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## Morphology and molecular phylogeny of *Haplozoon praxillellae* n. sp. (Dinoflagellata): A novel intestinal parasite of the maldanid polychaete *Praxillella pacifica* Berkeley

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### Abstract

The genus *Haplozoon* comprises a group of endoparasites infecting the intestines of polychaete worms. Comparative studies using light microscopy, scanning and transmission electron microscopy, and small subunit rDNA have shown that these organisms are very unusual dinoflagellates. To date, there is only one species known from the Pacific Ocean, namely *Haplozoon axiothellae* Siebert. In this study, we describe *Haplozoon praxillellae* n. sp. from the intestine of the Pacific maldanid polychaete *Praxillella pacifica* Berkeley. The parasites are relatively small, oblong and about 35–125 µm in length, consisting of the trophocyte (anterior-most compartment), rectangular gonocytes and bulbous sporocytes. The trophocyte bears an attachment apparatus with a prominent ‘suction disc’ and numerous stylets. We were able to detect spherical vesicles near the ventral surface of each gonocyte. The whole organism is covered with thecal barbs of different shape and size, except for the caudal end of the posterior-most sporocyte, which is instead covered with hexagonal or pentagonal alveoli. A continuous membrane encloses the whole pseudocolony. Molecular phylogenetic data, host specificity and morphological differences clearly distinguish *H. praxillellae* n. sp. from *H. axiothellae*.

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**Keywords:** Alveolata; Dinoflagellate; *Haplozoon*; Parasite; *Praxillella pacifica*

### Introduction

The genus *Haplozoon* is one of several genera of marine dinoflagellates that have evolved a parasitic way of life (Coats 1999). Haplozoans are endoparasites that infect the intestines of polychaete worms belonging to the family Maldanidae Malmgren, 1867 (Siebert 1973). All known *Haplozoon* species infect a single host species

(Coats 1999; Shumway 1924; Siebert 1973), and most of these species have been described from the Atlantic Ocean (Dogiel 1906; Shumway 1924). Only one species has been described and examined in detail from the Pacific Ocean, namely *Haplozoon axiothellae* Siebert, 1973, which infects the polychaete *Axiothella rubrocincta* (Johnston).

Prior to the implementation of electron microscopy and molecular phylogenetics, the classification of these parasites was uncertain. Dogiel (1906), for instance, classified the type species *Haplozoon armatum* as a mesozoan, and Calkins (1915) assigned a new species to

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the schizogregarines (Shumway 1924). Chatton (1919, 1938) first recognized them to be dinoflagellates, and subsequent studies using electron microscopy and molecular phylogenies of small subunit rDNA have shown that haplozoans are indeed strange dinoflagellates with several novel features associated with their parasitic mode of life (Leander et al. 2002; Saldarriaga et al. 2001; Shumway 1924; Siebert 1973; Siebert and West 1974).

The trophont stage of the haplozoans is chain-like and multinucleated, and different species can form three different configurations: pyramidal, pectinate and linear (Chatton 1919; Shumway 1924). The anterior end of these parasites contains dynamic, retractable stylets that penetrate the intestinal lining of their host. Leander et al. (2002) demonstrated distinctive surface features, such as thecal barbs, small polygonal plates and a longitudinal row of ventral pores. This previous study also provided evidence that haplozoans are not truly multicellular or colonial, but instead are enveloped by a single, continuous membrane and compartmentalized by internal sheets of alveoli. Here, we characterize the general morphology of *H. praxillellae* n. sp., a novel dinoflagellate that inhabits the intestines of a subtidal polychaete collected in the Pacific Ocean. We also sequenced the small subunit rDNA gene from *H. praxillellae* n. sp. and compared it with sequences derived from *H. axiothellae* and other dinoflagellates.

## Material and methods

### Collection and isolation of organisms

Specimens of *H. praxillellae* n. sp. were isolated from the intestines of the marine polychaete tubeworm *Praxillella pacifica* Berkeley, 1929, belonging to the family of Maldanidae Malmgren, 1867, so-called 'bamboo-worms'. Hosts were collected by dredge from the Imperial Eagle Channel (48°54'N, 125°12'W) near Bamfield Marine Station, Vancouver Island, Canada, in July 2007 at a depth of 80 m. The hosts were dissected within 3 days after collection.

Haplozoon parasites were isolated in seawater by teasing apart the intestine of *P. pacifica* under a dissecting microscope (Leica MZ6). The gut material was examined under an inverted microscope (Zeiss Axiovert 200) and parasites were removed by micro-manipulation and washed three times in seawater.

### Microscopy

Differential interference contrast (DIC) light micrographs were produced by securing parasites under a cover slip with vaseline and viewing them with an

imaging microscope (Zeiss Axioplan 2) connected to a colour digital camera (Leica DC500).

