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Phylogenomics shows that novel tapeworm-like traits of haplozoan parasites evolved from within the Peridiniales (Dinoflagellata)

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ABSTRACT

Haplozoans are intestinal parasites of marine annelids with bizarre traits, including a differentiated and dynamic trophozoite stage that resembles the scolex and strobila of tapeworms. Described originally as "Mesozoa", comparative ultrastructural data and molecular phylogenetic analyses have shown that haplozoans are aberrant dinoflagellates; however, these data failed to resolve the phylogenetic position of haplozoans within this diverse group of protists. Several hypotheses for the phylogenetic position of haplozoans have been proposed: (1) within the Gymnodiniales based on tabulation patterns on the trophozoites, (2) within the Blastodiniales based on the parasitic life cycle, and (3) part of a new lineage of dinoflagellates that reflects the highly modified morphology. Here, we demonstrate the phylogenetic position of haplozoans by using three single-trophozoite transcriptomes representing two species: *Haplozoon axiothellae* and two isolates of *H. pugnus* collected from the Northwestern and Northeastern Pacific Ocean. Unexpectedly, our phylogenomic analysis of 241 genes showed that these parasites are unambiguously nested within the Peridiniales, a clade of single-celled flagellates that is well represented in marine phytoplankton communities around the world. Although the intestinal trophozoites of *Haplozoon* species do not show any peridinioid characteristics, we suspect that uncharacterized life cycle stages may reflect their evolutionary history within the Peridiniales.

1. Introduction

The Dinoflagellata is a group of mostly single-celled eukaryotes, including over 2,400 described species with diverse morphologies, habitats and modes of nutrition (Gómez, 2012; Taylor et al., 2008). They include important members of aquatic communities worldwide, ranging from beneficial symbionts essential for reef-forming corals to harmful algae that are responsible for red tides (Hackett et al., 2004). Traditionally, dinoflagellate diversity has been classified based on comparative morphology and life cycles (Fensome et al., 1999). Several major groups have been recognized: Dinophysiales, Gonyaulacales, Gymnodiniales, Noctilucales, Peridiniales, Prorocentrales, Suessiales, and Syndiniales (Not et al., 2012; Saldarriaga et al., 2004). Despite their extreme morphological diversity, most dinoflagellates share some fundamental traits, such as a nucleus with permanently condensed chromosomes. This so-called "dinokaryon" lacks typical nucleosomal histones but has dinoflagellate viral nucleoproteins (DVNPs) that were obtained through horizontal gene transfer (Lin, 2011). Dinoflagellates also have cortical alveoli, which are flattened vesicles underneath the plasma membrane that are often filled with cellulose called "thecal plates". The specific arrangements of the cortical alveoli, called "tabulation patterns", differentiate the main subgroups of dinoflagellates. A recent phylogenomic study showed that thecate (=armoured) dinoflagellates form a monophyletic group consisting of the Dinophysiales, Gonyaulacales, Peridiniales, Prorocentrales, and Suessiales (also known as Symbiodiniales (Gómez, 2020) (Janouskovec et al., 2017).

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The Gymnodiniales, Syndiniales, and Noctilucales are known as athecate (=naked) dinoflagellates because they lack cellulosic plates within the cortical alveoli (Orr et al., 2012).

