CONVERGENT EVOLUTION OF CYTOSKELETAL TRAITS IN THE INTESTINAL PARASITES (MYZOZOA, PLATYPROTEUM) OF PEANUT WORMS

by

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Convergent evolution of cytoskeletal traits in the intestinal parasites (Myzozoan, *Platyproteum*) of peanut worms

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Abstract

The Myzozoa is a monophyletic group of primarily single-celled eukaryotes that have adopted diverse modes of nutrition, such as vampire-like predation, photoautotrophy, and parasitism. Most myzozoans fall into two major subgroups, dinoflagellates and apicomplexans, but several lineages fall outside of these groups, such as predatory colpodellids and parasitic squirmids. In this study, the 3-dimensional cytoskeletal organization of a squirmid lineage, namely *Platyproteum vivax*, was investigated with confocal laser scanning microscopy (CLSM).

*Platyproteum* inhabits the intestines of Pacific peanut worms (*Phascolosoma agassizii*) and has traits that are similar to other distantly related lineages of intestinal parasites within the Myzozoa called ‘gregarine apicomplexans’ (i.e., *Selenidium*), such as conspicuous feeding stages, called "trophozoites", capable of dynamic undulations via a system of longitudinal microtubules. More detailed characterizations of these traits will refine inferences about convergent evolution in the intestinal parasites of marine invertebrates. For instance, SEM and CLSM micrographs of *P. vivax* revealed the presence of an inconspicuous flagellar apparatus and a uniform array of longitudinal microtubules organized in bundles (LMBs). Extreme flattening of the trophozoites and a consistent orientation of the anterior end provided a reliable way to distinguish the dorsal and ventral surfaces. CLSM data also revealed a novel system of microtubules oriented in the flattened dorsoventral plane. Most of these ‘dorsoventral microtubule bundles’ (DVMBs) had a punctate distribution in dorsoventral view and were evenly spaced along a curved line spanning the longitudinal axis of the trophozoites. This configuration of microtubules is novel amongst myzozoans and is inferred to function in maintaining the flattened shape and potentially facilitate dynamic undulations via microtubule sliding. Overall, this study revealed novel traits in the trophozoites of *Platyproteum*, such as a flagellar apparatus and a system of DVMBs, that are consistent with phylogenomic data showing that this lineage of intestinal parasites is only distantly related to *Selenidium* and other marine gregarine apicomplexans. Therefore, similarities in the trophozoites of *Platyproteum* and *Selenidium*, such as relatively large trophozoites capable of dynamic undulations and uniform arrays of superficial microtubules, reflect convergent evolution within the intestines of marine invertebrates.
Lay Summary

The Myzozoa is a group of mostly single-celled eukaryotes that have diverse lifestyles, including vampire-like predators, algae, and parasites. Although most myzozoans fall into two major subgroups, namely dinoflagellates and apicomplexans, some fall outside of those groups, such as intestinal parasites called ‘squirmids’. In this study, the 3-dimensional structure of subcellular traits in a squirmid parasite, *Platyproteum vivax*, was investigated with a modern method called confocal laser scanning microscopy. This method provides a way to fluorescently stain and visualize components of the cells to evaluate similarities and differences with other organisms. These data revealed novel traits in *Platyproteum*, including an inconspicuous flagellar apparatus and a previously unknown system of subcellular structures, that are consistent with molecular data showing that this lineage of intestinal parasites evolved independently from other parasites that otherwise look similar. Therefore, similarities between *Platyproteum* and some apicomplexan parasites reflect convergent evolution within the intestines of marine invertebrates.
Preface

All chapters from this thesis are original works by Danja Currie-Olsen.

The protocol for Chapter 2 was adapted from works by Phil Angel (MSc, UBC) and Kevin Wakeman (PhD, UBC) and were redesigned for this project by Danja Currie-Olsen, Dr. Miki Fujita (UBC Bioimaging Facility) and Dr. Brian Leander. Sipunculid samples for Chapter 2 were collected by Danja Currie-Olsen. Imaging was carried out by Danja Currie-Olsen with assistance from UBC Bioimaging Facility staff members Dr. EunKyoung Lee (CLSM) and Derrick Horne (SEM). Analysis of the image data was performed by Danja Currie-Olsen and Brian Leander.