Ten of the isolated parasites were prepared for scanning electron microscopy. Individuals were deposited directly into the threaded hole of a Swinnex filter holder, containing a 5 µm polycarbonate membrane filter (Millipore Corporation, Billerica, MA), that was submerged in 10 ml of seawater within a small canister (2 cm diameter and 3.5 cm tall). A piece of Whatman filter paper was mounted on the inside base of a beaker (4 cm dia. and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4% OsO<sub>4</sub> and the beaker was turned over the canister. The parasites were fixed by OsO<sub>4</sub> vapours for 30 min. Ten drops of 4% OsO<sub>4</sub> were added directly to the seawater and the parasites were fixed for an additional 30 min. A 10 ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with CO<sub>2</sub>. Filters were mounted on stubs, sputter coated with 5 nm gold, and viewed under a scanning electron microscope (Hitachi S4700). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

### DNA isolation, PCR, cloning, and sequencing

Thirty-four individuals of *H. praxillellae* n. sp. were isolated from dissected hosts, washed three times in filtered seawater, and deposited into a 1.5 ml Eppendorf tube. DNA was extracted by using the total nucleic acid purification protocol as specified by EPICENTRE (Madison, WI, USA). The small subunit (SSU) rDNA was PCR amplified using puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada).

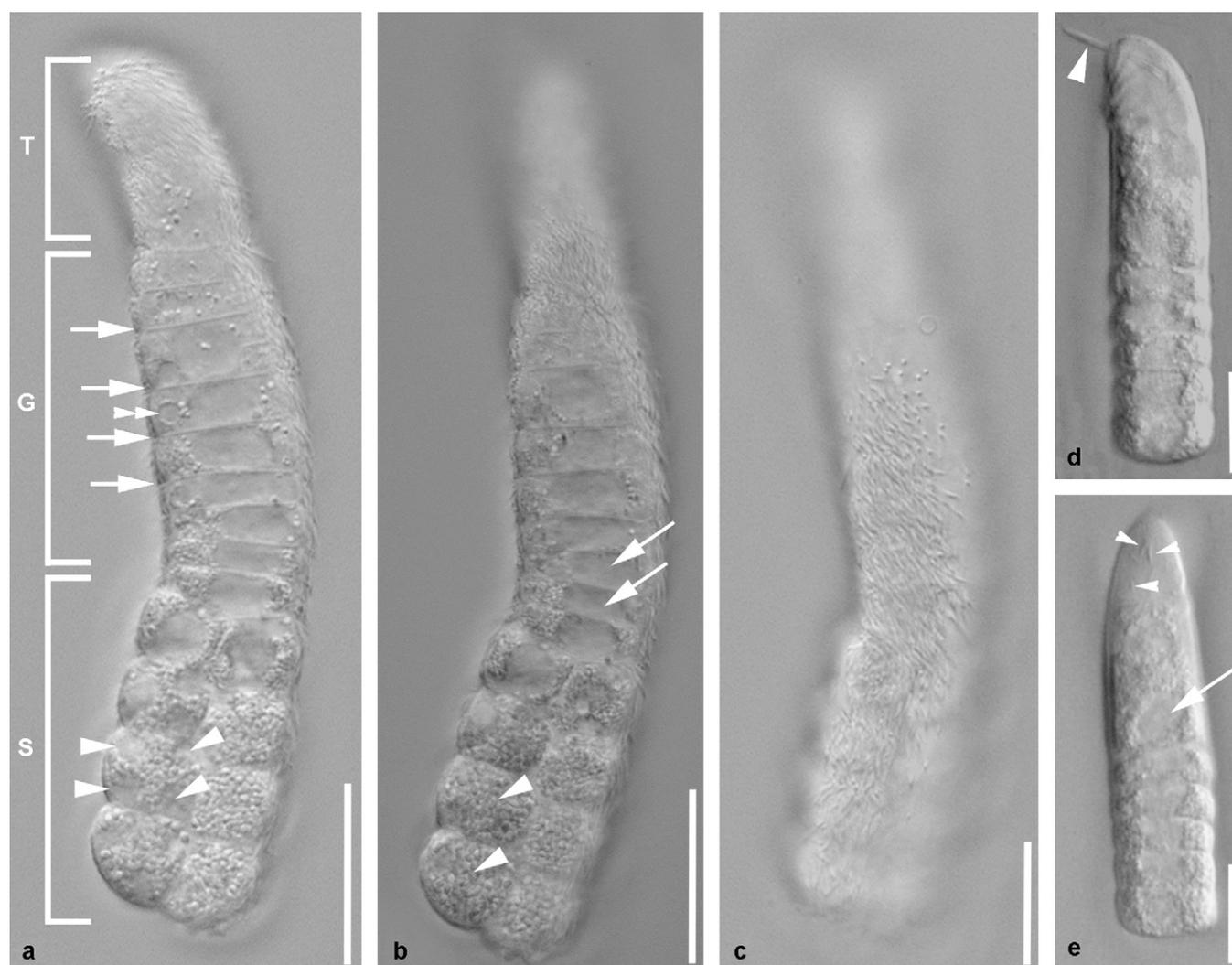
The SSU rDNA gene from *H. praxillellae* n. sp. was amplified in one fragment using universal eukaryotic PCR primers F1 5'-GCGCTACCTGGTTGATCCTGCC-3' and R1 5'-GATCCTTCTGCAGGTTCCACTAC-3' (Leander et al. 2003) and internal primers designed to match existing eukaryotic SSU sequences F2 5'-CGTACTTAACTGTATGGTGCACGATC-3' and R2 5'-TTTAAGTTTCAGCCTTGCCG-3'. PCR products corresponding to the expected size were gel isolated and cloned into the PCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Eight cloned plasmids were digested with EcoRI and screened for size. Two clones were sequenced with ABI bigdye reaction mix using vector primers and internal primers oriented in both directions. The SSU rDNA sequence was identified by BLAST analysis (GenBank Accession number EU598692).

