Molecular Phylogeny and Morphology of Haplozoon ezoense n. sp. (Dinophyceae): A Parasitic Dinoflagellate with Ultrastructural Evidence of Remnant Non-photosynthetic Plastids

Kevin C. Wakeman\textsuperscript{a,b,1}, Aika Yamaguchi\textsuperscript{c}, and Takeo Horiguchi\textsuperscript{d}

\textsuperscript{a}Institute for International Collaboration, Hokkaido University, Sapporo 060-0808, Japan
\textsuperscript{b}Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan
\textsuperscript{c}Kobe University Research Center for Inland Seas, Kobe 657-8501, Japan
\textsuperscript{d}Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

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This study describes a novel species of Haplozoon, H. ezoense n. sp., a dinoflagellate parasite isolated from the intestines of Praxillella pacifica (Polychaeta). Trophonts (feeding stages) of H. ezoense n. sp. were isolated and studied with scanning and transmission electron microscopy, and molecular phylogenetic analyses was performed using 18S rDNA and 28S rDNA. Trophonts had an average length of 120 \( \mu \)m, and were linear, forming a single longitudinal row comprising a trophocyte with a stylet, an average of 14 gonocytes (width = 10 \( \mu \)m), and bulbous cells that we concluded were likely sporocytes. The surface of H. ezoense n. sp. was covered with projections of the amphiesma. Sections viewed under TEM revealed multiple triple membrane-bound organelles reminiscent of relic non-photosynthetic plastids within the gonocytes. Haplozoon ezoense n. sp., H. praxillellae, and H. axiothellae formed a well-supported clade in the 18S rDNA datasets. The sequences of H. ezoense n. sp. differed from H. praxillellae, a species of Haplozoon isolated from the same host species in the Northeast Pacific, at 88/1,748 bases; and 155/1,752 bases from H. axiothellae. Concatenated 18S rDNA and 28S rDNA datasets were unable to resolve the deeper relationships of Haplozoon in the context of dinoflagellates.

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**Key words:** Alveolate evolution; anterior stylet; dinoflagellate phylogeny; marine parasite; transmission electron microscopy.

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\textsuperscript{1}Corresponding author; fax +81 11 706 4851
e-mail wakeman.k@oia.hokudai.ac.jp (K.C. Wakeman).

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**Introduction**

Haplozoon Dogiel, 1906 is a highly divergent and specialized parasitic dinoflagellate lineage primarily described from the intestines of mal-danid polychaetes (bamboo worms). Haplozoon
trophonts (or feeding stages) are the most conspicuous life stage of this parasite. They form rows of multiple cells comprised of a trophocyte with a single or multiple stylet/s that protrude and retract, multiple gonocytes, and terminal sporocytes; trophocytes can measure hundreds of microns (Shumway 1924; Siebert 1973), and can also be covered with peculiar projections of the amphiesma (syn. thecal barbs/spines) (Leander et al. 2002; Siebert and West 1974). This unique morphology was likely the reason that *Haplozoon* was initially classified as a metazoan (mesozoan) (Dogiel 1906) and a gregarine apicomplexan (Calkins 1915), before being recognized as a bizarre parasitic dinoflagellate (Chatton 1919; Shumway 1924).

The first molecular data from *Haplozoon*, the SSU rDNA from *H. axiothellae*, was generated by Saldarriaga et al. (2001). Although the molecular phylogenies from Saldarriaga et al. (2001) were largely unresolved, the work and other subsequent analyses (Gómez and Skovgaard 2015; Rueckert and Leander 2008) did support the conclusion that *Haplozoon* is not a close relative to other known groups of parasitic dinoflagellates, and suggested that *Haplozoon* likely descended from a photosynthetic ancestor. More recent work has begun to catalogue some of the diversity, both molecular and morphological, from areas around the North-eastern Pacific (Leander et al. 2002; Rueckert and Leander 2008). Still, relatively limited information is available for *Haplozoon*, and consequently little is known about its biology or biogeographic distribution. With little exception, most descriptions of *Haplozoon* are from the East Atlantic, and no information exists on this species from the West Pacific.

To this end, this study describes *Haplozoon ezoense* n. sp. from Praxillella pacifica Berkeley, 1929, a maldanid polychaete worm from the Northwestern Pacific island of Hokkaido, Japan. Trophocytes of *H. ezoense* n. sp. were isolated for molecular analyses using 18S rDNA and 28S rDNA, as well as morphological work using scanning and transmission electron microscopy.

