolutionary history and further distribution. Lastly, we formally describe this new species by combining this phylogenetic context with previous morphological descriptions.

2 | Materials and Methods

2.1 | Isolation of Cysts

Cysts were extracted from *A. japonicus* specimen as described in Unuma et al. (2020). Briefly, a green morphotype of *A. japonicus* was collected by gaff fishing at Hamajima (34°29' N, 136°76' E), Mie, Japan, on December 17, 2010, then transported to the Mie Prefectural Fish Farming Center. Once in aquaculture, the ovaries were examined under a stereoscopic microscope for the presence of cysts. Once found, cysts were manually extracted and stored in ethanol at −30°C until DNA extraction. Histological imaging was performed as described in Unuma et al. (2020). Briefly, a portion of the infected ovaries was fixed in Davidson's solution (Bell and Lightner 1988). The tissue was then embedded in paraffin wax and stained using hematoxylin and eosin.

2.2 | DNA Extraction and Low-Input Metagenomic Sequencing

DNA was extracted from combined cysts using the QIAGEN DNeasy Blood & Tissue Kit (Germantown, Maryland, USA) with an overnight proteinase K digestion step. Extracted DNA was quantified using a QIAGEN Qubit 4 Fluorometer with the Qubit 1X dsDNA HS Assay Kit. Extracted DNA was then brought to the Center for Genome Technology at the John P. Hussman Institute for Human Genomics (University of Miami Miller School of Medicine), where it was prepared for sequencing using the NEBNext Ultra II DNA Library Prep Kit with 100 ng of input and 3 cycles of PCR. DNA libraries were sequenced on an Illumina Novaseq X (San Diego, California, USA) targeting 50 M 150×150 paired-end reads. Paired-end reads were trimmed of adapters and low-quality (< 20 Q-score) reads using Fastp (Chen et al. 2018). Trimmed reads were assembled into a metagenome using Megahit with default settings (Li et al. 2015).

2.3 | Extraction of *Rrn* Operon and Phylogenetics

To retrieve the complete *rrn* operon (including the 18S + 28S rRNA genes), a hidden Markov Model (HMM) profile was generated

based on an alignment of published microeukaryotic *rrn* operons (Supporting Information S1A). This profile was then used with nhmmer (Wheeler and Eddy 2013) to extract any matching sequences from the assembled metagenome, resulting in the recovery of the majority of the 28S rRNA and ITS portions of the operon. The 18S rRNA gene was recovered using barrnap v0.9 (Seemann 2018) and then combined with the previously extracted 28S and ITS regions to assemble the complete *rrn* operon.

A *rrn* operon tree of Apicomplexa was generated using previously recovered 18S and 28S rRNA genes from representative apicomplexan lineages (Supporting Information S1A). Whole *rrn* operon sequences longer than 500bp and shorter than 15,000bp were sorted and clustered at 99%, then aligned using MAFFT v7.480 (Katoh and Standley 2013). The alignment was then trimmed using Trimal v1.4.rev15 (Capella-Gutiérrez et al. 2009) with a gap threshold of 30%, a similarity threshold of 0.001, and a minimum conservation of 30%. ModelTest-NG v0.1.7 (Darriba et al. 2020) was run on the trimmed alignment to determine the optimal substitution model for phylogenetic reconstruction. A maximum-likelihood tree was then constructed using RAXML v8.2.12 with the General Time Reversible model with a GAMMA+P-Invar model of rate heterogeneity ('-m GTRGAMMAIX') and 1000 bootstrap replicates (Stamatakis 2014).

2.4 | Organelle Assemblies and Phylogenetics

The mitochondrial genome of this parasite was assembled by first identifying mitochondrial contigs within the metagenomic assembly using tblastn v2.13.0 (Camacho et al. 2009) against an apicomplexan mitochondrial gene database from Bonacolta et al. (2024). Using this approach, a contig representing the incomplete mitochondrial genome of the parasite was identified and utilized as an input seed for Novoplasty v4.3.3.pl (Dierckxsens et al. 2017), which assembled a complete mitochondrial genome from the raw sequencing reads. MFANNOT was used for mitochondrial genome annotation (Lang et al. 2023). The *cox1*, *cox3*, and *cob* genes were then extracted and aligned using MUSCLE v3.8.1551 (Edgar 2004) with those from other apicomplexans (plus a dinoflagellate outgroup; Supporting Information S1B). Gene alignments were combined using CONCATENATOR v0.2.1 (Vences et al. 2022) and then trimmed using Trimal v1.4.rev15 (Capella-Gutiérrez et al. 2009) with a gap threshold of 20%. This gap threshold was chosen by manually inspecting the output from a range of thresholds to ensure sufficient information was retained while removing poorly aligned regions. IQ-TREE with the C60 + LG + GAMMA model and 1000 bootstrap replicates was run to generate a phylogenetic reconstruction of apicomplexan mitochondrial evolution (Nguyen et al. 2015). This model was chosen because it performed better than other, less complex models (Baños et al. 2024).