### Molecular phylogenetic analysis

The new sequence from *Haplozoon praxillellae* n. sp. was aligned with 63 alveolate SSU rDNA sequences using MacClade 4 (Maddison and Maddison 2000) and visual fine-tuning. Maximum likelihood (ML) and Bayesian methods under different DNA substitution models were performed on the 64-taxon alignment containing 1589 unambiguous sites. All gaps were excluded from the alignments before phylogenetic analysis. The  $\alpha$ -shape parameter was estimated from the data using the general-

time-reversible (GTR) model for base substitutions (Posada and Crandall 1998) and a gamma-distribution with invariable sites (eight rate categories,  $\alpha = 0.432$ , fraction of invariable sites = 0.241). The ML bootstrap analyses were performed using PhyML\* (Guindon and Gascuel 2003; Guindon et al. 2005) on 100 resampled data under an HKY model using the  $\alpha$ -shape parameter and transition/transversion ratio (Ti/Tv) estimated from the original data set.

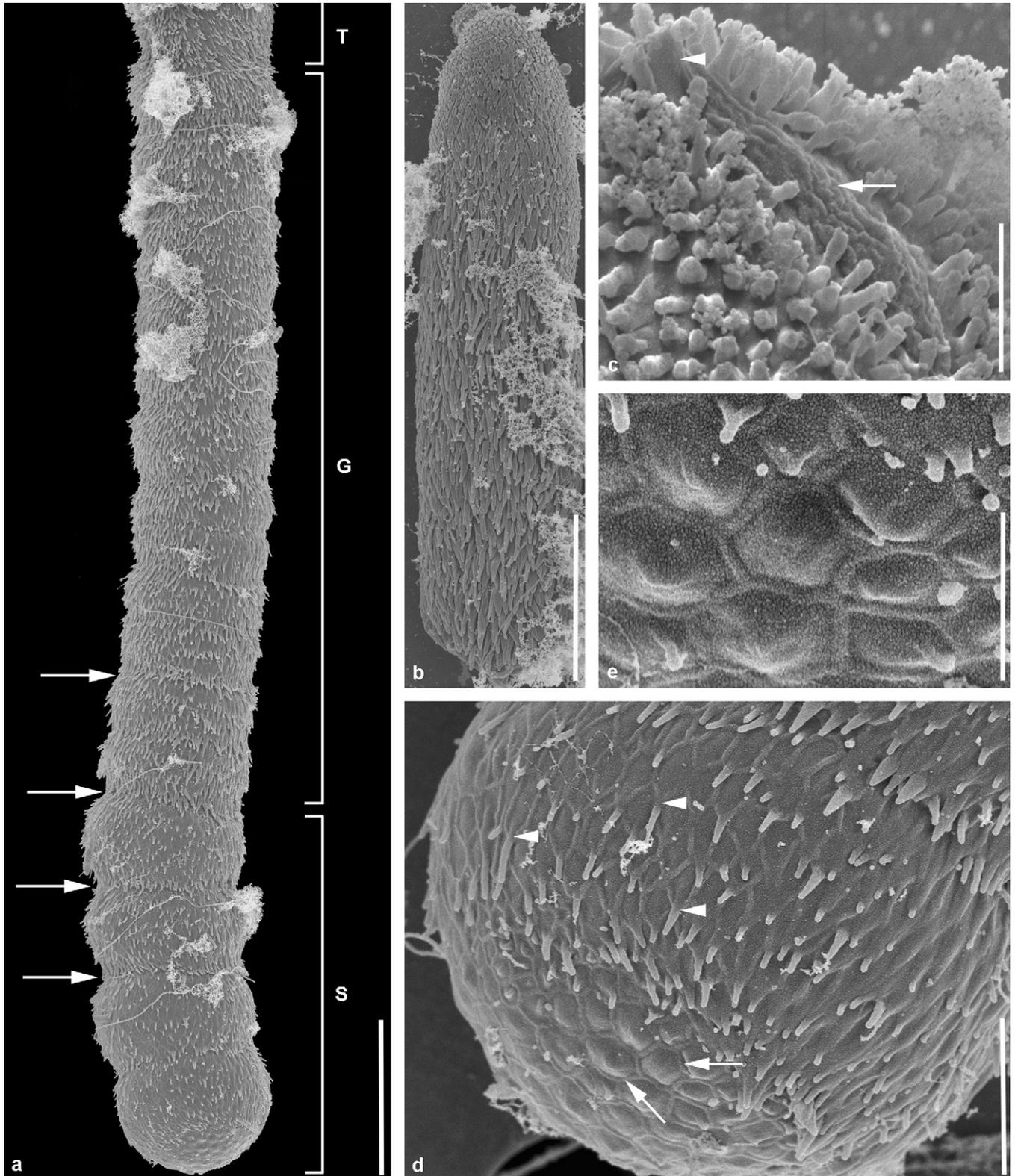
We also examined the 64-taxon data set with Bayesian analysis using the program MrBayes 3.0 (Huelsenbeck