Parasitism has evolved multiple times throughout the evolution of dinoflagellates, and has been observed in diverse hosts, including animals and other protists, representing at least 35 dinoflagellate genera (Cachon and Cachon, 1987; Coats, 1999). One of the most interesting but poorly understood lineages of parasitic dinoflagellates are haplozoans, which infect the intestines of marine maldanid annelids (bamboo worms) (Shumway, 1924). The feeding stages or "trophozoites" (=trophonts) of haplozoans have a bizarre combination of traits that are more similar to tapeworms than to dinoflagellates, which reflects a compelling case of convergent evolution (Leander 2008). These traits include the absence of flagella, an attachment apparatus consisting of hooks and suckers, a surface covered in microtrich-like thecal barbs, and a strobila-like arrangement of differentiated compartments (Leander, 2008) that appears multicellular. The trophozoites consist of three functionally differentiated regions: a trophomere, gonomeres, and sporomeres (Angel et al. 2021). The dynamic trophomere at the anterior end contains a stylet that probes the host gut epithelium, a few non-motile stylets, and an adhesive apparatus that is used for attachment. A string of gonomeres is located behind the trophomere and forms most of the length of the trophozoite. Several sporomeres are located at the posterior end of the trophozoite and are thought to be responsible for reproduction through the release of flagellated dinospores that leave one host to infect another (Shumway, 1924). The differentiated multicellular appearance of haplozoans was difficult to interpret and led early researchers to hypothesize that they might be members of either (1) the now defunct Mesozoa (Dogiel, 1906) or (2) colonial gregarine apicomplexans (Calkins, 1915) or (3) a distinct lineage called the "Haplozooidea" (Neresheimer, 1908; Poche, 1913). Chatton (1920) was the first to recognize similarities between haplozoans and dinoflagellates and placed the genus *Haplozoon* within the Gymnodinida. Shared characteristics between *Haplozoon* and dinoflagellates were supported later by studies using transmission electron microscopy (TEM, (Siebert and West, 1974) and scanning electron microscopy (SEM, Leander et al. 2002), such as the presence of permanently condensed chromosomes, tubular mitochondrial cristae, and cortical alveoli containing thecal material.

Although several molecular phylogenetic studies of SSU and LSU rDNA sequences have shown that haplozoans are highly modified dinoflagellates (Rueckert and Leander, 2008; Saldarriaga et al., 2001; Wakeman et al., 2018; Yamamoto et al., 2020), the phylogenetic position of these parasites within the Dinoflagellata remains unknown. *Haplozoon* was classified within the Blastodiniales along with other parasitic dinoflagellates for convenience rather than on the basis of any shared characteristics (Fensome et al., 1999; Skovgaard et al., 2007). It was also suggested that *Haplozoon* might have a close affinity to gymnodinioids because they share a tabulation pattern of numerous and relatively small, polygonal alveoli across the surface of the trophozoite stage (Leander et al., 2002). This is consistent with a few molecular phylogenetic studies that showed *Haplozoon* branching early among dinoflagellates, albeit with weak statistical support (Rueckert and Leander, 2008; Wakeman et al., 2018). However, the presence of thecal material within the cortical alveoli in the four species examined with TEM so far (*H. axiothellae*, *H. ezoense*, *H. gracile*, and *H. pugnus*) do not support their affinity to athecate lineages like the Gymnodiniales (Landers, 2000; Siebert and West, 1974; Wakeman et al., 2018; Yamamoto et al., 2020).

A total of 17 species have been described within *Haplozoon*. Among them, 12 species are known from the Atlantic (Calkins, 1915; Dogiel, 1907, 1906; Shumway, 1924), and only five species (*H. axiothellae*, *H. paraxillellae*, *H. ezoense*, *H. gracile*, and *H. pugnus*) have been characterized with modern methods (e.g., EM and molecular phylogenetic analyses), which are all from the Pacific Ocean (Angel et al., 2021; Rueckert and Leander, 2008; Siebert, 1973; Siebert and West, 1974; Wakeman et al., 2018; Yamamoto et al., 2020). Molecular phylogenetic analysis of SSU rDNA sequences from the five *Haplozoon* species listed above strongly supported their monophyly, but their phylogenetic position within dinoflagellates remains unresolved (Yamamoto et al., 2020). Multi-gene phylogenomic analysis of single-cell transcriptomes is expected to resolve deeper relationships, especially of rare organisms that cannot be cultured (Cooney et al., 2020). Here, we obtained three different transcriptomes representing two species of *Haplozoon* collected on the northeastern and northwestern coasts of the Pacific Ocean: *H. axiothellae* (USA), *H. pugnus* (Canada) and *H. pugnus* (Japan). By inferring a phylogenomic tree using 241 genes, we were able to demonstrate that these enigmatic parasites have an unexpected position within the tree of dinoflagellates.

