

Research Article

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Coinfection of slime feather duster worms (Annelida, *Myxicola*) by different gregarine apicomplexans (*Selenidium*) and astome ciliates reflects spatial niche partitioning and host specificity

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Abstract

Individual organisms can host multiple species of parasites (or symbionts), and one species of parasite can infect different host species, creating complex interactions among multiple hosts and parasites. When multiple parasite species coexist in a host, they may compete or use strategies, such as spatial niche partitioning, to reduce competition. Here, we present a host–symbiont system with two species of *Selenidium* (Apicomplexa, Gregarinida) and one species of astome ciliate co-infecting two different species of slime feather duster worms (Annelida, Sabellidae, *Myxicola*) living in neighbouring habitats. We examined the morphology of the endosymbionts with light and scanning electron microscopy (SEM) and inferred their phylogenetic interrelationships using small subunit (SSU) rDNA sequences. In the host ‘*Myxicola* sp. Quadra’, we found two distinct species of *Selenidium*; *S.* cf. *mesnili* exclusively inhabited the foregut, and *S. elongatum* n. sp. inhabited the mid to hindgut, reflecting spatial niche partitioning. *Selenidium elongatum* n. sp. was also present in the host *M. aesthetica*, which harboured the astome ciliate *Pennarella elegantia* n. gen. et sp. *Selenidium* cf. *mesnili* and *P. elegantia* n. gen. et sp. were absent in the other host species, indicating host specificity. This system offers an intriguing opportunity to explore diverse aspects of host–endosymbiont interactions and competition among endosymbionts.

Introduction

It is widely thought that virtually every free-living species is infected by at least one parasite species, highlighting the substantial contribution of parasites to biodiversity (Poulin and Morand, 2000). Numerous examples exist of a single host species hosting multiple parasite species, as well as instances where one parasite species either inhabits multiple host species or requires multiple host species to complete its life cycle; all of these possibilities make it challenging to tease apart host–parasite interactions (Rigaud *et al.*, 2010; Pilosof *et al.*, 2015). When multiple parasite species coexist in one host individual, competition for resources is expected (Dobson, 1985; Mideo, 2009), because parasites by definition rely on their host for survival during at least part of their life cycle. Niche partitioning is an outcome of parasites avoiding competition when they contemporaneously co-occur within one host individual (Bashey, 2015). Detailed observations of locations within a host combined with comparative molecular data have revealed several different cases of niche partitioning among parasites occurring within the same host individual (Wakeman *et al.*, 2014; Mohapatra *et al.*, 2022).

Annelids play important ecological roles in marine environments as both predators and prey for other animals, and also contribute to sediment bioturbation (Hutchings, 1998). Furthermore, annelids host a diverse range of animal and protistan symbionts (Peoples *et al.*, 2012), where gregarine apicomplexans are particularly common within the host intestines (Leander, 2008; Schrével *et al.*, 2016; Rueckert and Horák, 2017; Paskerova *et al.*, 2018; Iritani *et al.*, 2021). Gregarines are commonly known as parasites; however, they might show a broader spectrum of symbioses ranging from mutualism to parasitism (Rueckert *et al.*, 2019). Gregarines are known for their host specificity and are typically horizontally transmitted through the faecal–oral route (Leander, 2008; Lynn, 2008; Desportes and Schrével, 2013). The infestation process in a new host animal begins when the host ingests oocysts (syn., sporocysts) from the environment. Then, four or more haploid sporozoites (the infectious stage) emerge from an oocyst and penetrate the gut epithelial cells of the host. The sporozoites develop into larger feeding cells, called ‘trophozoites’, that emerge from host cells and occupy extracellular spaces within the host, such as the intestinal lumen. Two mature trophozoites subsequently pair up in a process known as ‘syzygy’ (the mating stage) to form a connected pair of gamonts that are eventually enveloped by a cyst wall, forming a ‘gametocyst’. The two gamonts within the gametocyst undergo a series of cell divisions to form female and male gametes that fuse together to produce a fleeting diploid sporoblast (i.e. zygote). Each sporoblast develops into an oocyst, within which meiosis occurs

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to from four (or more) resting sporozoites. Therefore, each gametocyst can be filled with hundreds of oocysts that ultimately leave the host *via* the faeces and become distributed in the surrounding sediments. Trophozoites are the most conspicuous, dynamic and morphologically diverse stage in the life cycle of gregarines, so they commonly form the basis for species discovery and identification (Leander, 2008).

Since the early stages of gregarine research, it has been well-known that multiple infections of gregarines and other protists can occur within an individual annelid host (Ray, 1930; Fowell, 1936). In the modern era, small subunit (SSU) rDNA sequencing has become a widely used method for species identification and confirming the presence of multiple parasite species within a host (Leander *et al.*, 2003; Wakeman *et al.*, 2014; Diakin *et al.*, 2016; Rueckert and Horák, 2017; Iritani *et al.*, 2021; Paskerova *et al.*, 2021). Some detailed studies have demonstrated that different parasites can occupy distinct locations within the host's body or different life stages of the host. For instance, in the yellow mealworm *Tenebrio molitor*, three gregarine species were exclusively detected in the larval stage, while one species exclusively infected the adult stage, indicating temporal niche partitioning (Clopton *et al.*, 1992). In the case of the common heart urchin *Echinocardium cordatum*, five gregarine species were discovered, with three inhabiting the intracoelomic space and 2 found within the intrahemal space (Coulon and Jangoux, 1986). In the spaghetti worm *Thelepus japonicus*, *Selenidium terebelle* and *S. melongena* occupied the intestinal and coelomic spaces, respectively, demonstrating another case of spatial niche partitioning (Wakeman *et al.*, 2014).

In addition to gregarines, other parasitic alveolates (e.g. dinoflagellates, ciliates, protococcidians, rhytidocystids and squirmids) have also been discovered in marine annelids (Kozloff, 1961; Leander, 2006; Leander and Ramey, 2006; Park *et al.*, 2023; Paskerova *et al.*, 2023). Among ciliates, several species of astomes have been documented in marine annelid hosts (Caullery and Mesnil, 1907; Awerinzew, 1908; De Puytorac, 1954; Sauvadet *et al.*, 2017). As their name suggests, astomes lack a cytostome (i.e. a cell mouth) (De Puytorac, 1954; Lynn, 2008) and are thought to be commensal inhabitants within the intestines of diverse animals, including molluscs, turbellarians, newts and frogs (Fokam *et al.*, 2011; Obert and Vďačný, 2019). While more than 50 genera from 9 families of astomes are known, molecular phylogenetic data remain limited and are only available for astomes that inhabit oligochaetes (Fokam *et al.*, 2011; Obert and Vďačný, 2019, 2021), polychaetes (Sauvadet *et al.*, 2017), turbellarians (Rataj and Vďačný, 2018; Rataj *et al.*, 2022) and gastropods (Zhang and Vďačný, 2022). Among marine species, SSU rDNA sequences are currently accessible for only a few species of *Durchoniella* (astome ciliate) that infect cirratulid polychaete *Cirriformia* (Sauvadet *et al.*, 2017).

Myxicola Koch in Renier 1847 (Polychaeta: Sabellidae), which comprises more than 20 named species of feather duster worms, is a group of marine annelids known for producing gelatinous substances and exhibiting remarkable regenerative abilities (Stabili *et al.*, 2014; Licciano *et al.*, 2015). While exploring various symbionts in marine annelid hosts collected from Harriot Bay, Quadra Island (British Columbia, Canada), we identified two different *Selenidium* species (Apicomplexa, Gregarinida) and one astome ciliate species in two species of *Myxicola*: *Myxicola* sp. Quadra (resembling *M. infundibulum*) and *M. aesthetica*. We utilized SSU rDNA sequences, light microscopy (LM), histology and scanning electron microscopy (SEM) to characterise the endosymbionts found in these *Myxicola* hosts. We compared the traits of the *Selenidium* species described in this study with the traits of *Selenidium mesnli* Brasil, 1909, which was described over 100 years ago from individuals of *Myxicola infundibulum* collected

in Europe (Brasil, 1909; Ray, 1930; Reed, 1933). Based on comparative morphology and molecular phylogenetic data from the symbionts observed in this study, we identify *Selenidium* cf. *mesnli* and establish one new species of gregarine apicomplexans and one new species of astome ciliates from the Pacific Ocean: *Selenidium elongatum* n. sp. and *Pennarella elegantia* n. gen. et sp.