A condensed version of all Chapters will be compiled and submitted for publication.
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List of Abbreviations

ATP – Adenosine triphosphate
BSA – Bovine serum albumin
CLSM – Confocal laser scanning microscopy
DIC – Differential interference contrast
DVMBs – Dorsoventral microtubule bundles
EGTA – Ethyl glycol tetraacetic acid (Egtazic acid)
e.g. – exempli gratia
EM – Electron microscopy
HIGS – Heat inactivated goat serum
IMC – Inner membrane complex
LM – Light microscopy
LMBs – Longitudinal microtubule bundles
ml – milliliter(s)
mm – millimeter(s)
mM – millimolar
NA – Numerical aperture
nm – nanometer(s)
PBS – Phosphate-buffered saline
PBT – Phosphate-buffered Triton X-100
PFA – Paraformaldehyde
PM – Plasma membrane
rDNA – ribosomal Deoxyribonucleic acid
SEM – Scanning electron microscopy
SSU – Small subunit
TEM – Transmission electron microscopy
µL – microliter(s)
µm – micron(s)/micrometer(s)
Glossary

Alveoli – Flattened vesicular sacs producing an inner membrane complex.

Apical Complex – Complex cell invasion apparatus positioned near the anterior end of apicomplexan cells made of cytoskeletal elements modified into novel structures.

Axoneme – The core strand of a cilium or a flagellum, composed of microtubules in a cylindrical arrangement.

Centrin – Calmodulin-like Ca\(^{2+}\)-binding protein present in essentially all eukaryotic cells, associated with centrosomes and basal bodies.

Epicytic Folds – Fixed (permanent) longitudinal or transverse folds arranged on the surface of gregarine trophozoites formed by the plasma membrane.

Gametocyst – A structure in which gametes are formed.

Inner Membrane Complex – One or more flattened vesicular sacs, also named alveoli, that are visible as double-membranous structures underneath the plasma membrane.

Mucron – A bulbous attachment apparatus found on the anterior end of apicomplexan gregarines.

Pellicle – In apicomplexans, the three-layered structure comprising the plasma membrane and the underlying inner membrane complex.

Sporozoites – Spindle-shaped haploid cells residing within oocysts, equipped with an apical complex used to infect tissues of a host.

Trophozoites – Relatively large feeding cells that develop from sporozoites in apicomplexan life cycles.

Tubulin – Protein forming the building blocks of microtubules, alpha and beta tubulin subunits form heterodimers which bond with other heterodimers to make the cylindrical structure of microtubules.

Ultrastructure – Fine cell structure which can only be seen at high levels of magnification.
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Sipunculid specimens were collected on the traditional territory of the Huu-ay-aht First Nations, a Nuu-chah-nulth Nation and member of the Maa-nulth Treaty Society. Sipunculid specimens were also collected on the traditional, ancestral, and unceded territory of the T’Sou-ke and Scia’new Nations.

Data collection and analysis for this project was conducted on the traditional, ancestral and unceded territory of the xʷməθkʷəy̓əm (Musqueam) people.

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Chapter 1: Literature Review

1.1 Overview of major myzozoan lineages

The Myzozoa is a subgroup within the Alveolata, which is a diverse clade of microbial eukaryotes united by the presence of an organized arrays of vesicles (alveoli) located below the plasma membrane and distinct openings in the cell surface called ‘‘micropores’’ (Patterson, 1999). Most alveolates fall into three major subgroups: ciliates (Doflein, 1901), apicomplexans (Levine, 1970) and dinozoans (Biitschli, 1885 emend. Adl et al., 2005) (Fig. 1). Apicomplexans, chrompodellids, squirmids, and dinozoans collectively form the Myzozoa, which refers to a vampire-like mode of feeding on single-celled prey, called myzocytosis, that is found in several modern species and in their most recent common ancestor (Cavalier-Smith and Chao, 2004; Leander, 2007; Leander and Keeling, 2003). A variety of microscopic imaging and molecular phylogenetic techniques have been utilized for discovering species within the Myzozoa and for inferring patterns of ultrastructural character evolution associated with multiple independent transitions from free-living to parasitic modes of nutrition (Mathur et al., 2019, Janouskovec et al., 2015; Yokouchi et al., 2022; Moore et al., 2008; Myl’nikov, 2009). Further investigation of myzozoans will provide important insights into the unity and diversity of the group, including novel cases of convergent evolution at the ultrastructural level.
Figure 1. Synthetic tree reflecting the current phylogenetic framework of myzozoans (ciliates included as the outgroup). The tree shows that the Squirmida (Platyproteum spp., Filipodium and Digydulum) branch separately from gregarines and form the sister lineage to a clade consisting of apicomplexans and chrompodellids. The myzozoan ancestor is inferred to have been a free-living, biflagellated, myzocytosis-feeding predator. Parasitism evolved independently multiple times within the Myzozoa, the origins of parasitism of major lineages are indicated (orange). Additional gains and losses of parasitism reside within the green groups (parasitic, non-parasitic and undetermined lineages). Flagella were also lost in the most recent ancestor of apicomplexans.