2. Materials and methods

2.1. Sample collection

This study included isolates from two different *Haplozoon* species: *H. axiothellae* and *H. pugnus*. A single trophozoite of *H. axiothellae* was isolated from the intestines of *Axiothella rubrocincta* that was collected in Argyle Lagoon, San Juan Island, Washington, USA in September 2022 (Fig. 1A). Two different isolates of *H. pugnus* were collected from two distant locations in the Pacific Ocean: Japan and British Columbia, Canada. In Japan, four trophozoites of *H. pugnus* were isolated from *Nicomache* sp. collected near Akkeshi Marine Station in October 2020 (Fig. 1B). In Canada, a single trophozoite of *H. pugnus* was isolated from *Nicomache* sp. collected from Stanley Park, British Columbia, during the low tide in August 2022 (Fig. 1C). The *Haplozoon* isolates were rinsed in autoclaved seawater at least 3 times, and then a single trophozoite was placed in a 0.2-ml tube containing 2 μl of cell lysis buffer (0.2% TritonX-100 + RNase Inhibitor) by using a hand-drawn glass pipette. The single trophozoites were stored in a −80 °C freezer until they were used for reverse transcription and cDNA amplification using the SmartSeq2 protocol (Picelli et al., 2014). The cells of *H. axiothellae* (USA) and *H. pugnus* (Canada) were processed at the University of British Columbia, while the cells of *H. pugnus* (Japan) were processed at Hokkaido University and the Okinawa Institute of Science and Technology. cDNA concentrations were measured using a Qubit Fluorometer.

2.2. Single-cell transcriptomics

For the samples collected along the eastern Pacific coast, sequencing libraries were prepared using the Illumina Nextera XT protocol. The libraries were sequenced on an Illumina NextSeq 500 with 150 bp paired-end reads by the Sequencing and Bioinformatics Consortium at the University of British Columbia. The libraries for the four *H. pungus* from Japan were prepared using the NEBNext Ultra II (NEB) kit. The libraries were barcoded and sequenced with paired-end reads (2 × 150 bp) on Illumina NovaSeq6000 by the Sequencing Section at the Okinawa Institute of Science and Technology. FastQC version 0.11.9 was used to assess the quality of transcriptome raw reads (Andrews, 2010). Raw reads were corrected by Rcorrector version 1.0.4 (Song and Florea, 2015), and then the adapters used for the SmartSeq2 protocol and Illumina sequencing were trimmed using Trimmomatic version 0.39 (Bolger et al., 2014). The trimmed reads of *H. pugnus* from Japan were pooled before assembly. The final three assemblies were made using rnaSPAdes version 3.13 (Bushmanova et al., 2019): *H. axiothellae* (USA), *H. pugnus* (Canada), *H. pugnus* (Japan). Transcriptome completeness was assessed using BUSCO version 5.4.3, compared against the Alveolata lineage database (Simão et al., 2015). The raw reads and the contigs were deposited to Sequence Read Archive (SRA) (BioProjectID: PRJNA985051) and Mendeley data (doi: 10.17632/kdgmfb8k55.1).