Materials and methods

Sample collection and light microscopy

Various numbers (10–40) of specimens of *Myxicola* (Fig. 1) were collected by SCUBA diving in November 2021; January, February, April, October and November 2022; and in March and August 2023. Two different species of *Myxicola* were present in these collections: '*Myxicola* sp. Quadra' (resembling *M. infundibulum*) and *M. aesthetica*. Specimens of *Myxicola* sp. Quadra were discovered on the sediment near Cove Beach (50°07'00.8"N 125°13'20.9"W), while specimens of *M. aesthetica* were found on ropes connected to a raft located ~10–20 m away from the collection site of *Myxicola* sp. Quadra (50°07'01.5"N 125°13'22.0"W). The two species of *Myxicola* were clearly distinguished by their size. *Myxicola* sp. Quadra measured 5–10 cm in length and only a single individual was found within a gelatinous incasement. In contrast, *M. aesthetica* specimens measured 1.5–3 cm in length, and most individuals ($n = 10–30$) were discovered within a mass of gelatinous material (Fig. 1A–D).

The collected animals were either dissected at a marine station (e.g., the Hakai Institute) near the collection sites or transported to a laboratory at the University of British Columbia (UBC), Canada. The animals were dissected using fine-tipped forceps and a scalpel, after which the intestine was carefully placed into a clean Petri dish containing filtered seawater. The intestine was opened, starting from the anterior end towards the posterior end, using fine-tipped forceps and a scalpel. Individual symbiont cells for PCR analysis were isolated using a hand-drawn glass pipette and subsequently washed three times in filtered seawater. Endosymbionts with different morphotypes were then separated and transferred to a glass slide for examination under differential interference contrast (DIC) microscopy. A Sony ILC-7RM4A digital camera attached to Zeiss Axio Scope A1 compound microscope and Zeiss Axiocam 503 colour camera attached to Zeiss AxioPlan 2 microscope or Axiovert 200 inverted scope were used for micrographs at the marine station and at UBC, respectively.

Scanning electron microscopy and histology

Individual cells of *Selenidium* spp. and astome ciliates were picked up using a hand-drawn glass pipette and subsequently transferred to a Swinnex filter holder fitted with an isopore membrane (pore size = 10 µm), which was filled with filtered seawater. The cells were fixed with osmium vapour for 10 min, followed by the addition of 6–7 drops of 4% osmium tetroxide directly into the Swinnex filter holder for post-fixation for 10 min. The osmium-fixed cells were rinsed twice with filtered seawater. Ethanol dehydration was performed in a series, starting with 30–50%, 70%, 80%, 85%, 90%, 95%, and finally 100% ethanol. The membrane was transferred to a metal basket submerged in 100% ethanol for critical point drying using the *Tousimis autosamdri* 815B Critical Point Dryer. Once dried, the membrane with specimens was placed onto a stub and a 2 nm layer of platinum gold was sputter coated using either the Cressington 208HR high-resolution sputter coater or the Leica EM ACE600 Coater. Images were captured using Zeiss Crossbeam XB350. SEM processing and imaging were conducted at the UBC Bioimaging Facility.

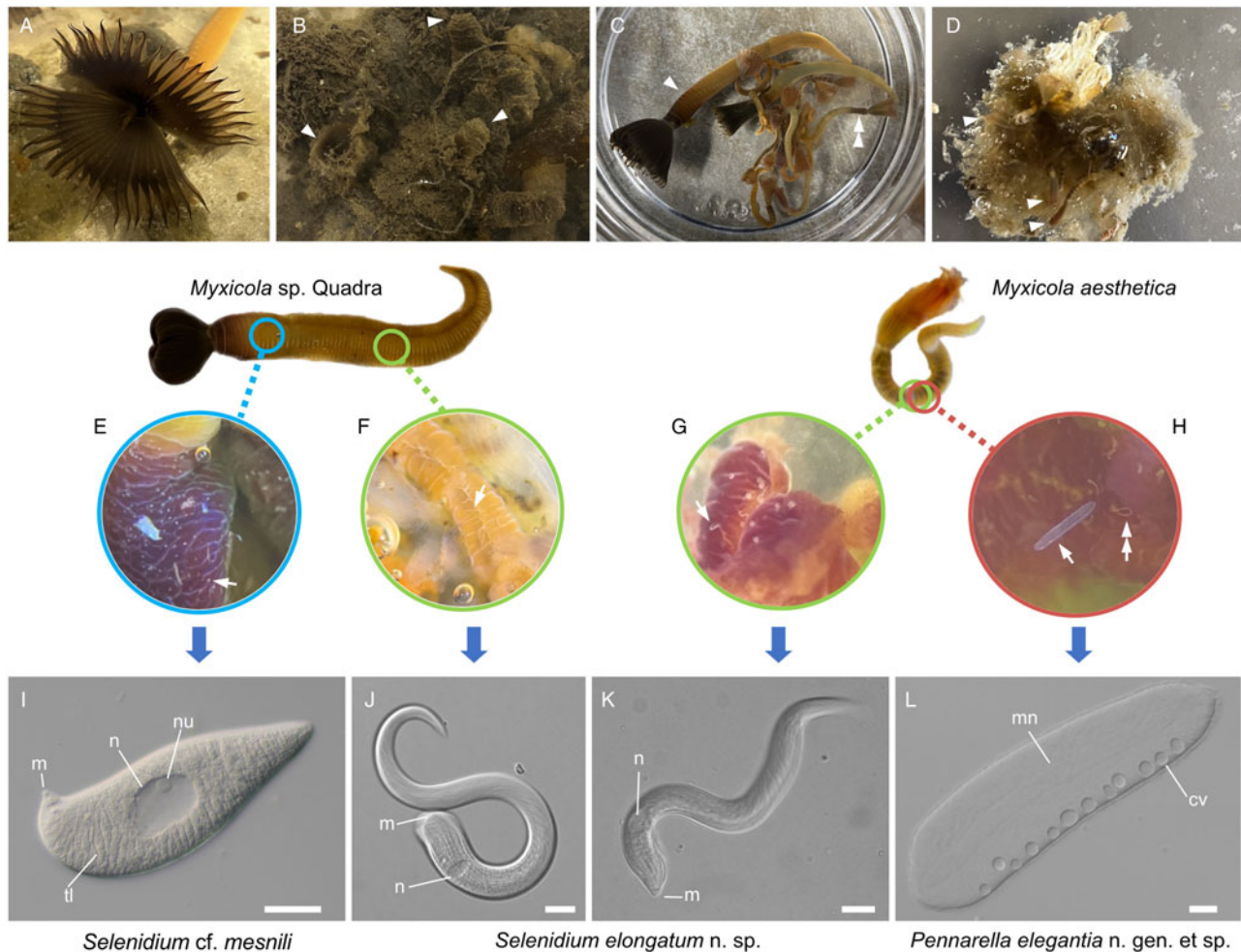


Figure 1. Two gregarine species and one ciliate species found in two *Myxicola* host species. (A) A live specimen of *Myxicola* sp. *Quadra* placed in a container filled with seawater. The brown-coloured crown, used for respiration and food collection, is visible. (B) Specimens of *Myxicola* sp. *Quadra* in gelatinous substance produced by them. A few individuals are indicated with arrowhead. (C) The two *Myxicola* species investigated in this study. *Myxicola* sp. *Quadra* (single arrowhead) is larger than *M. aesthetica* (double arrow head). (D) A colony of *M. aesthetica* in the gelatinous substance produced by them. A few individuals are indicated with arrowhead. (E) A large number of small, lemon-shaped trophozoites of *Selenidium* cf. *mesnili* (arrow) were visible on the epithelium of the foregut of *Myxicola* sp. *Quadra*. (F) Several long trophozoites of *Selenidium elongatum* n. sp. (arrow) were found in the mid to hindgut of *Myxicola* sp. *Quadra*. (G) Several *Selenidium elongatum* n. sp. (arrow) were attached to the epithelium of the gut of *M. aesthetica*. (H) Free swimming *Pennarella eleganta* n. gen. et sp. (arrow) inhabited the gut of *M. aesthetica*. *Selenidium elongatum* n. sp. coinfecting the same host are visible in the background (double arrowhead). I. Differential interference contrast (DIC) light micrograph of *Selenidium* cf. *mesnili* (scale bar = 5 μ m). (J) DIC light micrograph of *Selenidium elongatum* n. sp. from *Myxicola* sp. *Quadra* (scale bar = 20 μ m). (K) DIC light micrograph of *Selenidium elongatum* n. sp. from *M. aesthetica* (scale bar = 20 μ m). (L) DIC light micrograph of astome ciliate *Pennarella eleganta* n. gen. et sp. from *M. aesthetica* (scale bar = 20 μ m). n, nucleus; nu, nucleolus; m, mucron; tl, transverse lines; mn, macronucleus; cv, contractile vacuoles.