Contigs corresponding to apicoplast genes were identified using BLASTn v2.13.0 against the NCBI database. After failing to get an assembly using Novoplasty as detailed for the mitochondrial genome above, these contigs were used as seed input for GetOrganelle v1.7.7 (Jin et al. 2020) to generate a fragmented apicoplast genome. A second round of GetOrganelle v1.7.7 using the fragmented assembly as a seed

yielded a less fragmented but still incomplete apicoplast genome. MFANNOT was used to annotate the genes we could recover from this assembly, and then the genes were aligned with their apicomplexan counterparts using MUSCLE v3.8.1551. The alignments were then trimmed using Trimal v1.4.rev15 with a gap threshold of 60% using the same methodology mentioned above. The apicoplast phylogenomic tree was constructed from the concatenated trimmed alignments using IQ-TREE with the C60 + LG + GAMMA model and 1000 bootstrap replicates.

The plastid 16S rRNA gene (SSU) was recovered using barrnap v0.9 and combined with other apicoplast-derived SSUs. Sequences between 500 and 15,000 bp were sorted and clustered at 99.5% using VSEARCH v2.25.0 (Rognes et al. 2016). They were then aligned using MAFFT v7.520 and trimmed using Trimal v1.4.rev15 under the following conditions: a gap threshold of 30%, a similarity threshold of 0.001, and a minimum conservation of 30%. ModelTest-NG v0.1.7 was run on the trimmed alignment to determine the optimal substitution model for phylogenetic reconstruction. Then, a maximum-likelihood tree was built with RAXML v8.2.13, using the General Time Reversible model with a GAMMA+P-Invar model of rate heterogeneity ('-m GTRGAMMAIX') and 1000 bootstrap replicates.

2.5 | Microbiome Screening and Evolutionary Placement Analysis

The apicoplast SSU of the sea cucumber apicomplexan was searched against the IMNGS server (a massive, continuously updated database of 16S rRNA gene metabarcoding studies; Lagkouvardos et al. 2016) for any sequences with >90% similarity. Matching sequences were then evolutionarily mapped to the apicoplast SSU tree constructed earlier using RAXML's evolutionary placement algorithm. The General Time Reversible model with a gamma-distributed rate variation (GTR+CAT) and a minimum bootstrap support threshold of 20% were used to generate the EPA placement tree. Those sequences that clustered with the sea cucumber parasite were considered as likely the same parasite (or cyst stage of the parasite). A small selection of additional studies spanning other echinoderms and ocean regions was screened (Table S1) for the sea cucumber parasite apicoplast SSU using the same approach to ensure a comprehensive microbiome search.

3 | Results

The complete *rrn* operon could be recovered from the metagenomic assembly of the recovered cysts. Phylogenetic analysis using this recovered *rrn* operon places the sea cucumber parasite, hereafter referred to as *Apostichocystis gudetama*, as sister to the clade, which includes the corallicolids and ichthyocolids (+90% bootstrap; Figure 1). This clade of strictly marine parasites rests sister to the Eimeriorina suborder of Coccidia (Figure 1). The protococcidians *Eleutheroschizon* spp. branch basal to those clades (Figure 1).

Contigs corresponding to organelle genomes were successfully recovered for *A. gudetama*. The complete 6.74 kb mitochondrial