Results

General Morphology and Surface Ultrastructure of *Haplozoon ezoense* n. sp.

Individuals were comprised of trophocytes (anterior), gonocytes (middle), and bulbous cells that were interpreted as sporocytes (posterior). The average length of individuals observed was 120 μm (n = 30), but lengths varied (range = 65–150 μm) (Fig. 1A–D); average width of the gonocytes and trophocytes was 10 μm (n = 50) and 13 μm (n = 30), respectively. On average, individuals were comprised 14 gonocytes (n = 30), but their number ranged from 8 to 18. Nuclei appeared spherical, with a diameter of 5 μm (n = 20) Gonocytes were only observed to be linear (i.e., forming a single, longitudinal row) (Fig. 1A–C). In some cases, the chain of cells were broken (Fig. 1D). The trophocyte of *H. ezoense* n. sp. had a single, distinct stylet which protracted and retracted continuously at even intervals lasting a little over 1 sec (Figs 1A, B; 2A–I; Supplementary Material Fig. S1A–F; Supplementary Material Videos 1, 2). The tip of the stylet appeared to bend slightly as it retracted into the trophocyte (Fig. 2C–D), and parts of the suction disc appeared to form a bulge during this process (Supplementary Material Fig. S1D–F; Supplementary Material Video 2).

The surface of *H. ezoense* was covered with amphiesmal projections. These hair-like projections were dense, and short around the trophocyte, and became longer, but sparse, progressively down the gonocytes (Fig. 3A, B). A single stylet was seen protruding from some fixed cells (Fig. 3C, D). The stylet seemed to slightly twist or spiral, having distinct lines running down its length (Fig. 3D). At the base of the stylet there was a bare patch on the trophocyte, the adhesive apparatus (Fig. 3C). In one of the fixed cells, a fine, flagellar-like filament was observed to be projecting from the tip of the stylet (Fig. 3E).

Transmission Electron Microscopy of the Trophonts

Starch granules were abundant within gonocytes (Fig. 4A–C). In dividing gonocytes, starch granules lined the sides of the cell, while the starch was located at either the anterior or posterior end of non-dividing cells (Fig. 4A, B). Starch was also observed in trophocytes (Fig. 6C). Mature junctions were visible between gonocytes. A single layer of large, ovoid amphiesma separated the gonocytes (Figs 4D, 5A). Some part of the amphiesma form projections on the surface of the cell and these projections are tightly fused with other parts of the amphiesma that are more leveled. An internal pellicle was also clearly visible beneath the amphiesmal vesicles (Fig. 4D). Within the gonocytes we observed nuclei, Golgi apparatus, and mitochondria with tubular cristae (Figs 4D, 5A–D). The nucleus was typical for dinoflagellates and
Figure 1. Light micrographs showing the general morphology of Haplozoon ezoense n. sp. A–C. Trophocytes (T) containing a stylet (S) that protracted and retracted. Refractive fibers (F) were observed throughout the trophocyte. A single chain of gonocytes (G) extended from the trophocyte, each with a nucleus (N). Some gonocytes had mature junctions (J), while others had developing junctions (D), in the process of cytokinesis. Bulbous cells that were interpreted to be sporocytes (Sp) were positioned at the end of the chain of gonocytes. D. A chain of gonocytes where a gonocyte has become dislodged from the chain. Note the mucilaginous (Mu) film. Scales: A–D = 10 μm.

contained thick chromosomes (Figs 4A–B, 6A). Extranuclear spindles were regularly observed in dividing cells (Fig. 6A). In the trophocyte, we were able to observe two ‘arms’ (Fig. 6C), as well as electron-dense fibres (Fig. 6B). Cross-sections through the stylet showed that the stylet had a spiral organization with a distinct, electron-dense core (Fig. 6C, D). Putative non-photosynthetic plastids were observed throughout the cytoplasm of many of the gonocytes. These organelles were always bound by three distinct membranes, and were partially filled with a similar consistency of electron-dense structures, being roughly spherical or oblong and ranging in size between 200 nm and 750 nm at their widest parts. The spacing between membranes varied from 10 nm to 25 nm (Figs 7B, 8A–D).