For histological observations, a whole intestine was isolated from a specimen of *Myxicola* sp. *Quadra*. The anterior and mid-to-hind gut parts were then separately placed into 1.5 mL tubes. The tissues were fixed in 4% paraformaldehyde (PFA) overnight and stored in 70% ethanol at 4°C until they were sent to the Diagnostic & Research Histology Laboratory at UBC for histological analysis. The tissues were embedded in paraffin blocks, and 4 μ m-thick sections were stained with haematoxylin and eosin (H&E). Imaging was performed using a Zeiss Axiocam 503 colour camera attached to a Zeiss Axioplan 2 microscope.

PCR and sequencing

Trophozoites representing different morphotypes of *Selenidium* were individually isolated from the host, washed 3 times in filtered seawater and placed into an Eppendorf tube. 10 μ L of distilled water was added to each tube containing a single trophozoite of *Selenidium*. The tubes were then incubated at 56°C for 3 min to obtain template DNA for PCR. To obtain SSU rDNA sequences of *Selenidium*, two consecutive PCR runs were conducted for nested PCR. For the first round of

PCR, 22 μ L of distilled water, 0.5 μ L each of forward and reverse primers (GF2; 5'-TGCCTAC CTGGTTGATCC-3' and SSUR4; 5'-GATCCTTCTGCAGGTT CACCTAC-3') and 2 μ L of DNA template were added in a 0.2 mL tube containing a PCR bead (Cytiva PuReTaq Ready-To-Go™ PCR Beads; Cytiva, Global Life Sciences Solutions, Marlborough, MA, USA). PCR reactions were conducted with the following conditions: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 90 s and a final extension at 72°C for 7 min. The second round of PCR was conducted using 23 μ L of distilled water, 0.5 μ L each of forward and reverse primers (GF5; 5'-CCTGGTTGATCCTGCCAG-3' and SEL_R1; 5'-GAAACCTT GTTACGACTTCTCC-3'), 1 μ L of PCR product from the first round of PCR, and a PCR bead with the following conditions: an initial denaturation at 95°C for 3 min, 26 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 90 s and a final extension at 72°C for 7 min. SSU rDNA sequences of astome ciliates were obtained using the primer pair GF5 and SSUR4 with the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 90 s and a final extension for 7 min at 72°C.

To obtain COI sequences of the host specimens, 12.3 μ L of distilled water, 4 μ L of reaction buffer, 0.8 μ L of each forward and reverse primer (LCO1490 and HCO2198; Folmer et al., 1994), 0.1 μ L of MyTaq (Bioline) and 2 μ L of DNA were used. PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 40 s and a final extension for 10 min at 72°C. Afterward, 5 μ L of PCR product from each reaction was loaded onto a 1.5% agarose gel stained with GelRed™ (Biotium) and subsequently purified using ExoSAP-IT™ (Applied Biosystems). The purified PCR products were sent to the UBC sequencing and bioinformatics facility for sequencing.

Molecular phylogenetic analyses

Three separate DNA sequence datasets were created for *Selenidium*, astome ciliates and *Myxicola* hosts, respectively. *Selenidium* is not a monophyletic group when inferred from SSU rDNA sequences but consists of several genetically divergent lineages (Rueckert and Horák, 2017). The *Selenidium* sequences obtained in this study were highly similar to those of the main *Selenidium* clade based on a BLAST search. The sequences of *Selenidium* showed identities ranging from 93.75% to 98.64% with '*S. cf. mesnili*' (JN857968) and 92.44% to 93.90% with *S. sabellae* (MF882906). They demonstrated a 90.55–91.94% similarity with *S. pendula* (MF882901), the type species of the genus *Selenidium*. Therefore, for the *Selenidium* dataset, one SSU rDNA sequence representing each *Selenidium* species of the 'main' clade (i.e. the clade containing the type species of the genus) available in GenBank was included, and two blastogregarine sequences were used as outgroups. As a result, 25 SSU rDNA sequences were included in the dataset. For astome ciliates, one SSU rDNA sequence representing each astome ciliate species available in GenBank, as well as sequences representing major groups of Oligohymenophorea were included. As a result, 53 SSU rDNA sequences were included including three sequences for outgroups. For the *Myxicola* hosts, all COI sequences of the genus *Myxicola* available in GenBank (21 sequences), as well two sequences for outgroups, were included. In all three datasets, DNA sequences were aligned using the MAFFT algorithm (Katoh and Standley, 2013) within Geneious Prime. Ambiguous sites were subsequently removed for the SSU rDNA alignments using Gblocks (Castresana, 2000). As a result, alignments of 1574 bp, 1595 bp and 685 bp were used for further analyses for *Selenidium*, astome ciliates and *Myxicola*, respectively.

All phylogenetic analyses were conducted using CIPRES Science Gateway v3.3 (Miller et al., 2010). Maximum likelihood (ML) trees were inferred using RAxML v8.2.12 with GTRCAT approximation (Stamatakis, 2014). A rapid bootstrap analysis was conducted with 1000 replicates. The best-fitting model of nucleotide evolution for each dataset was determined based on the corrected Akaike information criterion (AICc) using jModelTest v2.1.6 (Darriba et al., 2012). As a result, GTR + I + G was chosen as the best model for *Selenidium* and astome datasets, and HKY + I + G was chosen for *Myxicola*. For Bayesian tree inference, two independent runs, consisting of four chains each, were simultaneously conducted for 1 000 000 generations with a sampling frequency of 1000. A stop rule was applied to terminate the MCMC generations as soon as the standard deviation of split frequencies fell below 0.01. The initial 25% of samples were discarded. The resulting trees were visualized using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Coinfection and distribution of endosymbionts in the host

At least 20 individuals of *Myxicola* sp. Quadra and 30 individuals of *M. aesthetica* were observed. Nearly all (>90%) individuals of

Myxicola sp. Quadra examined in this study hosted two different morphotypes of *Selenidium* trophozoites: a short morphotype (= *S. cf. mesnili*) and a long morphotype (= *S. elongatum* n. sp.). Within *Myxicola* sp. Quadra, the foregut region was heavily infested with the short morphotype of *Selenidium*, which appeared as white dots under a stereoscope (Fig. 1E). These short morphotype cells were either embedded in or attached to the gut epithelium. In contrast, only the long morphotypes of *Selenidium*, which resembled nematodes under a stereoscope, were observed in the mid to hindgut region of the host, where they were attached to the gut epithelium (Fig. 1F). Histological sections (Figs. 2B and 2C) confirmed the presence of the two morphotypes and their locations in the epithelial cells in the anterior part of the gut (for the short morphotype) and in the gut lumen of the mid-posterior part (for the long morphotype) in the host, consistent with the observations made using light microscopy. In *M. aesthetica*, one morphotype of *Selenidium* (= *S. elongatum* n. sp.) trophozoites (Fig. 1G) and one astome ciliate species (= *Pennarella elegantia* n. gen. et sp.) (Fig. 1H) almost always occurred together within the gut of a single host individual. No specific distribution patterns of *Selenidium* trophozoites and the astome ciliates along the gut were observed; however, the *Selenidium* trophozoites were attached to the gut epithelium, while the astome ciliates were freely swimming within the gut lumen (Fig. 1G and H).