genome, containing a potentially trans-spliced *cox1* gene (Figure 2), was assembled with Novoplasty using a mitochondrial contig identified from the metagenomic assembly as a seed. A multi-gene tree incorporating *cox1*, *cox3*, and *cob* presents a contrary phylogeny to the *rrn* operon tree with *A. gudetama* branching sister to *E. duboscqi*, although with weaker support (87% bootstrap; Figure 2). Together, this clade is sister to coccidians (Figure 2). A fragmented apicoplast genome assembly (22.39 kb) was recovered, containing the following genes: *rpoB*, *sufB*, *rps4*, *rnl*, *rns*, *rpl4*, *rpl2*, *rps19*, *tufA*, *rps12*, *rps11*, *rpl36*, *rps5*, *rpl6*, *rps8* (Figure 3A). A 31-gene apicoplast phylogeny generated using this new genetic information showed *A. gudetama* once again branching sister to the protococcidian *E. duboscqi* with stronger support than the mitochondrial gene tree (95% bootstrap; Figure 3B). Notably, the corallicolids and ichthyocolids branch distinctly away from *A. gudetama* and coccidians in this phylogeny (Figure 3B). Taking the SSU of the apicoplast and generating a phylogenetic tree incorporating other recovered apicoplast SSUs as well as environmental sequences correlating to SSU from apicomplexan-related lineages (ARLs) shows *A. gudetama* branching deep within Apicomplexa and not correlating to any known ARL (Figure 3C). It still branches near *E. duboscqi* with sufficient surrounding bootstrap support (Figure 3C). Searching the IMNGS server, which contains operational taxonomic unit (OTU) data from thousands of metabarcoding studies, for any sequences with 90% similarity to the apicoplast SSU derived from the sea cucumber parasite revealed 5 closely related OTUs with low overall abundance in the environment. Of these, 4 OTUs were mapped onto the branch with *A. gudetama*'s apicoplast SSU using an evolutionary placement algorithm (Figure 4A). All 4 OTUs originate from marine ecosystems around Australia. 2 OTUs (representing 4 total sequences) were recovered from the encrusting coral, *Plesiastrea versipora*, found near Sydney, Australia. The other 2 OTUs (representing 2 total sequences) were retrieved from marine waters around Rottneest Island, Australia (Figure 4B).

4 | Discussion

Through deep genomic sequencing of apicomplexan cysts originating from the ovaries of the Japanese sea cucumber (*A. japonicus*), we were able to recover additional genetic information to reveal a rather complicated phylogenetic placement of this commercially relevant parasite, which we have named *Apostichocystis gudetama*. All phylogenies originating from organelle data consistently show *A. gudetama* as closely related to but distinct from *E. duboscqi* (Figures 2 and 3), a protococcidian that infects marine polychaete worms (Valigurová et al. 2015). However, due to the fast-evolving nature of AT%-rich apicoplast genomes (Muñoz-Gómez et al. 2019), along with the more limited taxonomic sampling of deep-branching apicoplasts, the usefulness of plastid-encoded genes for resolving phylogenetic relationships within Apicomplexa is limited (Mathur et al. 2021). On the other hand, the *rrn* operon phylogeny, which includes the broadest taxonomic sampling and more closely matches apicomplexan nuclear-gene phylogenies (Mathur et al. 2023), shows support for a close relation of *A. gudetama* to the clade of ichthyocolids and corallicolids (Figures 1 and 2). Given the recent findings that these three clades (*Eleutheroschizon* spp., corallicolids, and ichthyocolids) are closely related to each other