**Fig. 1.** Differential interference contrast (DIC) light micrographs showing the general morphology of *Haplozoon praxillellae* n. sp. (a–c) Micrographs of one individual taken at different focal planes. (a) Left lateral view, showing the three fundamental units: an anterior trophocyte (T), the midregion consisting of one row of nine rectangular gonocytes (G) and the posterior two rows of eight bulbous sporocytes (S) with multiple nuclei (arrowheads). The mature junctions in between gonocytes are visible (arrows). A spherical vesicle (double arrowhead) is located close to the ventral membrane in gonocytes. The position of these vesicles is suggestive of pusules (scale bar = 20  $\mu$ m). (b) Focal plane through the gonocytes and sporocytes showing large nuclei (arrows) in the gonocytes and high granulation in the sporocytes (arrowheads) (scale bar = 20  $\mu$ m). (c) Focal plane through the surface of the pseudocolony showing the abundance and distribution of thecal barbs (scale bar = 15  $\mu$ m). (d) A smaller pseudocolony without sporocytes. The micrograph shows a protruded stylet (arrowhead) at the anterior end of the trophocyte (scale bar = 15  $\mu$ m). (e) The same individual showing additional 'reserve' stylets (arrowheads) within the trophocyte, which are smaller than the retractable stylet. The nucleus (arrow) of the trophocyte is located at the posterior end (scale bar = 15  $\mu$ m).

and Ronquist 2001). The program was set to operate with GTR, a gamma-distribution, and four Monte Carlo Markov chains (MCMC; default temperature = 0.2). A total of 2,000,000 generations were calculated

with trees sampled every 100 generations and with a prior burn-in of 200,000 generations (2000 sampled trees were discarded). A majority-rule consensus tree was constructed from 18,000 post-burn-in trees with PAUP\*



4.0 (Swofford 1999). Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

## Results and discussion

### General morphology

Most individuals of *Haplozoon praxillellae* n. sp. consisted of all three fundamental units described by Shumway (1924): the anterior trophocyte, a midregion of gonocytes and the posterior sporocytes (Figs. 1a–c 2a). The compartments were mostly arranged in a single linear row, but some of the individuals had two rows of sporocytes as can be seen in Fig. 1a, b. Size (35–125  $\mu\text{m}$  in length and 8–20  $\mu\text{m}$  in width) and the morphology of individual specimens showed a high degree of variation. The smallest stage observed consisted of only the trophocyte, while some parasites consisted of the trophocyte followed by 14 gonocytes and four to eight sporocytes.