Two different morphotypes of selenidium trophozoites

The most notable differences between the two morphotypes of *Selenidium* in *Myxicola* sp. Quadra were the cell lengths, the location of the nucleus and the presence or absence of cytoplasmic transverse lines (Fig. 1I and J, and Supplementary Fig. 1A and B). The short morphotype of *Selenidium* (= *S. cf. mesnili*) in *Myxicola* sp. Quadra was 35 μ m (26–45 μ m, n = 13) long and 13 μ m (8–23 μ m, n = 13) wide. The transverse lines only seen in the short morphotype in *Myxicola* sp. Quadra were due to granules in the cytoplasm and were therefore only visible in the light micrographs (Fig. 1I). Although some intermediate-sized cells were observed, the position of the nucleus in the two morphotypes was distinct, with the short morphotype having the nucleus in the middle part of the trophozoite, while the long morphotype (= *S. elongatum* n. sp.) having the nucleus near the anterior end of the trophozoite. The nucleus of the short morphotype was ellipsoidal and longitudinally elongated, measuring 8.4 μ m long (6–10 μ m, n = 5) and 4.3 μ m wide (4–5 μ m, n = 5). The long morphotypes of *Selenidium* observed in both *Myxicola* sp. Quadra (Fig. 1J) and *M. aesthetica* (Fig. 1K and Supplementary Fig. 1C) were characterized by a relatively large cell size: 144 μ m (98–200 μ m, n = 6) long and 13 μ m (8–23 μ m, n = 7) wide in *Myxicola* sp. Quadra and 140 μ m (100–185 μ m, n = 6) long and 25 μ m (20–30 μ m, n = 6) wide in *M. aesthetica*. Additionally, the nucleus of the long morphotype was ellipsoidal and elongated transversely, positioned between the anterior end and the middle part of the trophozoite in both host species (Fig. 1J and 1K), with 6 μ m long (5–10 μ m, n = 8) and 12 μ m wide (7–18 μ m, n = 6) wide in *Myxicola* sp. Quadra, and 12 μ m long (8–18 μ m, n = 5) and 18.4 μ m wide (18–20 μ m, n = 5) in *M. aesthetica*. These trophozoites moved by twisting and bending. Moreover, the morphological traits of the long morphotypes of *Selenidium* in both hosts were indistinguishable when examined with SEM (Fig. 3A and 3B). The long morphotypes from both hosts displayed rounded or pointed mucrons (depending on their movement) along with 20–28 longitudinal epicytic folds and numerous epicytic transverse striations near the anterior or middle part of the cell (Fig. 3D and 3E). Because epicytic transverse striations were also visible on the short morphotype of

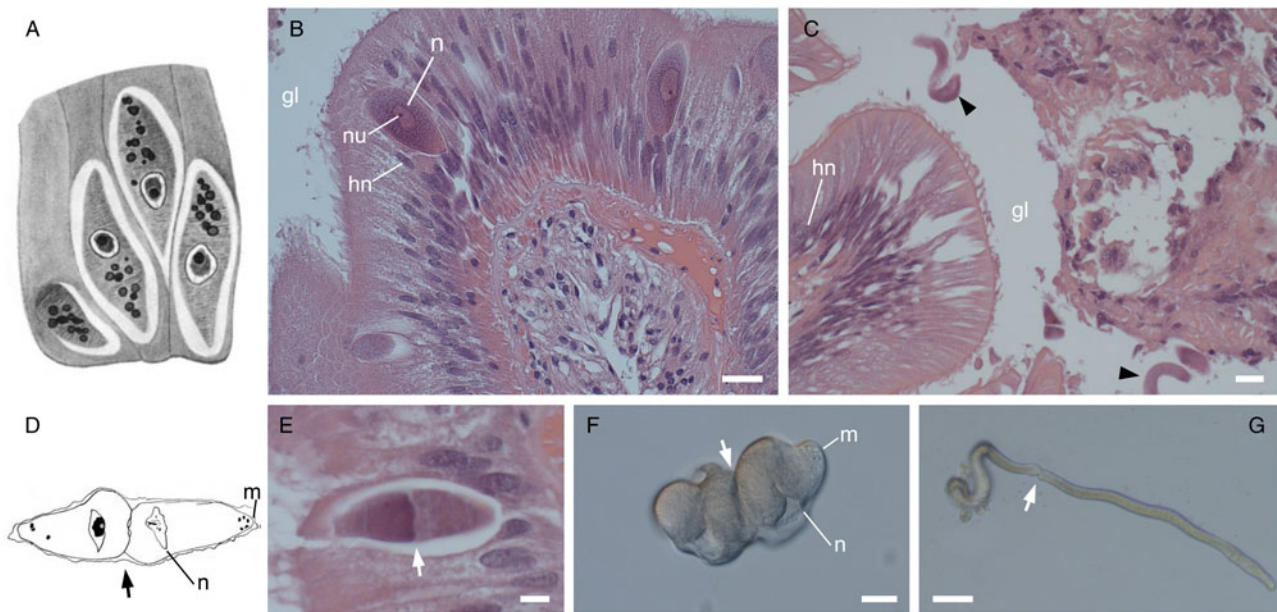


Figure 2. Comparison of trophozoites and syzygy of *Selenidium* from *Myxicola* using light micrographs (LMs) and previously reported drawings. (A) A drawing of trophozoites of *Selenidium mesnili* inside the epithelial cells of host specimens of *Myxicola infundibulum* collected in the UK (modified from Ray, 1930). (B) A histological section of the anterior part of the gut of *Myxicola* sp. Quadra (Canada) showing trophozoites of *Selenidium* cf. *mesnili* inside host epithelial cells (Scale bar = 20 μ m). (C) A histological section of the mid-part of the gut of *Myxicola* sp. Quadra (Canada) showing trophozoites of *Selenidium elongatum* n. sp. (black arrowheads) in the gut lumen (Scale bar = 20 μ m). (D) A drawing of *S. mesnili* gametocytes in syzygy from *M. infundibulum* hosts collected in the UK (modified from Reed, 1933). The gamonts are connected (arrow) by their posterior ends (Scale bar = 20 μ m). (E) A histological section showing gametocytes of *S. cf. mesnili* from *Myxicola* sp. Quadra (Canada) in syzygy within a host epithelial cell. The syzygy junction is indicated by the arrow (Scale bar = 10 μ m). (F) Native observations under DIC of the gamonts of *S. cf. mesnili* from *Myxicola* sp. (Quadra) in syzygy and connected (arrow) by their posterior ends (Scale bar = 10 μ m). (G) Native observations under DIC showing syzygy of *Selenidium elongatum* n. sp. from *Myxicola* sp. Quadra (Scale bar = 20 μ m). The syzygy junction is indicated by the arrow. B, C and E were obtained from a single host individual, while F and G were obtained from another single host individual. n, nucleus; nu, nucleolus; hn, nucleus of the host; gl, gut lumen; m, mucron.

Selenidium with SEM. (Fig. 3C), the most notable distinction between the two morphotypes under the SEM was relative cell length.

Astome ciliates

Ciliates were observed swimming freely within the gut lumen of the host (Fig. 1H). The elongated cells were 267 μ m (217–387 μ m, $n = 6$) long and 70 μ m (50–86 μ m, $n = 6$) wide, with their surface densely covered by a multitude of cilia (Fig. 4). A macronucleus of 207 μ m (150–320 μ m, $n = 4$) long and 21 μ m (20–25 μ m, $n = 4$) wide was clearly visible, but a micronucleus was not detected in the light micrographs (Fig. 4A and Supplementary Fig. 1D). Typically, between 6 and 12 contractile vacuoles were arranged in a single row (Fig. 4A); however, sometimes, a second row of only 1–3 contractile vacuoles was visible on the opposite side of the cell. No attachment apparatus, such as a hook or an apparent thigmotactic area, was observed (Fig. 4A–C, and Supplementary Fig. 2).

Molecular phylogeny

Selenidium

Both ML and Bayesian analyses were conducted on the alignment of 1,574 bp from 25 taxa. The results showed that all of the *Selenidium* sequences obtained in this study belong to the largest *Selenidium* clade that includes the type species, *S. pendulum* (Fig. 5). All the *Selenidium* sequences obtained in this study, along with the sequence reported as *Selenidium* cf. *mesnili* (GenBank ID: JN857968) from *Myxicola* sp. collected in Victoria (Canada), formed a monophyletic group with strong support (BS = 100 and PP = 1). The long morphotype *Selenidium* from *Myxicola* sp. Quadra, the *Selenidium* isolated

from *M. aesthetica* and the *Selenidium* isolated from an unverified species of *Myxicola* (reported as '*Myxicola infundibulum*' in Wakeman and Leander, 2012) exhibited high pairwise genetic similarities ranging between 97.8% and 99% (Table 1). However, the short morphotype of *Selenidium* from *Myxicola* sp. Quadra displayed less than 95% genetic divergence from the long morphotype of *Selenidium* (Table 1).

Astome ciliates

Our ML and Bayesian analyses of the alignment of 1595 bp from 53 taxa strongly supports the major subgroups within Oligohymenophorea, except for Scuticociliatia (Fig. 6). Astomes from oligochaetes (earthworms) and marine polychaetes formed a monophyletic group with strong support (BS = 94 and PP = 1). However, *Haptophrya planariarum*, a ciliate found in flatworm hosts, did not cluster with astomes from annelid hosts but instead was grouped with scuticociliatians. Based on comparisons of SSU rDNA sequences, the astome ciliate species from *M. aesthetica* was 93.7% identical to *Durchoniella* species. The astomes from *Myxicola* appears to be sister to the clade containing all other annelid-infecting astomes; however, their relationships were poorly resolved (Fig. 6).