Molecular Phylogeny of Haplozoon ezoense n. sp. Based on 18S and 28S rDNA Datasets

Deeper phylogenetic relationships of Haplozoon to other dinoflagellate groups were uncertain in all analyses conducted in this study (18S rDNA, 28S rDNA and the concatenated 18S-28S rDNA data) (Figs 9–11). The 18S amplified from H. ezoense n. sp. did group with the two other sequences available from Haplozoon, namely H. axiothellae and H. praxillellae, branching as a sister lineage to H.
Figure 2. Light micrograph still frames taken from a video showing the protraction and retraction of the stylet of *Haplozoon ezoense* n. sp. A–D. Frames showing the trophocyte (T) and a chain of gonocytes (G) with nuclei (N), separated by mature junctions (J); note the retracting tip (C and D) of the stylet (arrow) appears to bend. E–I. The process of protracting and retracting takes approximately 1 second. Scales: A–I = 2 μm.
Figure 3. Scanning electron micrographs (SEM) of Haplozoon ezoense n. sp. A. SEM images showing the general morphology of the trophocyte (T) with adhesive apparatus (Ad), chain of gonocytes (G), separated by mature junctions (J), and boulbous cells that are probable sporocystes (Sp). B. The cells were generally covered on the surface with amphiesmal projections (Am) (syn. thecal barbs/spines). C. High-magnification SEM showing the base of the stylet (S) at the interface of the adhesive apparatus (Ad) (syn. suction disc), surrounded by amphiesma (Am); relic precipitate (Pr) is also visible. D. High-magnification SEM of the stylet (S); the stylet seems to twist or spiral, having a distinct lines running down its length (arrows). E (and inset). High-magnification SEM of the apical end of the trophocyte (T) covered with amphiesma (Am) with the stylet (S) slightly projecting; a flagellar-like fibril (F) can be seen extending from the tip of the stylet. Scales: A = 10 μm; B = 1 μm; C = 1 μm; D, E = 2 μm (E inset = 300 nm).
Figure 4. Transmission electron micrographs (TEM) of *Haplozoon ezoense* n. sp. A, B. Longitudinal-sections through the chain of gonocytes. Amphiesmal (Am) projections can be observed on the surface of the cells. Each gonocyte has a nucleus (N), and starch granules (St) are distributed generally along the sides and ends of the gonocytes. Mature junctions (J) separating the gonocytes can be observed. Developing gonocytes (cells undergoing cytokinesis) (D), have a nucleus that appears pinched on each side. C. High-magnification TEM cross-section of the gonocytes showing mature junctions (J), amphiesma (Am), starch granules (St) and nuclei (N). D. High-magnification longitudinal-section near the mature junction (J) between two gonocytes. Amphiesma vesicles appear to interlock (arrowhead). The internal pellicle (Ip) of the amphiesma is visible. Mitochondria (M) with tubular cristae were also visible. Scales: A, B = 5 μm; C = 2 μm; D = 500 nm.

*praxillellae*, with high support (Fig. 9). Both the 28S and concatenated datasets showed *H. ezoense* as a sister lineage to *Noctiluca*. However, the statistical support for the relationship between *Haplozoon* to *Noctiluca* garnered low support (Figs 10 and 11).

**Discussion**

There are currently 14 recorded species of *Haplozoon* that have been isolated predominately from bamboo worms. Most of these species have been described from locations around Scandinavia and Europe, and are based primarily on line drawings using light microscopy. Three of these, *H. lineare*, *H. clymenidis*, and *H. ariciae*, share a general morphological characteristic with *H. ezoense* n. sp. in that they are all linear (having their cells in a single, longitudinal row). *Haplozoon ariciae* has cells that are at angles to the next, and not directly stacked like the cells of *H. ezoense* n. sp. *Haplozoon cl-
Table 1. Morphological comparisons between *Haplozoon* species that have been isolated from either the same host species as *Haplozoon ezoense* n. sp. or share a similar morphological (linear) organization. The novel species described in this study, *H. ezoense* n. sp., is highlighted in bold font.