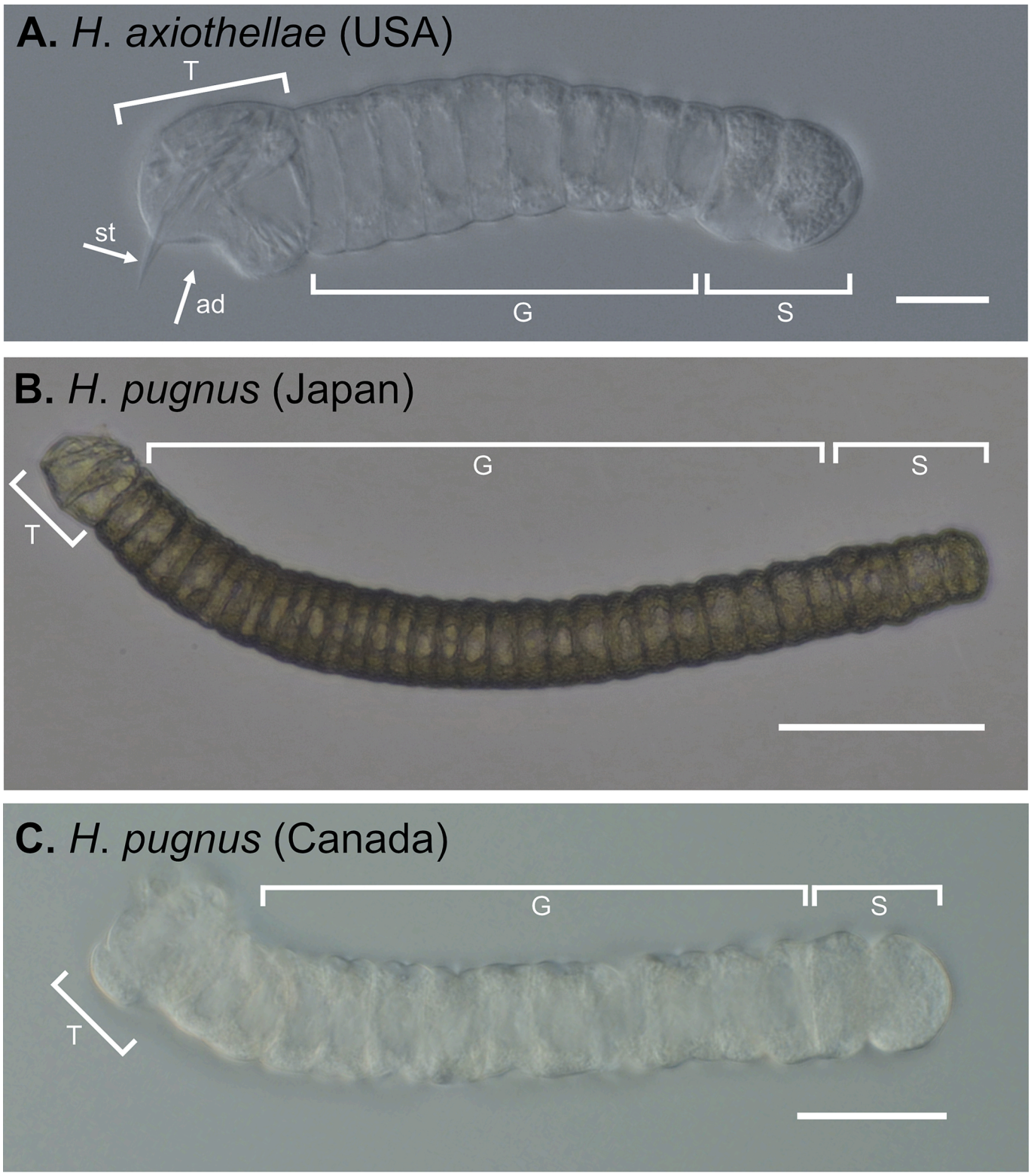

Fig. 1. Light micrographs of the Haplozoon species investigated in this study. A: Haplozoon axiothellae from Argyle Lagoon, WA, USA (the image was obtained from Angel et al. 2021). The stylet (st) and the adhesive disk (ad) are indicated with arrows. B: Haplozoon pugnus from Akkeshi Marine Station, Japan. C: Haplozoon pugnus from Stanley Park, British Columbia, Canada. A trophomere (T), gonomeres (G), and sporomeres (S) are shown. Scale bars = 20 μm.