Myxicola hosts

Both ML and Bayesian analyses of the alignment of 685 bp of 23 taxa showed similar topologies (Fig. 7). *Myxicola* sp. Quadra showed 100% identity with two COI sequences obtained from Bamfield, Canada (HM473471-2; Carr et al., 2011). All six COI sequences of *M. aesthetica*, including the Quadra specimen from the Northwest Pacific, were 100% identical to each other. It is notable that COI sequences identified as '*Myxicola infundibulum*' are divergent and not shown to be monophyletic. Instead, all of the sequences from '*M. infundibulum*' collected

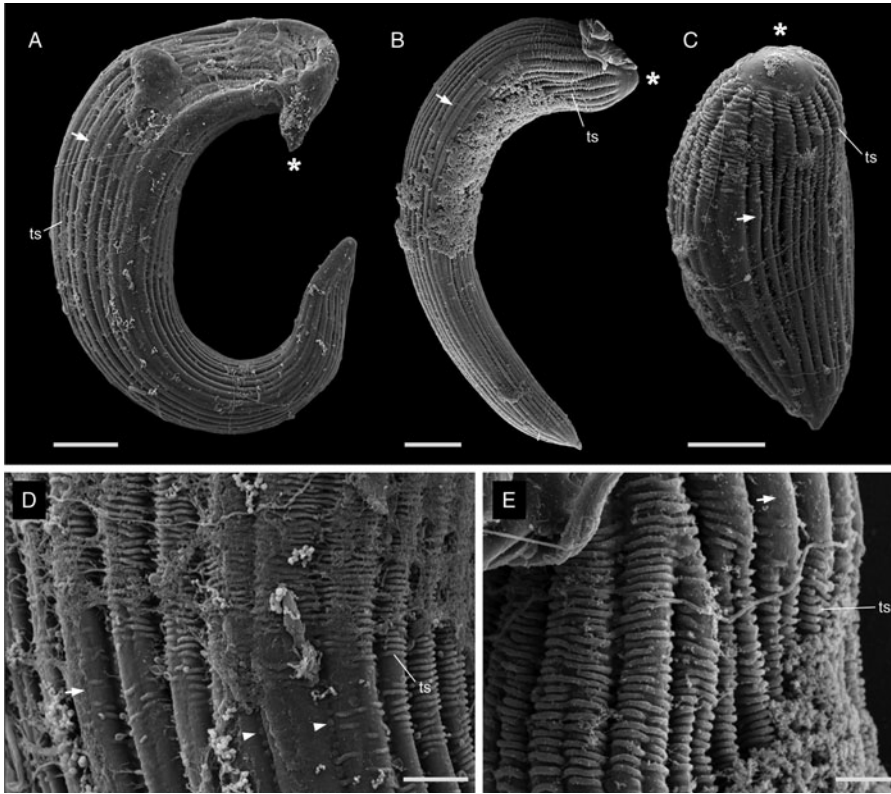


Figure 3. Scanning electron micrographs (SEM) of the trophozoites of *Selenidium elongatum* n. sp. and *Selenidium* cf. *mesnili*. (A) A trophozoite of *Selenidium elongatum* n. sp. obtained from *Myxicola aesthetica* showing a pointed mucron (*), longitudinal epicytic folds (arrow) and transverse striations (ts) (scale bar = 10 μm). (B) A trophozoite of *Selenidium elongatum* n. sp. from *Myxicola* sp. Quadra showing a rounded mucron (*), longitudinal epicytic folds (arrow) and transverse striations (ts) (scale bar = 10 μm). (C) A trophozoite of *Selenidium* cf. *mesnili* from *Myxicola* sp. Quadra showing the mucron (*), longitudinal epicytic folds (arrow) and transverse striations (ts) (scale bar = 5 μm). (D) High magnification SEM of a trophozoite of *Selenidium elongatum* n. sp. from *M. aesthetica* showing longitudinal epicytic folds (arrow), transverse striations (ts) and micropores within the epicytic grooves (arrowhead) (scale bar = 3 μm). (E) High magnification SEM of *Selenidium elongatum* n. sp. from *Myxicola* sp. Quadra showing transverse striations (ts) at the anterior end of the trophozoites. (scale bar = 2 μm).

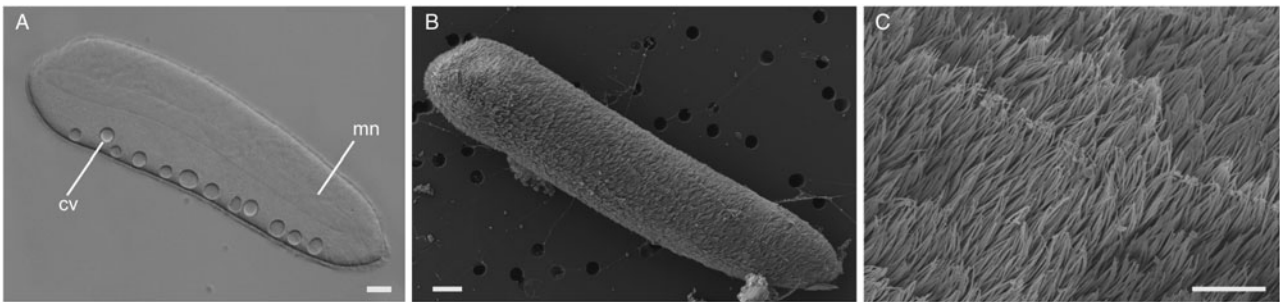


Figure 4. Light and scanning electron micrographs (SEM) of the astome ciliate *Pennarella eleganta* n. gen. et sp. (A) A differential interference contrast (DIC) light micrograph showing a long, central macronucleus (mn) and a single row of contractile vacuoles (cv) (scale bar = 20 μm). (B) Low magnification SEM showing a single astome cell (scale bar = 20 μm). (C) High magnification SEM of the same cell showing the uniform array of cilia (scale bar = 4 μm).

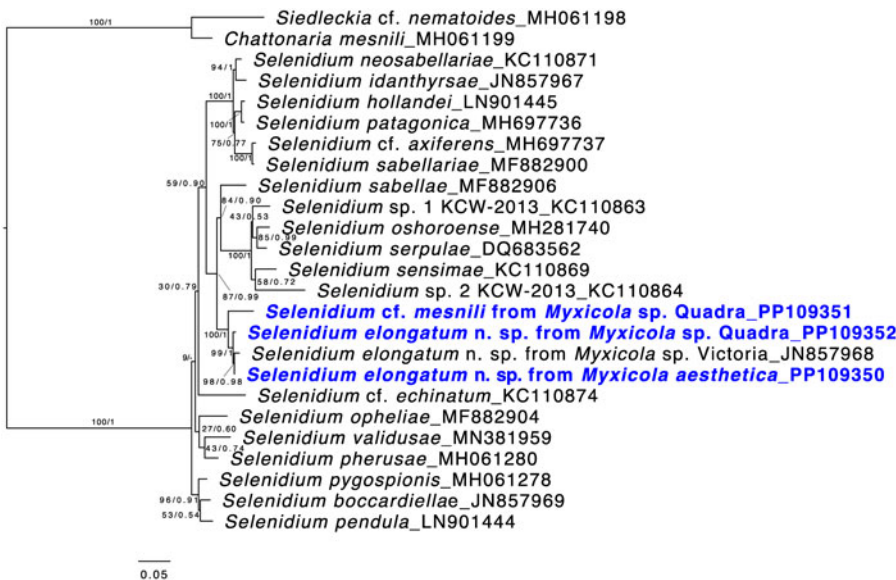


Figure 5. Maximum likelihood (ML) tree of the main lineage of *Selenidium* that includes the type species *S. pendula*. This tree was inferred from 25 small subunit (SSU) rDNA sequences and 1,574 unambiguously aligned sites using RAXML. Posterior probabilities (PP) and ML bootstrap supports (BS) are shown. Sequences from *Selenidium* obtained in this study are highlighted in bold blue font. All *Selenidium* species from *Myxicola* hosts form a monophyletic group with maximal support. Additionally, the clade containing all SSU sequences of *Selenidium elongatum* n. sp. (the long morphotype) is strongly supported, while the SSU sequence from *Selenidium* cf. *mesnili* (the short morphotype) is distinct from the others.