<table>
<thead>
<tr>
<th>Haplozoon ezoense n. sp</th>
<th>H. praxillelae</th>
<th>H. axiothelae</th>
<th>H. lineare</th>
<th>H. clymenidis</th>
<th>H. ariciae</th>
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</thead>
<tbody>
<tr>
<td><strong>Host</strong></td>
<td><em>Praxillella pacifica</em></td>
<td><em>Praxillella pacifica</em></td>
<td><em>Axiothella rubrocincta</em></td>
<td><em>Clymene lumbricalis</em></td>
<td><em>Aricia norvegica</em></td>
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<tr>
<td><strong>General morphology</strong></td>
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<td><strong>Trophocyte morphology</strong></td>
<td><em>Trophocyte round or elongate with short, hair-like amphiesma; 1 stylet; arms present</em></td>
<td><em>Trophocyte elongated; with long hair-like amphiesma; lacking neck region; 1 style with reverse stylets; arms absent</em></td>
<td><em>Trophocyte round/elongated with hair-like amphiesma; base narrows to neck region; 1 stylet with reserve stylets; arms present</em></td>
<td><em>Trophocyte either rounded or elongated when stretch; no neck visible; 1 arching stylet</em></td>
<td><em>Trophocyte elongated; trophocyte narrows forming neck region; 1 stylet</em></td>
</tr>
<tr>
<td><strong>Gonocyte morphology</strong></td>
<td><em>Long, hair-like amphiesma, central nucleus</em></td>
<td><em>Long, hair-like amphiesma; central nucleus</em></td>
<td><em>Gonocytes become smaller as they retreat from the trophocyte; nucleus central</em></td>
<td><em>Central nucleus</em></td>
<td><em>Posterior gonocytes angled to each other</em></td>
</tr>
<tr>
<td><strong>Sporocyte Morphology</strong></td>
<td><em>1-2 putative sporocytes; rounded; with fewer hair-like amphiesma</em></td>
<td><em>Many sporocytes (up to 8); some with 4 nuclei; shorter hair-like amphiesma</em></td>
<td><em>Sporocytes in a single row with fewer hair-like amphiesma; rounded</em></td>
<td><em>Putative sporocytes in a single row; rounded</em></td>
<td><em>Putative sporocytes angled to each other</em></td>
</tr>
<tr>
<td><strong>“epibionts”</strong></td>
<td>Not observed</td>
<td>Not observed</td>
<td>Observed</td>
<td>Data not available</td>
<td>Data not available</td>
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<tr>
<td><strong>Source</strong></td>
<td><em>Rueckert and Leander (2008)</em></td>
<td><em>Schiller (1933–1937)</em></td>
<td><em>Leander et al. (2002); Siebert (1973)</em></td>
<td><em>Schiller (1933–1937)</em></td>
<td><em>Schiller (1933–1937)</em></td>
</tr>
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Figure 5. Transmission electron micrographs (TEM) of *Haplozoon ezoense* n. sp. A–C. High-magnification TEM through the mature junction (J) separating gonocytes. Enlarged amphiesma (Am) were observed along the junction. Golgi apparatus (G) were observed near the nuclei (N). D. Mitochondria (M) and starch (St) were observed in the cytoplasm, near the edges of the cells. Scales: A–D = 500 nm.

*menidis* was described to possess a distinct arching stylet that seems to differ from that of *H. ezoense* n. sp., and *H. lineare* possesses multiple stylets, while *H. ezoense* n. sp. appeared to have a single stylet. The gonocytes of *H. lineare* tapered in size towards to posterior end of the cell, while the gonocytes of *H. ezoense* n. sp. appeared to be consistent in size. Furthermore, all these species came from different host, and don't share a geographic locality with *H. ezoense* n. sp. (Schiller 1933–1937; Table 1). We concede that it has not been established how specific *Haplozoon* parasites are with their host species, and to what extent geographic location might inform species diversity. However, there is very little overlapping information available for this group, and to this end, the addition of molecular work will help determine taxonomically relevant characteristics.
Figure 6. Transmission electron micrographs (TEM) of *Haplozoon ezoense* n. sp. A. High-magnification TEM cross-section through a gonocyte undergoing cytokinesis. The nucleus (N) and extranuclear spindles (Ns) are visible. B. High-magnification TEM cross-section through the trophocyte, showing electron dense fibres (F) and starch granules (St). C. TEM cross-section though a trophocyte (T) in a region of the cell showing the adhesive apparatus (Ad), arms (Ar), amphiesma (Am), stylet (S), and starch granules (St). The junction (J) separating the trophocyte and gonocyte (G) is visible. D. High-magnification cross-section of the trophocyte showing the stylet (S), as well as amphiesma (Am); note that the stylet appears to have a central, electron-dense core (C). Scales: A, B = 500 nm; C = 2 µm; D = 500 nm.