2.3. Species identification

Identification of the bamboo worm hosts and the *Haplozoon* parasites was primarily achieved with 18S rDNA sequences extracted from the transcriptomes using barrnap version 0.9 (Seemann, 2013). In addition, COI sequences of the bamboo worm hosts collected from the USA and Canada were obtained (the COI sequences from *Nicomache personata* and *Nicomache* sp. harbouring *H. pugnus* from Japan are already available; Yamamoto 2020). Tissue from the hosts from which single trophozoites of *Haplozoon* were obtained was used to extract total genomic DNA using

DNeasy Blood & Tissue Kit (Qiagen). For PCR, 12.3 µl of distilled water, 4 µl of reaction buffer, 0.8 µl of each forward and reverse primer (LCO1490 and HCO2198; Folmer et al., 1994), 0.1 µl of MyTaq (Bioline), and 2 µl of DNA were used. PCR conditions were: 94 °C initial denaturation for 5 min, 35 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 40 s, and final extension for 10 min at 72 °C. 5 µl of PCR product from each PCR reaction was run on a 1.5% agarose gel. PCR products were purified using ExoSap, and then the purified PCR products were sent to and sequenced by Sequencing and Bioinformatics Consortium at the University of British Columbia, Canada, using the same primers used for PCR amplification. SSU rDNA and COI sequences were deposited into GenBank (Accession IDs: OR144108-9, OR145347-8).

2.4. Multi-gene phylogeny

Open reading frames (ORFs) in the three transcriptome assemblies were identified using TransDecoder version 5.5.0 (Haas et al., 2013). These ORFs (in peptide sequences) were blasted against the SwissProt database to find possible matches to known proteins (Poux et al., 2017). Protein alignments of 263 genes from previous studies were used as queries for blast searches through our peptide transcriptomes (Burki