Table 1. Pairwise genetic distance of the small subunit rDNA sequences obtained from the *Selenidium* species inhabiting *Myxicola* host species

	<i>S. cf. mesnili</i> from <i>Myxicola</i> sp. Quadra	<i>S. elongatum</i> from <i>Myxicola</i> sp. Quadra	<i>S. elongatum</i> from <i>M. aesthetica</i>	<i>S. elongatum</i> from <i>Myxicola</i> sp. Victoria (JN857968) ^a
<i>S. cf. mesnili</i> from <i>Myxicola</i> sp. Quadra		94.9	94.8	93.6
<i>S. elongatum</i> from <i>Myxicola</i> sp. Quadra	94.9		99	97.8
<i>S. elongatum</i> from <i>M. aesthetica</i>	94.8	99		98.6
<i>S. elongatum</i> from <i>Myxicola</i> sp. Victoria (JN857968)	93.6	97.8	94.9	

^aReported as *S. cf. mesnili* in Wakeman and Leander (2012).

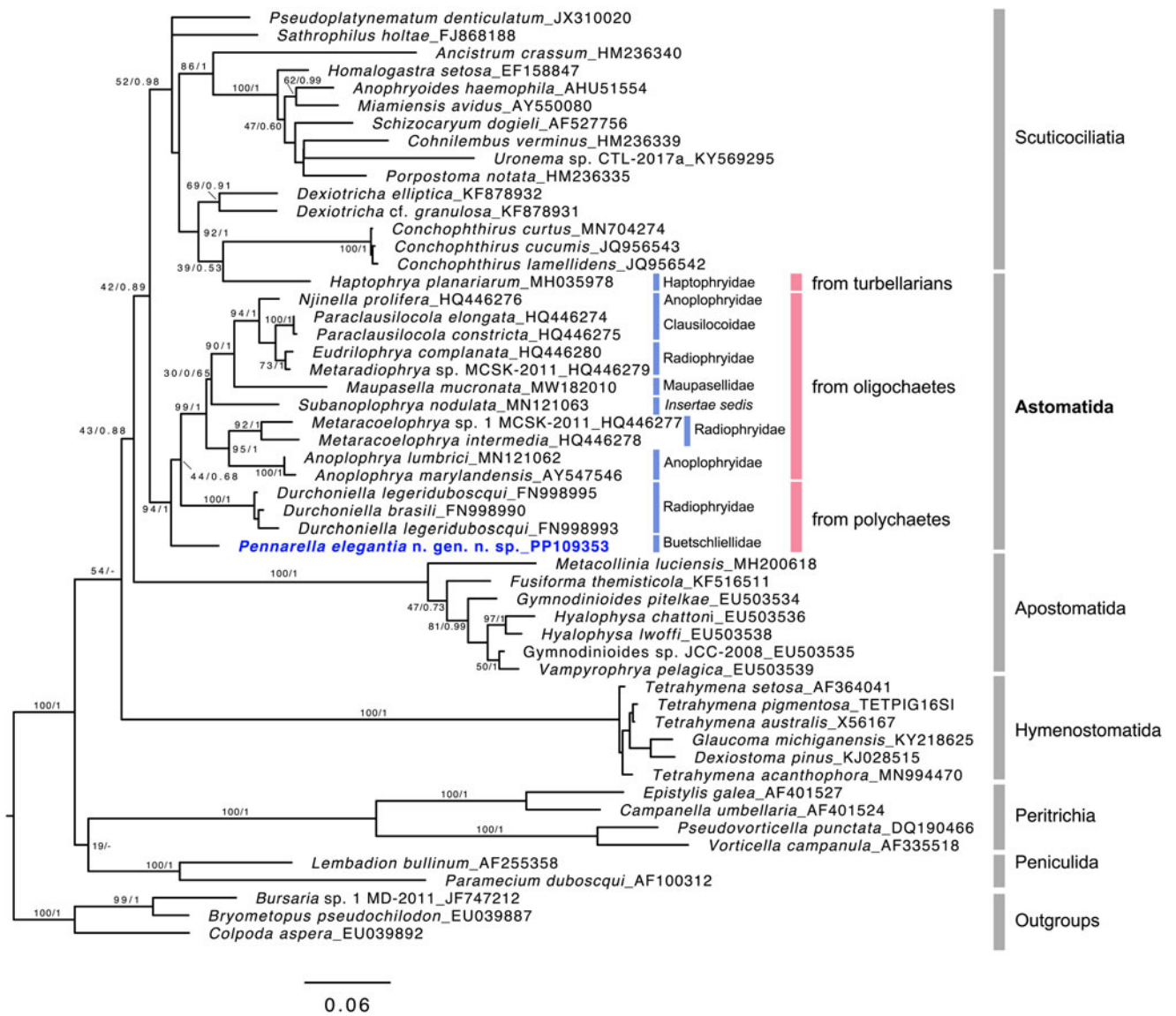


Figure 6. Maximum likelihood (ML) tree of oligohymenophorean ciliates showing the phylogenetic position of the new astome *Pennarella elegantia* n. gen. et sp. This tree was inferred from 53 small subunit (SSU) rDNA sequences and 1,595 unambiguously aligned sites using RaxML. Posterior probabilities (PP) and ML bootstrap supports (BS) are shown. The SSU rDNA sequence of the astome ciliate obtained in this study is highlighted in bold blue font. Major groups of Oligohymenophorea are marked with grey bars. Family names (blue bars) and host group information (pink bars) of astomes are shown. Although Astomatida is not a monophyletic group, the clade containing all of the annelid-infecting species is strongly supported, with polychaete-infecting species (i.e. *Pennarella elegantia* n. gen. et sp. and *Durchoniella* spp.) branching before the well supported oligochaete-infecting astome ciliates.