More recently, *Haplozoon praxillellae*, was described from the Eastern Pacific Ocean (*Rueckert and Leander 2008*). *Haplozoon praxillellae* and *H. ezoense* n. sp. were isolated from the same maldanid host species, *Praxillella pacifica*, albeit from different sides of the Pacific Ocean. These two isolates are superficially similar in that amphiesmal projections cover a large portion of the gonocytes surface and, to a lesser extent, cells that were interpreted to be sporocytes. After viewing multiple cells under light and transmission electron microscopy, it was determined that *H. ezoense* n.
sp. possesses a single stylet, while *H. praxillellae* was reported to have multiple (reserve) stylets within the trophocyte. We did find that there were numerous small fibril-like refractive/electron-dense structures in the trophocyte of *H. ezoense* n. sp., but none of these seemed to be the appropriate size of a stylet; we were also unable to find ultrastructural evidence of multiple stylets when viewing sections under TEM. It seems plausible that these small bodies could be immature (forming) stylets, however, the sheer number and distribution (they tended to form more of a matrix; see Figs 1A–C and 6B) of these objects throughout the trophocyte might contradict this view. Moreover, *H. praxillellae* was reported to lack ridges or ‘arms’ on the trophocyte, while *H. ezoense* n. sp. appears to have ‘arms’ on the trophocyte, something that was also observed in *H. axiathellae* (Leander et al. 2002; Siebert and West 1974).

The repeated movement of the stylet was recorded from two individuals (See Figs 2 and 3; also Supplementary Material videos). How *Haplozoon* facilitates the process of protruding and retracting of the stylet has not been studied, and we are limited in the conclusions we can make in this area, with just two videos. However, this specific subject along with how this dinoflagellate has become streamlined and specialized to this type parasitic lifestyle is the topic of future work. The videos showed that the stylet protrudes and retracts in regular intervals, lasting just over 1 second. In TEM sections taken through the trophocyte, we...
Figure 8. High-magnification transmission electron micrographs of the putative remnant plastids in *Haplozoon ezoense* n. sp. Parts of the membrane where the 3 membranes are visible are highlighted with a white dashed box and are shown with arrows. Scales: A–D = 100 nm.

were only able to find evidence of a single stylet. The stylet had two spiral arms with an electron dense central core. While protruding and retracting, the stylet itself seemed ridged, but the tip appeared to bend at times. Interestingly, one of the fixed cells prepared for SEM had what looked like a filament (or flagellum) that stuck out from the end of the stylet. This was only observed on one cell, and
it might very well be an artifact of fixation. It is known that the styelt is hollow at the center (Siebert and West 1974), and we were not able to find any evidence for any type of microtubule, filament, or flagellum in the TEM sections from previous studies or in this work. Future work is currently underway to further understand the components of the trophocyte and its utility.

In the more than 30 individuals that were observed under light and electron microscopy, none had gonocytes or sporocytes that subdivided into multiple rows/chains (i.e., all individuals observed were linear); on the other hand, Haplozoon praxillellae possessed both single rows of gonocytes as well as sporocytes that subdivided into rows of two (Rueckert and Leander 2008). It is challenging to speculate on the significance of such morphological characteristics, as only a few Haplozoon lifecycles have been explored so far (Shumway 1924), and definitions researchers use to describe cell types can vary. One example of this is the use of sporocyte. Some researchers define sporocytes as containing 4 nuclei (Siebert and West 1974), while other have preferred a definition that describes them only as bulbous, and granulated (Leander et al. 2002; Rueckert and Leander 2008; Siebert 1973) that only mature sporocytes possess 4 nuclei. In this work, we have used the definitions of Leander et al. (2002) and Rueckert and Leander (2008), as their data was most consistent with what we observed in H. ezoense n. sp., while acknowledging that different definitions exist.