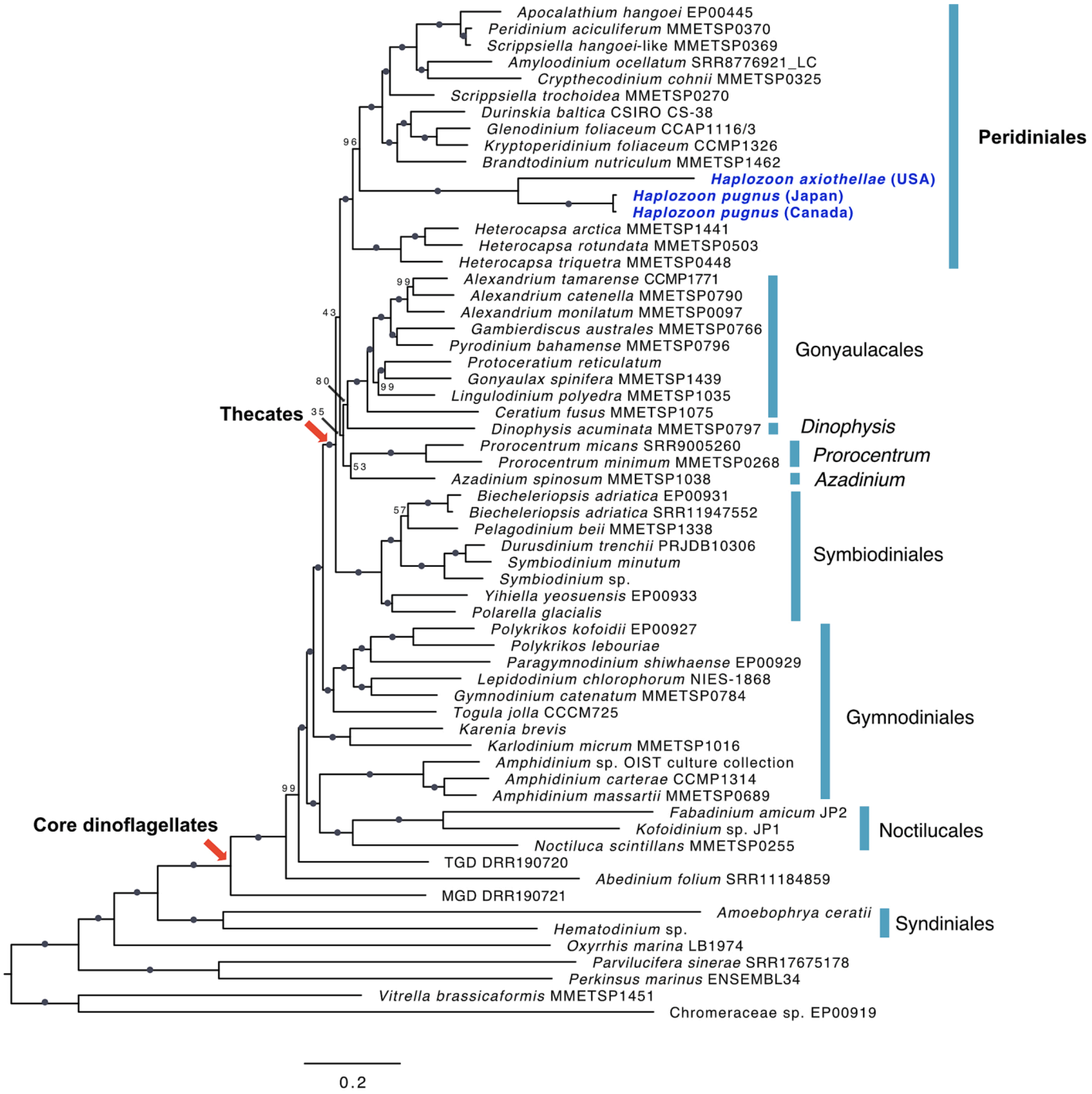

Fig. 2. Maximal Likelihood (ML) phylogeny of dinoflagellates inferred by IQ-tree based on 241 genes translated into protein sequences. Branches with maximal bootstrap support are marked with dark grey circles, and the rest are shown with numbers. *Haplozoon axiothellae* (USA), *H. pugnus* (Japan), *H. pugnus* (Canada) are highlighted in bold blue. Major dinoflagellate subgroups are shown with vertical bars (blue). The most common ancestors of the thecates and core dinoflagellates are indicated with arrows (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2016; Cooney et al., 2020). After adding new sequences to the existing datasets, alignments for each gene were made using MAFFT version 7.481 (Katoh and Standley, 2013), and then they were trimmed using trimAL version 1.4 (Capella-Gutiérrez et al., 2009). Single gene trees were inferred by IQTREE version 1.6.12 with the LG4X model (Nguyen et al., 2015). Individual trees were visually inspected to identify and remove contaminants or paralogs. SCaFoS was used to select taxa and genes with highest coverage (with > 60% presence across taxa) and to be included in the final concatenated alignment (Roure et al., 2007). A maximum likelihood tree based on the selected 241 genes was inferred by IQTREE with the LG + C60 + F + G4 model (Nguyen et al., 2015).

3. Results and discussion

3.1. The multi-gene phylogeny demonstrates that haplozoans evolved within the Peridiniales

The transcriptome assemblies from *H. axiothellae* (USA), *H. pugnus* (Canada), and *H. pugnus* (Japan) included 10,765, 27,805 and 53,394 peptides, and the complete BUSCO scores for each transcriptome were 55.6%, 84.2%, and 52.1%, respectively. The ML tree based on 241 protein orthologs placed *Haplozoon* within Peridiniales with maximal support (Fig. 2). Overall, this tree topology is consistent with previous analyses of similar data (Cooney et al., 2020, 2022; Janouskovec et al., 2017; Sarai et al., 2020) showing dinoflagellate strains MGD and TGD (see Sarai et al. 2020 for details about these strains), and *Abedinium* as early branching lineages of dinoflagellates, Noctilucales (*Noctiluca*, *Fabadinium*, *Kofoidinium*) and *Amphidinium* as sister clades, Gymnodiniales as paraphyletic, and thecates as a robust monophyletic clade. The relationships among the major subgroups of thecates (Peridiniales, Gonyaulacales, Symbiodiniaceae, *Dinophysis*, *Prorocentrum*, and *Azadinium*) were not clearly resolved even in our multi-gene phylogeny (Fig. 2). The Peridiniales was strongly supported (BS = 100) with *Heterocapsa* as the earliest branching lineage, which was also shown in a previous study (Janouskovec et al., 2017). *Haplozoon* branched after *Heterocapsa*, forming the sister clade to all other Peridiniales in the analysis. Although the *Haplozoon* species had relatively long branches and were not closely grouped with any other taxa in our tree, their phylogenetic placement within the Peridiniales was robust (Fig. 2).