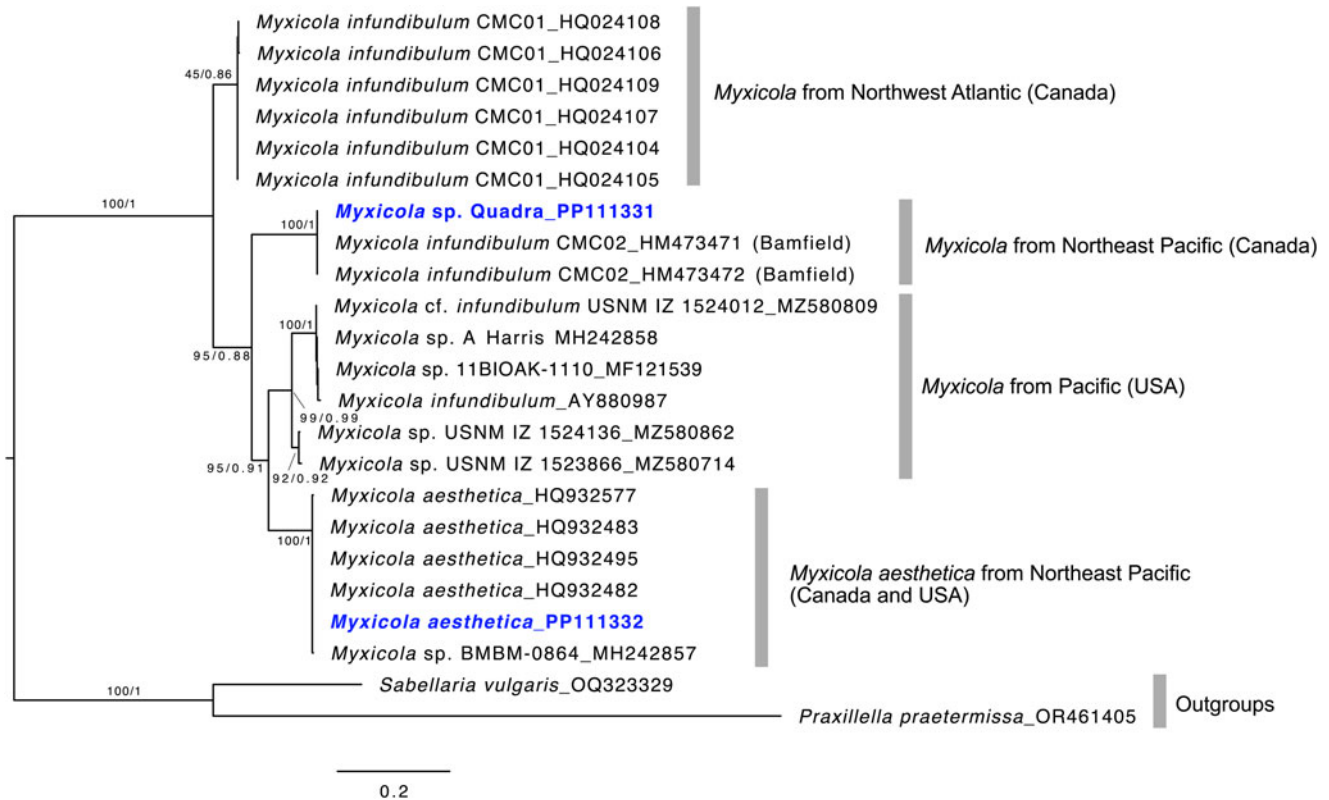


Figure 7. Maximum likelihood (ML) tree of slime feather duster worms (*Myxicola*) including all available COI sequences of the genus available in GenBank. This tree was inferred from 23 COI sequences of 685 sites using RAxML. Posterior probabilities (PP) and ML bootstrap supports (BS) are shown. Sequences obtained in this study from slime feather duster worms are highlighted in bold blue font. The COI sequence of *Myxicola aesthetica* obtained in this study is grouped with other sequences of *M. aesthetica* that were also collected from Northeast Pacific Ocean. COI sequences from specimens identified as '*M. infundibulum*' do not form a monophyletic group, suggesting the presence of cryptic species. *Myxicola sp. Quadra* forms a distinct clade along with specimens of '*M. infundibulum*' collected from Bamfield (Canada), indicating that these likely represent a new species that can be distinguished from other *M. infundibulum*-like host species.

in the Pacific Ocean grouped together with *M. aesthetica* and *Myxicola sp. Quadra*, which were also obtained from the Pacific Ocean, rather than with the '*M. infundibulum*' sequences collected from the Atlantic Ocean (Fig. 7).

Discussion

1, 2 or 3 species of *Selenidium*?

Gregarines are highly diverse at both morphological and molecular phylogenetic levels (Leander, 2008; Diakin *et al.*, 2016; Schrével *et al.*, 2016; Rueckert and Horák, 2017; Mathur *et al.*, 2019; Paskerova *et al.*, 2021). When multiple closely related species, especially congeneric species, are found in the same host species or even within one individual, distinguishing them requires careful examination, as different morphotypes might reflect different environmental conditions or developmental stages of the same species (Rueckert *et al.*, 2011).

Based on our examination of morphology, the two morphotypes of *Selenidium* trophozoites observed in *Myxicola sp. Quadra* were different in several aspects: infection location within the host, location of the nucleus within the trophozoite, trophozoite length and the presence or absence of cytoplasmic transverse lines visible under the light microscope (Table 2). Although detailed surface features of these two morphotypes examined with SEM did not reveal any noticeable differences, except for their cell lengths (Fig. 3), the pairwise distance and phylogenetic positions inferred from SSU rDNA sequences demonstrated that the two morphotypes represent different species (Table 1 and Fig. 5). The long morphotype of *Selenidium* from both *Myxicola sp. Quadra* and *M. aesthetica* were indistinguishable with SEM (Fig. 3A and 3B), which is consistent with their highly

similar (99%) SSU rDNA sequences (Table 1 and Fig. 5). Therefore, we identified two *Selenidium* species in this study: one (the long morphotype = *S. elongatum* n. sp.) that infected both *Myxicola sp. Quadra* and *M. aesthetica*, and one (the short morphotype = *S. cf. mesnili*) found only in *Myxicola sp. Quadra*.

Comparison of the 2 morphotypes of *Selenidium* with *S. mesnili*

Several authors have studied *Selenidium mesnili* Brasil, 1909, which infects *Myxicola infundibulum* originally collected in Europe (France and the UK) in the early 1900s (Brasil, 1909; Ray, 1930; Reed, 1933). Based on morphological characters, the short morphotype infecting *Myxicola sp. Quadra* highly resembles *S. mesnili* described in Europe: its location in the host, cell length, the position of the nucleus and the presence of cytoplasmic transverse lines. Trophozoites of *S. mesnili* described by Brasil (1909) measured 75–85 µm long and 20–25 µm wide and were found in the host's epithelium. He mentioned the 'zebroid' nature of the cytoplasm (= cytoplasmic transverse lines) and the presence of 'grains' near the anterior end of the living cell (see Fig. 2D and 2F). Ray (1930) also noted these characteristics, along with their limited distribution in the anterior part of the gut, and that they spend most of their life cycle within host epithelial cells. The cell length of *S. mesnili* reported by Ray (1930) was 50–90 µm, with a width of 20–30 µm. Therefore, when comparing the short and long morphotypes observed in this study with *S. mesnili* from Europe, the short morphotype is more similar to *S. mesnili* based on the aforementioned characteristics.

Although all these host specimens were identified as '*Myxicola infundibulum*', it is likely that *Myxicola infundibulum* actually

Table 2. Comparison of traits in the *Selenidium* species inhabiting *Myxicola* host species

	<i>S. mesnili</i> (Brasil, 1909)	<i>S. cf. mesnili</i> (this study)	<i>S. elongatum</i> n. sp. (this study)	<i>S. elongatum</i> n. sp. (this study)	<i>S. elongatum</i> n. sp. (Wakeman and Leander, 2012) ^a
Geographical location	France and the UK	Quadra Island, Canada	Quadra Island, Canada	Quadra Island, Canada	Victoria, Canada
Host species	<i>Myxicola</i> <i>infundibulum</i>	<i>Myxicola</i> sp.	<i>Myxicola</i> sp.	<i>M. aesthetica</i>	<i>Myxicola</i> sp.
Position within the host	Foregut, embedded in the epithelium or gut tissue	Foregut, embedded in the epithelium or gut tissue	Midgut to hindgut, attached to the gut epithelium	Midgut to hindgut, attached to the gut epithelium	NA
Trophozoite length (µm)	75–85 × 20–25	26–45 × 8–23	98–200 × 7–17	100–184 × 20–30	85–157 × 18–24
Location of the nucleus within the trophozoite	Middle	Middle	Anterior half	Anterior half	Anterior half
Cytoplasmic transverse lines visible under the light microscope	yes	yes	no	no	no
Epicytic transverse strations visible on the SEM	NA	yes	yes	yes	Yes

^aReported as *S. cf. mesnili* in Wakeman and Leander (2012).

consists of multiple cryptic species with subtle morphological differences and different geographical ranges (Putignano *et al.*, 2023). Unfortunately, molecular phylogenetic data from *Myxicola infundibulum* and *S. mesnili* collected from the type location in Europe are not available. However, considering that none of the currently recognized species of *Myxicola* have the same number of radiole pairs (one of the important taxonomic traits for the group) as *Myxicola* sp. Quadra (i.e. 21), it is likely that this host species is a new species that differs from *M. infundibulum* sensu stricto from the type location in Europe, which has 37 radiole pairs (Putignano *et al.*, 2023). Consequently, the European hosts from which the trophozoites of *S. mesnili* were collected, namely *M. infundibulum*, likely represent a different species from the host *Myxicola* sp. we collected on Quadra Island, Canada. Despite their significant morphological similarities, without molecular data of the type species, we cannot confirm that the short morphotype *Selenidium* is indeed *S. mesnili*. Therefore, we identify the short morphotype *Selenidium* in *Myxicola* sp. Quadra as *Selenidium* cf. *mesnili*.

The SSU rDNA sequences of the long morphotype of *Selenidium* from the two *Myxicola* host species obtained from Quadra Island were 97.8% and 98.6% identical to the SSU rDNA sequence from '*Selenidium* cf. *mesnili*' obtained from *Myxicola* sp. collected in Victoria, Canada (GenBank ID: JN857968), which also exhibited the long morphotype, with a length of 85–159 µm and a width of 18–24 µm (Wakeman and Leander, 2012). The clade containing these sequences was strongly supported in our phylogenetic analyses (BS = 99 and PP = 1) and was shown as sister to the short morphotype (Fig. 5). Therefore, we propose a new species of *Selenidium* for the long morphotype (obtained from *Myxicola* sp. Quadra, *M. aesthetica* and *Myxicola* sp. Victoria) that is clearly distinguished from *S. mesnili*: *Selenidium elongatum* n. sp.

Astome ciliates

Understanding the evolutionary relationships among mouthless ciliates has been a challenge due to the importance of oral structures in ciliate taxonomy (Lynn, 2008). Molecular data has proven invaluable for elucidating the phylogenetic placement and

relationships among these morphologically streamlined ciliates (Fokam *et al.*, 2011; Sauvadet *et al.*, 2017; Obert and Vďačný, 2019). In fact, recent studies with molecular data suggested that loss of mouth occurred several times independently among oligohymenophoreans (Rataj and Vďačný, 2018; Zhang and Vďačný, 2022). Although 'Astomatia' is polyphyletic, SSU rDNA sequences indicate that astomes infecting annelid hosts (polychaetes and oligochaetes) form a monophyletic group (Fig. 6), consistent with findings from other studies (Sauvadet *et al.*, 2017; Zhang and Vďačný, 2022). However, SSU rDNA sequences for astomes infecting polychaete hosts are only available for one genus, namely *Durchoniella*, thus far.