Under TEM, we were able to view the tightly interlocking amphiesma. The junctions between gonocytes were tightly fused to the extent that distinguishing the amphiesma from one cell to the next in the chain was impossible. We also observed other structures in the gonocytes such as Golgi apparatus, and seldom mitochondria with tubular cristae. Similar to the report of Siebert and West (1974), trichocysts were not observed in Haplozoon. Under light microscopy we observed a gonocyte becoming dislodged from its linear row. In the light micrograph, a mucilaginous film can be seen. At the time, we interpreted this to be what Leander et al. (2002) referred to as a “common outer membrane” connecting the individual cells of Haplozoon. However, while inspecting the junctions between cells under TEM, we never observed any membrane layer connecting the cells. Instead, it seemed as though the cells were tightly fused together. Leander et al. (2002) never conducted TEM on Haplozoon, so further comparison is difficult, however, it might be that what Leander et al. (2002) interpreted as a common outer membrane is mucilage from perturbed cells, not an actual membrane connecting cells.

Similar to previous works that examined the 18S rDNA from Haplozoon axiothellae (Gómez and Skovgaard 2015; Saldarriaga et al. 2001) and H. praxillellae (Rueckert and Leander 2008), our study, which included 28S rDNA, was unable to resolve deeper relationships between Haplozoon and other major dinoflagellate groups. In the 28S rDNA and concatenated datasets, Haplozoon was an early branch, sister to Noctiluca, however, the statistical support on this node was low. In the 18S rDNA phylogeny H. ezoense n. sp. branched as a sister lineage to H. praxillellae, which corresponds to their similar morphology and host niche. The 18S rDNA sequence from H. ezoense n. sp. and H. praxillellae differed at 88 sites across 1,748 overlapping bases (5%), suggesting that geographic distance/separation influences species diversity between Haplozoon. While 18S rDNA and 28S rDNA serve as a convenient barcode for delimiting closing related species, its likely that more robust datasets using several protein coding genes will be necessary for resolving the position of Haplozoon among dinoflagellates (Janouškovec et al. 2017).

One of our more intriguing findings during the investigation into the ultrastructure of Haplozoon ezoense n. sp. were conspicuous triple membrane-bound organelles in most of the gonocytes which we concluded are probable remnant non-photosynthetic plastids. Similar structures vaguely appear to be present in H. axiothellae (see fig. 17 in Siebert and West 1974), although they were not recognized as potential remnant plastids at that time. We have reviewed the possibility that what we interpret to be remnant plastids could in fact be something else entirely such as autophagosomes or food vacuoles. If these were autophagosomes or food vacuoles it seems likely that we would have observed intermediate stages (i.e., stages with a

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**Figure 9.** Maximum-likelihood (ML) tree inferred from 18S rDNA sequences. Bootstrap values over 50% and Bayesian posterior probabilities (PP) over 0.50 are shown at the nodes (ML/PP). Thick branches indicate maximal support (100/1.00). The branches leading to the fast-evolving taxa are indicated by dashed and shortened by one half. The scale bar represents inferred evolutionary distance in changes/site. The novel sequence of Haplozoon ezoense n. sp. is highlighted in bold font. The genus Haplozoon is highlighted with a gray box.
Figure 10. Maximum-likelihood (ML) tree inferred from 28S rDNA sequences. Bootstrap values over 50% and Bayesian posterior probabilities (PP) over 0.50 are shown at the nodes (ML/PP). Thick branches indicate maximal support (100/1.00). The branches leading to the fast-evolving taxa are indicated by dashed and shortened by one half. The scale bar represents inferred evolutionary distance in changes/site. The novel sequence from *Haplozoon ezoense* n. sp. is highlighted in bold font. The genus *Haplozoon* is highlighted with gray box.
Figure 11. Maximum-likelihood (ML) tree inferred from concatenated 18S + 28S rDNA sequences. Bootstrap values over 50% and Bayesian posterior probabilities (PP) over 0.50 are shown at the nodes (ML/PP). Thick branches indicate maximal support (100/1.00). The branches leading to the fast-evolving taxa are indicated by dashed and shortened by one half. The scale bar represents inferred evolutionary distance in changes/site. *Haplozoon ezoense* n. sp. is highlighted in bold font. The genus *Haplozoon* is highlighted with a gray box.
different number of membranes or a difference in the content contained within the membranes. Furthermore, the presence of relic plastids has been studied among members of the myzooza (apicomplexans as well as dinoflagellates) and their close (photosynthetic) relatives, providing evidence that they share a common red algal origin with their closest sister lineages, apicomplexans and chromopodelids (Janouškové et al. 2010), and that some lineages of dinoflagellates have undergone plastid loss during the course of their evolution (Saldarriaga et al. 2001). It’s believed that these relic, non-photosynthetic plastids have retained key metabolic pathways, even among lineages of internal parasites (Janouškové et al. 2015), being indispensable in the life history of the organism. Therefore, the probability that there are remnant plastids that have been maintained among Haplozoon lineages is high. Nonetheless, our images provide only ultrastructural evidence for the existence of remnant non-photosynthetic plastids in Haplozoon, and further genetic work looking at plastid targeted genes and specific metabolic pathways would be needed to confirm their existence and function in this bizarre dinoflagellate parasite.