3.2. Are there traits in haplozoans that reflect their phylogenetic position within the Peridiniales?

The presence of thin theca plates in all four *Haplozoon* species examined with TEM so far (Siebert and West, 1974; Wakeman et al., 2018; Yamamoto et al., 2020) is consistent with their phylogenetic position within a clade of thecate dinoflagellates and the inference that thecal plates evolved once during dinoflagellate evolution (Janouskovec et al., 2017). The synapomorphies for the Peridiniales mostly relate to the specific tabulation patterns of their cortical alveoli, namely the presence of anterior intercalary plates, a small apical pore, and two subequal antapical plates (Not et al., 2012). These peridinioid morphological features, however, are not present in the trophozoites of *Haplozoon*. Rather, the trophozoite stage of *H. axiothellae and H. praxillellae* have a uniform array of relatively small and numerous polygonal alveoli that is similar to the tabulation patterns found in gymnodinioid dinoflagellates (Leander et al., 2002; Rueckert and Leander, 2008). However, despite similar tabulation patterns, our phylogenomic analysis does not support that haplozoans are gymnodinioids. It is important to emphasize, however, that the trophozoite stage of *Haplozoon* is highly modified in morphology, relative to other known dinoflagellates, reflecting their parasitic lifestyles within the intestines of animal hosts.

3.3. Dinospores: An elusive life history stage with the potential to corroborate the phylogenetic data

Several researchers have suggested the importance of examining the dinospore stage of parasitic lineages of dinoflagellates to more confidently infer phylogenetic relationships (Landsberg et al., 1994; Saldarriaga et al., 2004; Skovgaard et al., 2007). Observations of *Haplozoon* species have been limited to the relatively conspicuous trophozoite stage, except for one reported observation of a motile dinospore by Shumway in 1924. Shumway (1924) described the movement of flagellated dinospores in *H. clymenellae* as resembling members of the 'Gymnodinia', but he also mentioned that the dinospores were similar to those of *Oodinium*, *Apodinium*, and *Blastodinium* as described by Chatton (1920). Although the dinospores of *Blastodinium*, in particular, were previously interpreted to be 'gymnodinioid dinoflagellates' (Chatton, 1920; Taylor, 2004), peridinioid tabulation patterns were subsequently shown in the dinospores of *B. contortum* and *B. navicular*, demonstrating their phylogenetic affinity to Peridiniales (Skovgaard et al., 2007). Similarly, tabulation patterns in the dinospores of *Amyloodinium* cf. *ocellatum*, a pathogenic dinoflagellate parasite of marine fish, was shown to be similar to that of free-living Peridiniales based on SEM data (Landsberg et al., 1994). Although the transcriptome of *A. ocellatum* was available in a public database, the phylogenetic position of this species has never been analyzed with multigene data; our multigene phylogenetic analysis shows that like *Haplozoon*, *A. ocellatum* is firmly placed within Peridiniales (Fig. 2). Therefore, careful examination of the elusive dinospores of *Haplozoon* is expected to reveal the synapomorphic tabulation patterns for Peridinales and corroborate the molecular phylogenetic data reported here.