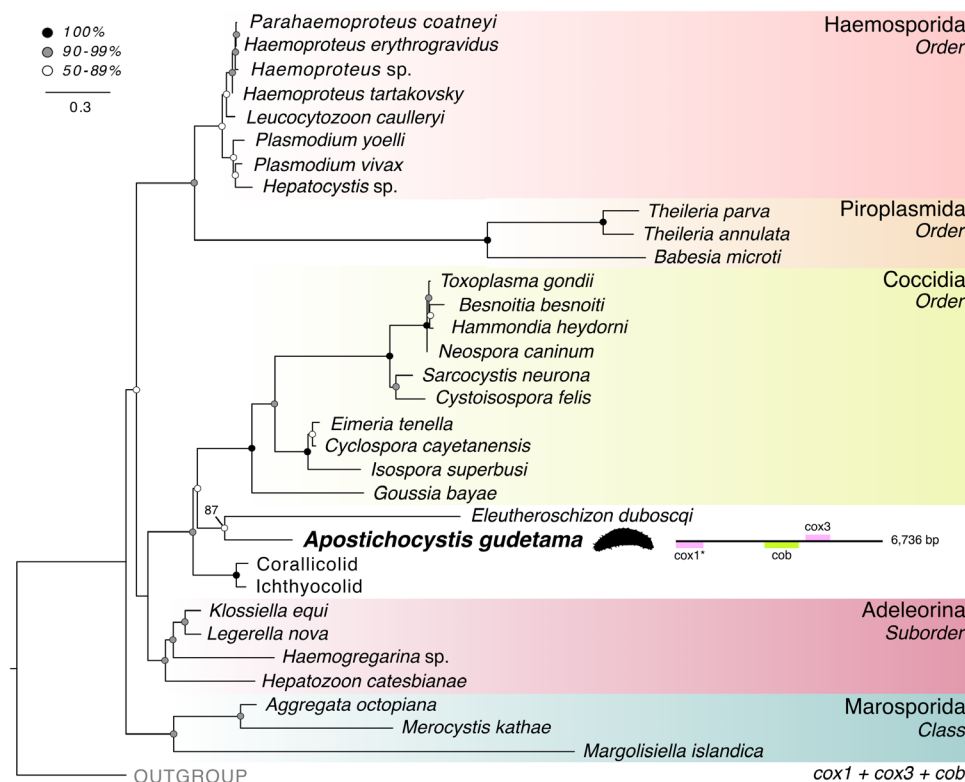


FIGURE 2 | Maximum-likelihood tree of apicomplexan mitochondria based on *cox1*, *cox3*, and *cob* using 1113 amino acid sites using IQ-TREE with the C60 + LG + GAMMA model and 1000 bootstrap replicates. Major clades of Apicomplexa and related lineages are color-coded. Stramenopiles were used as the outgroup. The gene arrangement of the *A. gudetama* mitochondrial genome alongside its placement on the tree. (See also Supporting Information S1B).

and branch together in phylogenomic trees (Jacko-Reynolds et al. 2025), likely all as protococcidians, it is safe to assume that *Apostichocystis* spp. likely branches within this clade as well. Together, these parasites encompass ecologically important apicomplexans, all found exclusively within marine hosts. Marine parasite host-divergence in the ocean and the genetic underpinnings of parasite evolution can be investigated within this lineage thanks to this expanded phylogenetic framework.

Diversification of apicomplexan lineages within marine hosts is common, as shown by the vast diversity of gregarines that infect marine hosts (Ode et al. 2024). The ichthyocolids and corallicolids are globally distributed, appearing in hosts from cold, upper latitudes and warm, tropical latitudes (Bonacolta et al. 2024; Trznadel et al. 2024), exemplifying the broad host-specific diversification common among apicomplexans (Morrison 2009). Relatedly, several sea cucumber-infecting apicomplexans have been described morphologically but lack molecular data (Massin et al. 1977; Fankboner and Cameron 1985; Jangoux 1986; Pomory and Lares 1998; Eeckhaut et al. 2004; Mueller 2017). Targeted sequencing of these parasites, as well as the gonads of other echinoderms, may lead to the characterization or expansion of the *Apostichocystis* clade and other existing apicomplexan lineages, as well as our understanding of the diversity of parasites in the ocean. To this end, the detection of *Apostichocystis*-related sequences in coral reefs and marine waters of Australia outside the known distribution of *A. japonicus* (Figure 4) hints at the possibility that other closely related apostichocystids may not be described.

Little is known about the biology and ecology of these closely related lineages of marine apicomplexan parasites. Known host-specificity varies, with *A. gudetama* and *E. duboscqi* only being described in association with a singular host (*A. japonicus* and *Scoloplos armiger*, respectively; Unuma et al. 2020; Valigurová et al. 2015). Corallicolids and ichthyocolids have much broader host ranges with corallicolids being recovered in the majority of sampled anthozoans as well as fireworms (Kwong et al. 2019; Bonacolta et al. 2025), and ichthyocolids being recovered from a variety of marine fish as well as gnathiids (Bonacolta et al. 2024; Sikkell et al. 2020). Morphologically, there is a large difference in size between *A. gudetama* and the rest of the mentioned apicomplexans. Gamont size is approximately 8 μ m in ichthyocolids and 20 μ m in *E. duboscqi* (Bonacolta et al. 2024; Valigurová et al. 2015). Corallicolids are approximately ~10 μ m (Kwong et al. 2019). All much smaller than the 250 μ m long cysts that *A. gudetama* forms. Regarding parasite development, *A. gudetama* grows and divides within the cytoplasm of the host cell, while *E. duboscqi* develops epicellularly.

Nuclear phylogenomics of *Apostichocystis* spp. could further support the Protococcidia as a marine-host-specific clade of apicomplexan parasites and shed light on our understanding of how the chlorophyll biosynthesis pathway has evolved within Apicomplexa. This pathway is hypothesized to have evolved unparsimoniously (Bonacolta et al. 2024; Jacko-Reynolds et al. 2025), having been lost multiple times independently throughout apicomplexan evolution. Corallicolids and *Eleutheroschizon* have retained elements of the chlorophyll