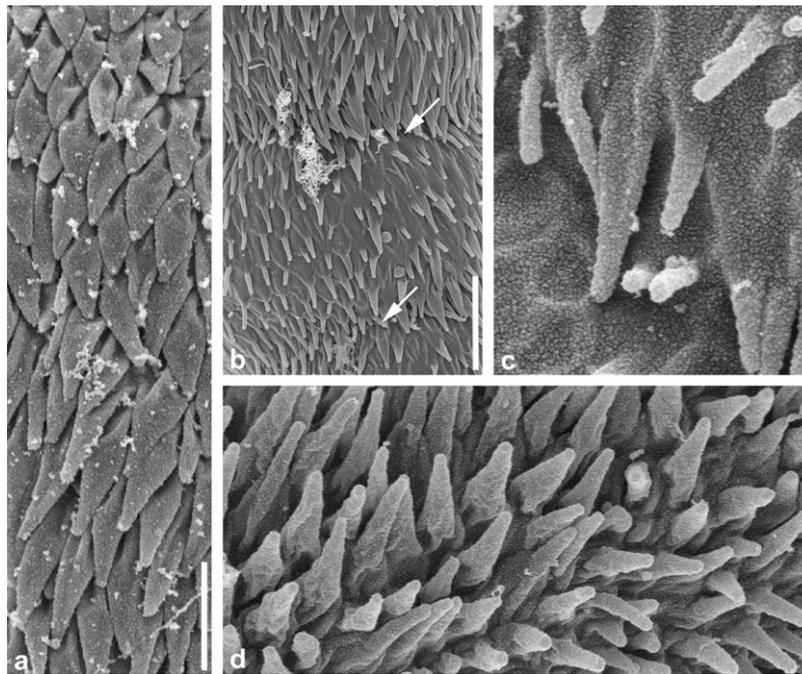
Compared to *H. axiothellae*, the trophocyte of *H. praxillellae* n. sp. is long (around 35  $\mu\text{m}$ ), slender and without a distinct neck region (Leander et al. 2002). On the ventral side of the anterior end, the trophocyte bears the attachment apparatus (Fig. 2c). This morphological feature is used to attach and hold on to the endothelial lining of the host's intestine. According to Siebert and West (1974) the attachment apparatus comprises a 'suction disc', a pair of 'arms' (thecal folds) and additionally stylets. The 'suction disc' was prominent and about 3  $\mu\text{m}$  in length (Fig. 2c). Associated 'arms', which play a role in the attachment process and nutrient uptake (Leander et al. 2002; Siebert and West 1974) were not detected. The trophocyte bears numerous small 'reserve' stylets (Fig. 1e). An individual bigger stylet (Fig. 1d) penetrates the host tissue by protraction and retraction. In Fig. 2c a protrusion can be seen at the anterior end of the 'suction disc', which we interpret to be the tip of the retractable stylet. The protracted stylet in Fig. 1d is about 8  $\mu\text{m}$  long. The entire trophocyte is

covered with thecal barbs that varied in shape and size depending on their location on the parasite (Figs. 2b, 3a). Fig. 1e shows the nucleus situated in the posterior third of the trophocyte, which corresponds to the TEM data of Siebert (1973) and Siebert and West (1974).

A string of rectangular gonocytes connects to the posterior end of the trophocyte. The 'primary gonocyte' is positioned next to the trophocyte and is formed by transverse division of the trophocyte (Shumway 1924). New gonocytes form by division of earlier primary gonocytes, and each gonocyte may contain one nucleus, two nuclei or more nuclei in divisional stages (Fig. 1b). Leander et al. (2002) showed that these different stages are also visible on the surface structure of *H. axiothellae*: relatively deep indentions reflect mature junctions and shallow indentions reflect immature junctions. Because the surface of *H. praxillellae* n. sp. is covered with thecal barbs, only the mature junctions were visible (Figs 1a, 2a). Nonetheless, the posterior-most gonocytes often contain two nuclei and these nuclei will eventually undergo karyokinesis without cell division to produce tetranucleate sporocytes (Siebert and West 1974).

The definition of the sporocytes by Shumway (1924) and Leander et al. (2002) differed compared to that given by Siebert (1973) and Siebert and West (1974). The latter two authors define sporocytes on the degree of cytoplasm granularity and the presence of four nuclei. Shumway (1924) and Leander et al. (2002), on the other hand, follow the definition that only the posterior-most compartment(s) with a bulbous shape and distinctive granulation, are sporocytes. We think that the definition by Shumway (1924) is most consistent with our observations. For instance, the light micrographs in Fig. 1a, b show eight bulbous sporocytes, with distinctly higher cytoplasm granularity and multiple nuclei when compared to the gonocytes. This is also supported by the scanning electron micrograph in Fig. 2a, which shows a clear difference between gonocytes and sporocytes; the latter are more bulbous in shape and have less coverage of thecal barbs. According to Shumway (1924), sporocytes detach from the pseudocolony, leave the host with the faeces and form four daughter