Oodinium, *Apodinium*, *Blastodinium*, *Amyloodinium* and *Haplozoon* were all classified within the Blastodiniales due to their parasitic lifecycle (Fensome et al., 1999; Skovgaard et al., 2007). However, the Blastodiniales has never been shown to be monophyletic with molecular phylogenetic data (Saldarriaga et al., 2004, 2001), and this taxon is now considered obsolete. Our phylogenomic tree shows that *Haplozoon* and *Amyloodinium* belong to the Peridiniales, although they were not grouped together (Fig. 2). Phylogenomic data is unavailable for the other members of the 'Blastodiniales', so their phylogenetic positions and interrelationships remain to be determined.

3.4. Host range and geographic distribution

The *Haplozoon* specimens collected from Canada and the USA were identified as *H. pugnus* (99.8% identical to the *H. pugnus* from Japan) and *H. axiothellae* (99.8% identical to the *H. axiothellae* collected from the same location; Argyle Lagoon, WA) based on the SSU rDNA sequences extracted from the transcriptomes. Because *H. pugnus* was previously found in two different species of *Nicomache* from Japan (*N. personata* and *N.* sp.) that showed 10% pairwise genetic distance in COI sequences (Yamamoto et al., 2020), a COI sequence was obtained from the host specimen from Canada for species identification and comparison. As a result, the bamboo worm collected from Canada was 90.1% and 94.7% identical to *N. personata* and *N.* sp., respectively, supporting that *N.* sp. from Canada is a distinct species from the two species collected from Japan. Therefore, *H. pugnus* has been found in three different *Nicomache* species collected on opposite sides of the Pacific Ocean. By contrast, two different species of *Haplozoon*, namely *H. ezoense* and *H. praxillellae*, identified with comparative morphology, were found from the same host species (*Praxillella pacifica*) from the northeastern and northwestern Pacific Ocean, respectively (Rueckert and Leander, 2008; Wakeman et al., 2018). Because bamboo worms are known to be diverse but difficult to identify based on morphology alone (De Assis and Christoffersen, 2011), reporting genetic information from both the polychaete hosts and the *Haplozoon* parasites is essential for understanding host specificity and the geographic distribution of haplozoans.

3.5. Concluding remarks

Haplozoan dinoflagellates are intestinal parasites of marine invertebrates with traits that resemble those of tapeworms, reflecting an example of convergent evolution at fundamentally different levels of organization (subcellular vs. multicellular) and over a vast phylogenetic distance (i.e., over 1 billion years). In order to better understand the origins of haplozoan traits, we generated three different transcriptomes representing two species of *Haplozoon* from the eastern and western coasts of the Pacific Ocean. Our phylogenetic analysis of a 241-gene alignment demonstrated that haplozoans do not group with members of the Gymnodiniales or with other 'Blastodiniales'. Instead, they evolved from within the Peridiniales, which have specific tabulation patterns of alveoli and are typically members of phytoplankton communities worldwide. Although the trophozoite stage of *Haplozoon* does not show any peridinioid synapomorphies, the ultrastructural traits of the elusive dinospore stage, such as tabulation patterns of cortical alveoli, is expected to corroborate the robust molecular phylogenetic data reported here.

CRediT authorship contribution statement

Eunji Park: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Elizabeth Cooney:** Conceptualization, Methodology, Software, Formal analysis, Writing – review & editing. **Yong Heng Phua:** Investigation, Writing – review & editing. **Takeo Horiguchi:** Resources, Writing – review & editing. **Filip Husnik:** Resources, Writing – review & editing. **Patrick Keeling:** Resources, Writing – review & editing. **Kevin Wakeman:** Investigation, Resources, Writing – review & editing. **Brian Leander:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Transcriptome raw reads are available in the NCBI SRA (BioProject ID: PRJNA985051), and assemblies are available at Mendeley Data, V1 (doi: 10.17632/kdgmfb8k55.1). SSU rDNA and COI sequences are available in GenBank with Accession IDs: OR144108-9 and OR145347-8.

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