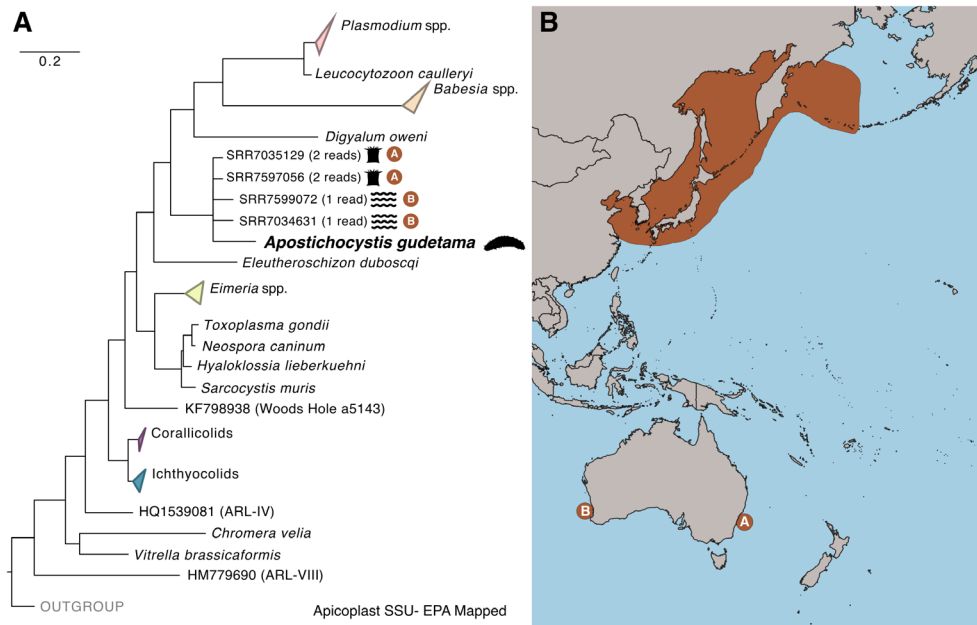


FIGURE 4 | (A) Maximum-likelihood tree of Apicomplexa using plastid 16S rRNA genes (16S) from Figure 3, with sequences from the Integrated Microbial Next Generation Sequencing (IMNGS) database with 90% similarity results placed to the *A. gudetama* apicoplast 16S rRNA gene with RAXML's evolutionary placement algorithm (EPA). (B) The geographic distribution of *A. japonicus* (orange shading) in comparison to the origins of the EPA-mapped, likely *Apostichocystis* spp. operational taxonomic units (OTUs), which are denoted with (A) and (B).

Etymology: N.L. n. *Apostichopus*, referencing the host species *Apostichopus japonicus*, and Gr. prefix *apo-* meaning “away from” or “separate,” combined with Gr. adj. *sticho-* meaning “orderly” or “arranged,” reflecting both the association with *Apostichopus* and the phylogenetic placement of the genus as potentially “disorderly” based on the inconsistencies between the *rrn* gene and organelle phylogenomics. N.L. n. *cystis*, from Gr. n. *kystis*, meaning “bladder” or “sac,” commonly used in the names of cyst-forming parasitic or symbiotic protists. Together, the name conveys the organism's biological and phylogenetic context.

Diagnosis: A distinct clade within Apicomplexa based on *rrn* operon, mitochondrial, and plastid (apicoplast) gene sequences.

Type species: *Apostichocystis gudetama* sp. nov.

Apostichocystis gudetama sp.

nov. Bonacolta et al. 2025

Zoobank ID: 15BFD54E-478C-41DD-99FB-4879AA354369.

Etymology: N.L. n. *gudetama*, inspired by the Japanese pop culture figure Gudetama, a character resembling a lazy egg, referencing the parasite's association with the ovaries of the Japanese sea cucumber (*Apostichopus japonicus*). The name reflects both the reproductive context of its host and the cultural connection to Japan.

Diagnosis: An unknown life stage invades the oocytes of female *A. japonicus*, where it forms ~2 1950 μm multi-cell cysts surrounded by a 50 μm transparent layer during host maturation. The sporozoites/merozoites (~40 μm in length) are then shed with the eggs from the gonopore during host spawning.

Type sequence: 18S rRNA gene sequence (PV105649) and 28S rRNA gene sequence (PV105650).

Type habitat: Marine; host-associated.

Type host: *Apostichopus japonicus* Selenka, 1867 (Metazoa; Echinodermata; Holothuroidea; Synallactida; Stichopodidae).

Type material: Cysts preserved in ethanol are stored at Tohoku University under the supervision of Dr. Tatsuya Unuma.

Acknowledgments

This study was supported by startup funds from the University of Miami Rosenstiel School of Marine, Atmospheric, and Earth Sciences, project PID2020-118836GA-I00 financed by MICIU/AEI/10.13039/501100011033, and project 2021 SGR 00420 financed by the Departament de Recerca i Universitats de la Generalitat de Catalunya.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI SRA at <https://www.ncbi.nlm.nih.gov/sra>, reference number PRJNA1227500.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.