**Fig. 2.** Scanning electron micrographs of *Haplozoon praxillellae* n. sp. showing general morphology and details of the attachment apparatus and thecal barbs. (a) Dorsal view of a parasite with a single row of compartments showing all three fundamental units: posterior end of the trophocyte (T), twelve gonocytes (G) and four more bulbous sporocytes (S). The thecal barbs are less numerous on the sporocytes when compared to the gonocytes. The indentions (arrow) of the surface at the junctions in between adjacent gonocytes and adjacent sporocytes are visible, although the theca is completely covered with thecal barbs (scale bar = 10  $\mu\text{m}$ ). (b) Higher magnification view of a detached trophocyte. The thecal barbs at the anterior end of the trophocyte are shorter in length and more flattened compared to the ones at the posterior end (scale bar = 8  $\mu\text{m}$ ). (c) High magnification lateral view of the attachment apparatus. The attachment apparatus shows a prominent 'suction disc' (arrow), which is about 3  $\mu\text{m}$  long. At the apical tip, part of the protruding stylet (arrowhead) is visible (scale bar = 1  $\mu\text{m}$ ). (d) High magnification view of the posterior-most sporocyte. The caudal tip is free of thecal barbs and shows that the theca is comprised of numerous, mostly hexagonal, thecal plates (arrows). The arrowheads indicate thecal barbs showing clearly that they each stem from a single thecal plate (scale bar = 2.5  $\mu\text{m}$ ). (e) High magnification view of the thecal plates at the caudal tip of the posterior-most sporocyte (scale bar = 1  $\mu\text{m}$ ).



**Fig. 3.** Scanning electron micrographs of *Haplozoon praxillellae* n. sp. showing details of thecal barbs. (a) High magnification view of thecal barbs at the anterior end of a trophocyte. Thecal barbs are broad, flat and arranged like overlapping scales (scale bar = 1  $\mu\text{m}$ ). (b) High magnification view of thecal barbs on adjacent sporocytes. The junction between two sporocytes (arrows) is covered by overarching thecal barbs (scale bar = 2.5  $\mu\text{m}$ ). (c) High magnification view of a junction between adjacent sporocytes (scale bar = 0.5  $\mu\text{m}$ ). No distinct line is visible in between the two sporocytes, indicating that the membrane across the junctions is continuous. (d) High magnification view of the thecal barbs (scale bar = 1  $\mu\text{m}$ ).

dinospores. Although we observed the tetranucleate condition in the sporocytes, dinospores were not observed in *H. praxillellae* n. sp.

### Surface structure

Without exception all individual parasites were covered with tapered thecal barbs that varied in shape and size. They were about 0.1–0.5  $\mu\text{m}$  wide at the base and between 0.2 and 2.0  $\mu\text{m}$  long (Figs 1c, 2a, 2b, 3a–d). Fig. 2d shows that each thecal barb stems from a single alveolar sac. Thecal barbs on the anterior end of some trophocytes were flattened and arranged like overlapping scales that appeared to be rhombic (Figs 2b, 3a). The barbs near the posterior end of the trophocyte had a more pointed tip and projected outward. Sporocytes showed the fewest and least developed thecal barbs (Fig. 2a, d). This stands in contrast to observations of sporocytes in *H. axiothellae*, which are covered by more thecal barbs than the gonocytes (Siebert and West 1974). While previous studies have shown that specific areas of *H. axiothellae* were free from thecal barbs (Leander et al. 2002; Siebert and West 1974), the observed individuals of *H. praxillellae* did not exhibit such areas, except for the caudal end of the posterior-most sporocyte (Fig. 2d, e).

As in *H. axiothellae* (Leander et al. 2002), surface areas with fewer thecal barbs and the caudal end of the posterior-most sporocyte had geometric patterns of small alveoli (Fig. 2d, e). The alveolar sacs possessed hexagonal or pentagonal margins, and were more longitudinally stretched on the sporocytes (0.4–0.8  $\mu\text{m}$  across) when compared to the alveoli on the caudal end of the posterior-most sporocyte (Fig. 2d). Moreover, the SEM data strongly supported the previous report that a continuous outer membrane encloses the entire pseudocolony (Leander et al. 2002). There are no visible furrows in the junctions between adjacent sporocytes (Fig. 3c).

A linear row of ventral pores like that described in *H. axiothellae* (Leander et al. 2002) was not detected on the gonocytes of *H. praxillellae*. However, this does not imply that they were absent in this species, because the presence of ventral pores was almost certainly obscured by thecal barbs. In fact, the presence of spherical vesicles near the ventral surface of each gonocyte provides indirect evidence that these pores were present (Fig. 1a). The shape and location of the ventral vesicles were reminiscent of the pusules known from other dinoflagellates (Hausmann et al. 2003). Leander et al. (2002) also pointed out that the pores could be derived from flagellar pores, which also serve as openings for pusules. Table 1 summarizes the characteristics observed in

**Table 1.** Summary of characteristics observed in *Haplozoon axiothellae* and the newly described *H. praxillellae* n. sp.