Taxonomic Summary

Class Dinophyceae Fritsch, 1927
Genus Haplozoon Dogiel, 1906

Haplozoon ezoense n. sp. Wakeman, Yamaguchi, and Horiguchi 2017

Description. Trophonts linear, with an average length of 120 μm; gonocytes rounded, but flattened on the connecting edges with a width of 10 μm. A single chain of gonocytes extended from the trophocyte to a single sporeocyte. Trophocytes generally knob-like containing two lobes near to the suction disc, with a single, large stylet, starch, and numerous refractive fibres. Surface of trophonts adorned with dense and short amphiesmal projections; density of amphiesmal projections decreased on posterior regions of trophonts.

DNA sequence 18S rDNA sequence (GenBank xxxxxx); 28S rDNA sequence (GenBank xxxxxx).

Type locality. Oshoro, Hokkaido, Japan (43°12′33.26″ N 140°51′30.67″ E). Host commonly found on the underside of large (~0.5 m diameter) rocks in the low intertidal to subtidal zones.

Type habitat. Marine

Type host. Praxillella pacifica Berkeley, 1929 (Annelida, Polychaeta, Maldanidae)

Location in host. Intestinal lumen

Hapantotype Trophonts on SEM stubs with a gold sputter coat have been stored in the algal and protist collection in the Faculty of Science at Hokkaido University (KCW_Haplo2).

Iconotype Fig. 1A

LSID 674AB21C-7E24-42F5-B230-50C174BCA270

Etymology The species name ‘ezoense’ refers to the old name of the North Island of Japan, Hokkaido, on which the parasite was found.

Methods

Collection of hosts and isolation of Haplozoon ezoense n. sp.: Praxillella pacifica Berkeley, 1929 (Annelida, Mal-danidae) was collected at low tide from rocks near the Hokkaido University Oshoro Marine Station, Oshoro, Hokkaido, Japan (43°12′33.26″ N 140°51′30.67″ E) from April – May 2016. Worms were held in cool seawater and transported to the laboratory where their intestines were dissected using forceps and razor blades. A total of 5 hosts were collected and dissected; all 5 were infected with Haplozoon ezoense n. sp. trophonts (feeding stages). Trophonts of H. ezoense n. sp. were isolated using hand-drawn glass pipettes, and subsequently washed (until clean) in filtered, autoclaved seawater for further morphological and molecular analysis.

Light microscopy, scanning electron microscopy and transmission electron microscopy: Differential interference contrast (DIC) images of the trophont stage of Haplozoon ezoense n. sp. were taken using a Zeiss Axioskop 2 Plus microscope connected to a Leica MC 120 HD digital camera. For scanning electron microscopy, individuals were transferred to a glass coverslip submerged in 2.5% glutaraldehyde in seawater on ice for 15 min. After washing the samples 3 times for 5 min in seawater, the coverslip was placed in 1% OsO4 for 30 min, and subsequently washed with distilled water and dehydrated through a graded series of ethanol washes (30%, 50%, 75%, 80%, and 100%) for 5 min at each step. Samples were critical point dried with CO2, sputter-coated with 5 nm gold and viewed using a Hitachi N-3000. For transmission electron microscopy, individual cells and small pieces of gut tissue were fixed in 2.5% glutaraldehyde in seawater on ice for 30 min, washed in seawater, and post fixed with 1% OsO4 on ice for 1.5 hours; both fixation steps were performed in the dark. Following the fixation with OsO4, samples were washed in seawater, and dehydrated through a graded series of ethanol washes (30%, 50%, 75%, 80%, and 100%), and acetone for 5 min at each step at room temperature. Samples were then placed in a 1:1 resin (Agar Low Viscosity Resin, Agar Sciences)/acetone mixture for 30 min, followed by 100% resin overnight at room temperature. Resin was exchanged the following day, and samples were polymerized at 68 °C for 32 hours. Samples were cut with a diamond knife, and viewed with a Hitachi-7400.