Polychaete-infecting astomes are classified into 7 genera in three families: Buetschliellidae (*Anoplophryopsis*, *Buetschliella*, *Herpinella* and *Rhizocaryum*), Intoshellinidae (*Spirobuetschliella*) and Radiophryidae (*Durchoniella* and *Hovasseiella*) (Caullery and Mesnil, 1907; Awerinzew, 1908; De Puytorac, 1954). Although astomes have been identified in several different families of polychaetes (e.g. Eunicidae, Spionidae, Ophelidae, Cirratulidae and Serpulidae), they have never been reported in hosts within the Sabellidae (e.g. *Myxicola*) (De Puytorac, 1954). The family Buetschliellidae is one of the 3 families that include species lacking attachment apparatus, and all the four genera within Buetschliellidae are from different families of polychaete hosts. Species within Buetschliellidae do not have attachment apparatus, such as a hook or a sucker, but do have a bare area without cilia near the anterior end, except for *Rhizocaryum*. Although *Rhizocaryum* is also completely covered by cilia, its size is considerably smaller (average of 60 µm long) compared to the astome ciliate we found.

Therefore, the astome ciliate observed in this study represents a new host range for the group, and the absence of an attachment apparatus or a 'thigmotactic area' at the anterior end is distinct from other known astomes found in marine annelid hosts. We establish a new genus within the family Buetschliellidae to accommodate the astome ciliate observed in this study (Figs 4 and 6). However, it should be noted that family-level classification of astomes has been chaotic, as gain or loss of organelles appear to have occurred multiple times due to adaptive radiation and host-driven diversification (Obert and Vďačný, 2021). An improved phylogeny, including taxa from diverse host groups

and environments, would be necessary to clarify the evolutionary relationships among astomes.

Coinfection, host specificity and niche partitioning

S. elongatum n. sp. (long morphotype) was found in both *Myxicola* species. However, *S. cf. mesnili* (short morphotype) and *Pennarella elegantia* n. gen. et sp. (astome ciliates) have never been observed in *M. aesthetica* and *Myxicola* sp. Quadra, respectively. This suggests that *S. cf. mesnili* (short morphotype) and the species of astome ciliates observed here are host specific. The two *Myxicola* species were collected from different types of habitats; *Myxicola* sp. Quadra is benthic, while *M. aesthetica* is typically found higher up in shallower waters (i.e. attached to suspended structures like submerged ropes). The distance between the two collection sites was ~10–20 m. Given the relatively short distance between these two sites and the fact that *S. elongatum* n. sp. (long morphotype) was shared by both host species, it seems likely that the cysts of these endosymbionts are present in both habitats. However, our understanding of their life cycles and cyst dispersal remains limited. If *S. cf. mesnili* (short morphotype) and *P. elegantia* exclusively infect *Myxicola* sp. Quadra and *M. aesthetica*, respectively, then this might be attributed to either specific host-endosymbiont cell recognition or suitable environment conditions for these particular symbionts. For instance, in both gregarines and astomes, previous studies have suggested that the localization of different endosymbiont species within the same host individual is correlated with factors such as ionic concentrations (e.g. pH) (Göhre, 1943; Nana and Zéphyrin, 2014). The fact that *P. elegantia* was not limited to certain areas of the host gut and was freely swimming in the host gut lumen could be explained by the lack of an attachment apparatus or competition for resources with other coinfecting endosymbiont species (i.e. *Selenidium*).

While coinfection by multiple gregarines in a single host is not uncommon, there is limited knowledge regarding the specific locations of these symbionts within the host's body (Coulon and Jangoux, 1986; Clopton *et al.*, 1992; Wakeman *et al.*, 2014). Three congeneric gregarine species (*Gregarina niphandroides*, *G. cuneata* and *G. polymorpha*) occupying different regions of the gut or found at different life stages was seen in *Tenebrio molitor*, yellow-mealworm beetles (Göhre, 1943; Clopton, 2009). Our study presents the first instance of sister gregarine species occupying distinct regions of the gut in the marine hosts. The mechanisms behind a symbiont preferences for certain cells, tissues and hosts remain unclear. Improved understanding of this study system might help explain interactions between hosts and symbionts, interactions among different symbionts, as well as the environmental factors influencing these interactions.

Taxonomic summary

Apicomplexa Levine, 1970

Archigregarinorida Grasse, 1953

Selenidiidae Brasil, 1907

Selenidium Giard, 1884

Selenidium elongatum n. sp. Park and Leander

Diagnosis. Trophozoite lengths vary, with some reaching considerable lengths (ranging from 50 to 180 × 15–25 µm). The nucleus is ellipsoid in shape and is positioned between the mid and the anterior end of the cell. The mucron can be either round or pointed and is free of folds. The cell surface displays 20–28 epicytic folds, and transverse striations can be visible in the anterior to middle part of the cell. These cells are capable of bending and twisting.

DNA sequence. SSU rDNA sequence has been deposited in GenBank (accession ID: PP109352)

Type locality. Hyacinthe Bay, Quadra Island, British Columbia, Canada (50°6'53"N, 125°13'29"W)

Type habitat. Marine

Type host. *Myxicola* sp. Quadra (accession ID for the host COI: PP111331)

Location in host. Mid to posterior region of the intestine

Iconotype. Figure 1K.

Zoobank Registration LSID. urn:lsid:zoobank.org:act:D5A5A5EB-87D4-43B1-AF89-212A81F4FEFB

Etymology. The trivial name 'elongatum' refers to the elongated shape of the trophozoites.

Remarks. This species was also found in the intestines of *M. aesthetica*.

Ciliophora Doflein, 1901

Oligohymenophorea de Puytorac *et al.*, 1974

Astomatia Schewiakoff, 1896

Astomatida Schewiakoff, 1896

Buetschliellidae de Puytorac in Corliss, 1979

Pennarella n. gen. Park and Leander

Diagnosis. The elongated cells measure 200–400 µm long and 50–90 µm wide, and they are completely covered by cilia without any attachment apparatus or thigmotactic area. A macronucleus is clearly visible, extending almost the entire length of the cell. These cells swim freely in the gut lumen.

Type species. *Pennarella elegantia* n. sp. Park and Leander

Etymology. 'Penn', a Latin word meaning 'feather', refers to the host family 'Sabellidae', commonly known as 'feather duster worms.'

Pennarella elegantia n. sp. Park and Leander

Diagnosis. The elongated cells measure 267 µm long (217–387 µm, *n* = 6) and 70 µm wide (50–86 µm, *n* = 6). A clearly visible macronucleus is 207 µm long (150–320 µm, *n* = 4) and 21 µm wide (20–25 µm, *n* = 4). A micronucleus may be absent. There are between 6 and 12 contractile vacuoles in a single row, although a second row can be present with a smaller number of vacuoles (1–3). The cell is entirely covered by cilia and lacks an attachment apparatus such as a hook or an apparent thigmotactic area. These cells swim freely within the host's gut lumen.

DNA sequence. SSU rDNA sequence has been deposited in GenBank (accession ID: PP109353)

Type locality. Hyacinthe Bay, Quadra Island, British Columbia, Canada (50°6'53"N, 125°13'29"W)

Type habitat. Marine

Type host. *Myxicola aesthetica* (accession ID for the host COI: PP111332)

Location in host. Gastrointestinal track of the host

Iconotype. Figure 4A

Zoobank Registration LSID. urn:lsid:zoobank.org:act:0FB773B2-BF65-4D92-94C9-550DAA0D4B69

Etymology. 'elegantia' refers to the elegant swimming movement of the ciliates.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182024000209>.

Data availability statement. All the sequences generated in this study are deposited in GenBank (Accession ID: PP109350-PP109353, PP111331-PP111332). LSID for this publication is urn:lsid:zoobank.org:pub:AC7EECEA-B5FD-42ED-AAB6-7888BA7EE125.

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Author contributions. EP and BSL conceived and designed the study. EP conducted data gathering and analyses. EP produced the figures and wrote the manuscript with input from BSL.

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Competing interests. The authors declare there are no conflicts of interest

Ethical standards. Not applicable.

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