	<i>Haplozoon axiothellae</i>	<i>Haplozoon praxillellae</i> n. sp.
Host	<i>Axiothella rubrocincta</i>	<i>Praxillella pacifica</i>
General morphology	Single row of main compartments Double rows of compartments in sporocyte (and sometimes gonocyte) sub-sections can occur	Single row of main compartments Double rows of compartments in sporocyte sub-sections can occur
Trophocyte morphology	Distinct neck region Barbs narrow and tapered	No neck region Barbs flattened and rhombic, or narrow or broad, tapered
Gonocyte morphology	Attachment apparatus present 'Suction disc' covered by barbs Movable stylet Nucleus in the posterior third Pores visible on the ventral surface No spherical vesicle described Area of junctions free of barbs (mostly) Central nucleus	Attachment apparatus present 'Suction disc' prominent (naked) Movable stylet Nucleus in the posterior third No pores visible (obscured by barbs?) Spherical vesicle near ventral surface Area of junctions covered by barbs Central nucleus
Sporocyte morphology	Bulbous shape Covered with well developed barbs Area of junctions free of barbs Multinucleate	Bulbous shape Covered with less developed barbs Area of junctions covered by barbs Multinucleate
Biconcave "epibionts"	Observed	Not observed
Molecular phylogeny	8.8% difference in the small subunit rDNA sequences	

*H. praxillellae* in comparison to those described for *H. axiothellae*.

### Molecular phylogeny

The 64-taxon data set consisting of taxa representing the overall diversity of dinoflagellates supported the establishment of a new *Haplozoon* species, based initially on comparative morphology and the host type (e.g. the two species were isolated from two different hosts collected at two different depths). The new sequence from *H. praxillellae* clustered together with *H. axiothel-*

*lae* with strong statistical support (Fig. 4). A total of 1762 base pairs were compared between *H. praxillellae* and *H. axiothellae*; 139 of these base pairs were different and there were 13 missing bases in one sequence or the other. A pairwise distance calculation based on the Kimura 2-parameter model (Kimura 1980) of 1749 base pairs (excluding the gaps) resulted in an 8.8% sequence divergence. Nonetheless, the phylogenetic position of the *Haplozoon* clade within the dinoflagellates remained unresolved (Leander et al. 2002; Saldarriaga et al. 2001). Previous SSU phylogenies of dinoflagellates showed *H. axiothellae* branching from within one of several *Gymnodiniales* clades with low support (Hoppenrath and Leander 2007; Saldarriaga et al. 2001); in our analyses, the two *Haplozoon* species branch together among the earliest lineages of dinoflagellates (Fig. 4). Future phylogenetic studies using other molecular markers (e.g. nucleus-encoded protein genes like heat-shock protein 90; Leander and Keeling 2004) are needed to improve our understanding of how *Haplozoon* is specifically related to other dinoflagellates.

### Species description

Alveolata Cavalier-Smith, 1991  
Myzozoa Cavalier-Smith and Chao, 2004  
Dinoflagellata Bütschli 1885