DNA extraction, PCR amplification, and sequencing of 18S rDNA: Single-cell (individual) isolates of Haplozoon ezoense n. sp. were placed in 0.2 ml PCR tubes. Total genomic
DNA was extracted following the manufacturers protocol using an Epicentre FFPE extraction kit (Epicentre, Madison, Wisconsin, USA). The primers SR1 5'-TAACGTGAGAGACCTCTGCCAG-3' and SRSTAK 5'-ACTACGAGCCTTTTAAACGC-3', as well as SR4 5'-AGGGCAAAGCTGTTGGCCAG-3' and SR12 5'-CCTTCCGAGGTTCACTAC-3' (Nakayama et al. 1996) were used to amplify 18S rDNA sequences using the following program on a thermocycler: Initial denaturing 94 °C 2:00 min; 35 cycles of 94 °C 0:30 s, 52 °C 0:30 s, 72 °C 1:30 min; final extension, 72 °C 7:00 min. In order to amplify 28S rDNA sequences, the primers 2SF1 5'-CGGCTGAATTTAGCATAT-3' and 2SR1 5'-CTTTGCGCGTGTTCAGAC-3' (Kogame et al. 1999) were used in a reaction following the same thermocycler program described above. In each PCR reaction, Econonag 2X Mastermix (Lucigen, Middleton, USA) was used, following the manufacturer’s protocols. PCR products were purified using a Qiagen PCR purification kit (Qiagen, Germantown, USA): 1 μl of purified product was used in a sequencing reaction with ABI BigDye Terminator v1.1 (Applied Biosystems, Massachusetts, USA) and subsequently purified with ethanol, before being eluted in 18 μl Hi-Di Formamide (Applied Biosystems, Massachusetts, USA) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). The two novel 18S rDNA sequences from *H. ezoense* n. sp. were deposited in NCBI’s GenBank (xxxxxxx and xxxxxx).

**Phylogenetic analyses:** The newly obtained 18S and 28S rDNA sequences from *Haplozoon ezoense* n. sp. were identified by BLAST. The 18S rDNA sequence was then aligned with 66 additional sequences, as well as apicomplexans and early alveolates as outgroups using “MUSCLE” (Edgar 2004; https://www.drive5.com/muscle/), with the default settings. A second alignment containing 58 taxa using 28S rDNA was constructed in the same way as described above. We also concatenated the 18S rDNA and 28S rDNA from 52 taxa for additional phylogenetic analyses. Taxa present in the trees were selected to represent a diverse set of dinoflagellates for which 18S and 28S rDNA was available. All alignments were fine-tuned using Mesquite version 3.11 (Maddison and Maddison 2015); unambiguous (gap) regions were omitted from analysis.

Gariøi.951-GUI (Zwicki 2006) was used to run Maximum-likelihood (ML) bootstrap analyses on all datasets. Jmodeltest 2.1.10 (Darriba et al. 2012; Guindon and Gascuel 2003) selected TIM2+I+G, TIM1+I+G and GTR+I+G model of nucleotide substitutions under Akaike Information Criteron (AIC) (Posada and Crandall 1998) for 18S, 26S and 18S +28S rDNA datasets, respectively. All ML bootstrap analyses were performed on 500 pseudo-replicates, with one heuristic search per pseudo-replicate (Zwicki 2006), using the same program set to each evolutionary model. All Bayesian analyses were performed using the program MrBayes 3.2.5 (Ronquist and Huelsenbeck 2003). The program was set to operate with GTR+I+G, and four Monte Carlo Markov Chains (MCMC) starting from a random tree. A total of 2,500,000, 3,000,000 and 137,000,000 runs were completed for 18S, 28S and 18S +28S rDNA datasets, respectively. Generations were calculated with trees sampled every 100 generations and the first 2,500, 7,500 and 342,500 trees in each run were discarded as burn-in. When the standard deviation of split frequencies fell below 0.01, the program was set to terminate. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees.

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**Appendix A. Supplementary Data**

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.protis.2018.04.008.

**References**


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