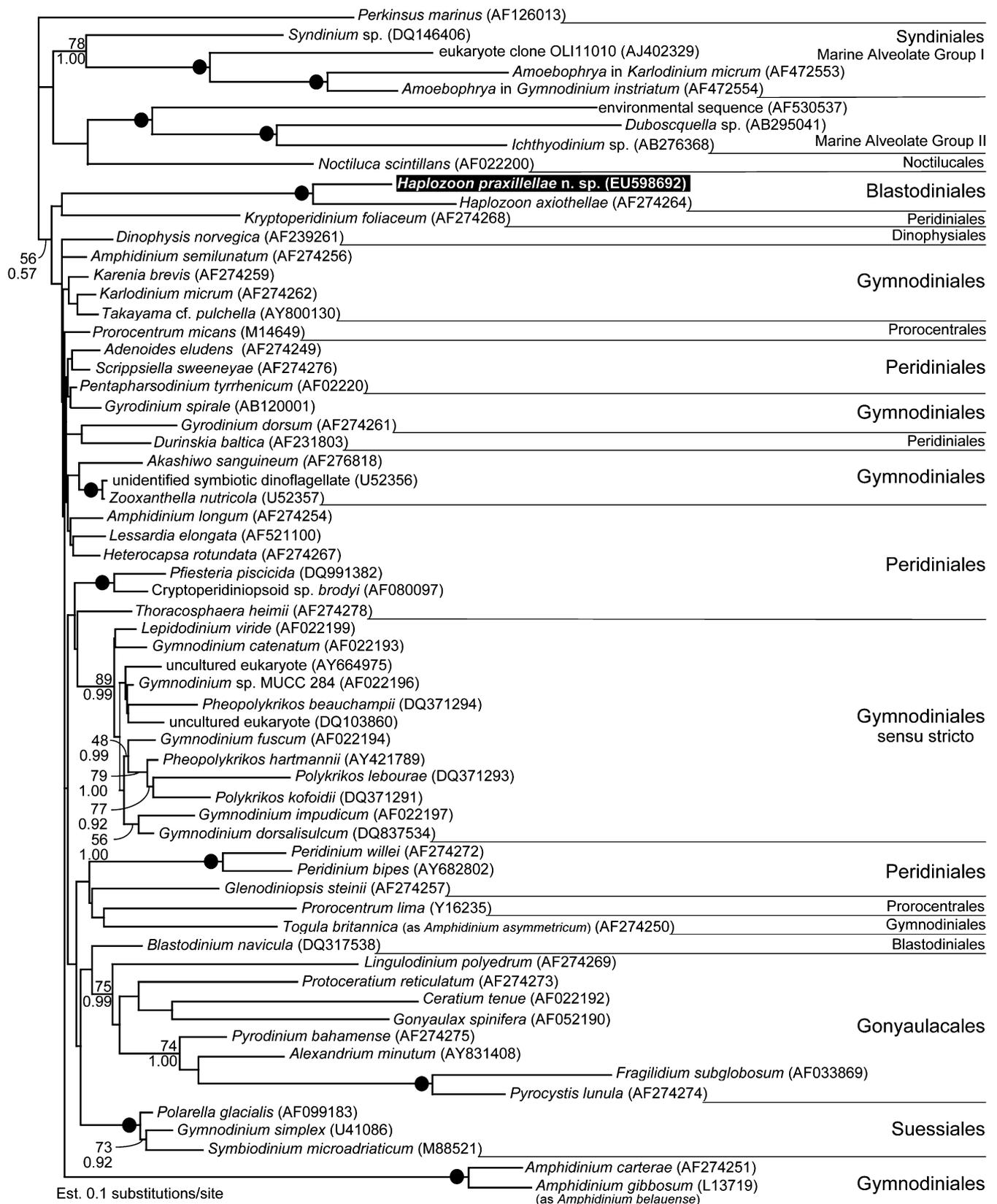
### *Haplozoon praxillellae* Rueckert and Leander n. sp.

#### Description

Linear trophonts are about 35–125 µm long and 8–20 µm wide, consisting of one trophocyte (anterior-most compartment), followed by one row of gonocytes and 1–2 rows of sporocytes. The trophocyte is relatively slender, about 30 µm long, and bears an attachment apparatus comprising a prominent 'suction disc', numerous small 'reserve' stylets and a large protruding stylet, about 8 µm long. The nucleus of the trophocyte is located in the posterior third of the compartment. The trophocyte is covered with thecal barbs; the anterior-most barbs are flattened and organized like overlapping scales. Gonocytes are rectangular in shape, each with a large central nucleus; more nuclei may be present during early stages of division. The cytoplasm of each gonocyte contains a spherical vesicle near the ventral surface. Sporocytes are bulbous with four nuclei and with a distinctly granular cytoplasm. Sporocytes are covered with fewer thecal barbs than gonocytes. The caudal end of the posterior-most sporocyte is free of thecal barbs and shows hexagonal or pentagonal alveoli.

#### Holotype

Fig. 1.



**Fig. 4.** Gamma-corrected maximum likelihood tree ( $-\ln L = 19694.26876$ ,  $\alpha = 0.432$ , fraction of invariable sites = 0.241, eight rate categories) inferred using the GTR model of substitution on an alignment of 64 SSU rDNA sequences and 1589 unambiguously aligned sites. Numbers at the branches denote bootstrap percentage (top) and Bayesian posterior probabilities (bottom). Black dots on branches denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. The sequence derived from this study is highlighted in the shaded box. The Gen-Bank accession numbers of all sequences included are shown in parentheses.

**Hapantotype**

Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada.

**Type locality**

Imperial Eagle Channel (48°54'N, 125°12'W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada; muddy sediments at a depth of 80 m.

**Habitat**

Marine.

**Etymology for the specific epithet**

Refers to the genus of the type polychaete host, *Praxillella pacifica* Berkeley.

**Type host**

*Praxillella pacifica* Berkeley (Metazoa, Annelida, Polychaeta, Maldanidae).

**Location in host**

Intestinal lumen.

**Acknowledgments**

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