There are a number of species that branch early within apicomplexans and dinozoans that shed light onto the traits present in the most recent common ancestor of myzozoans (Janouskovec et al., 2015; Kuvardina et al., 2002; Leander and Keeling, 2003; Mathur et al., 2019; Moore et al., 2008). For example, a group known as colpodellids (member of the chrompodellids), are biflagellated myzocytotic predators of other biflagellated predators (e.g.,
Bodo) and form an early branching lineage within apicomplexans (Kuvardina et al., 2002; Mathur et al., 2019) (Figs 1, 2A); colpodellids are also closely related to a group known as chromerids, which are photosynthetic coral symbionts (Moore et al., 2008; Obornik, et al., 2012). These two groups form a clade referred to as the chrompodellids that sits in between squirmids and apicomplexans in the tree of alveolates (Janouskovec et al., 2015; Mathur et al., 2019) (Fig 1.). Although myzocytosis-based feeding is present in several different lineages throughout the Myzozoa, most lineages within the group have either modified or lost myzocytosis as a feeding method. For example, it is estimated that roughly half of dinoflagellates are photosynthetic, while the rest are a mix of predators and parasites (Taylor, 1987) (Fig. 1). It is inferred that the most recent common ancestor of dinoflagellates did possess a specialized structure for myzocytosis, but this structure has since been lost or modified so significantly that it is unrecognizable (Larson, 1988; Leander and Keeling, 2003; Okamoto and Keeling, 2014; Schnepf and Winter, 1990). For instance, some predatory dinoflagellates use a tentacle-like structure known as a peduncle for myzocytosis-based feeding (Noren et al., 1999; Okamoto et al., 2012; Okamoto and Keeling, 2014).
The other major lineage of myzozoans, the Apicomplexa, are mostly comprised of obligate endoparasites with a few undetermined lineages included as well, that evolved from a free-living myzocytotic predator containing a plastid (Cavalier-Smith, 2004; Leander, 2008; Leander and Keeling, 2003; Janouskovec et al., 2015; Mathur et al., 2019). Apicomplexans contain major lineages of intracellular parasites (e.g., coccidians, haemosporidians, piroplasms), extracellular parasites (cryptosporidians), and extracellular parasites (gregarines) of animals (Diakin, 2018; Janouskovec et al., 2015). Among these lineages are important human and domestic animal pathogens, such as the *Toxoplasma gondii*, *Plasmodium*, and *Cryptosporidium*. Apicomplexans are characterized by their infective life cycle stage known as the sporozoite stage equipped with a host cell invasion apparatus known as the apical complex, consisting primarily of a tubulin-based conoid, polar rings and secretory organelles called rhoptries and micronemes (Desportes and Schrevel, 2013; Leander and Keeling, 2003; Okamoto and Keeling, 2014; Pacheco *et al.*, 2020; Portman and Slapeta, 2014). Structures that are homologous to components
of the apical complex have also been found in other myzozoan lineages, such as chrompodellids and perkinsids (Blackbourn et al., 1998; Brugerolle, 2002; Brugerolle and Mignot, 1979; Myl’nikov, 1991 & 2000; Perkins, 1976 & 1996; Simpson and Patterson, 1996). There are also lineages within the Apicomplexa that have highly modified or reduced versions of the apical complex (Aldeyarbi and Karanis, 2016; Leander, 2008; Leander and Keeling, 2003; Janouskovec et al., 2019; Rueckert and Leander, 2009; Simdyanov and Kuvardina, 2007; Wakeman et al., 2014a). Improved understanding of the earliest branching lineages within the Myzozoa will shed light on the gain, modifications and losses of the traits described above and on the transition from free-living photosynthetic ancestors to lineages of obligate parasites.

1.2 An introduction to gregarines and the taxonomic history of *Platyproteum vivax*

Gregarines form a group of myzozoans nested within the Apicomplexa, defined by the presence of a particular haploid feeding stage referred to as a trophozoite. This conspicuous extracellular stage exhibits exceptional diversity of sizes and shapes across various lineages (Desportes and Schrevel, 2013; Leander and Keeling, 2003; Leander, 2008; Mathur et al., 2019) (Figs 1, 2). Another characteristic that differentiates gregarines from other Apicomplexa is the use of syzygy, a process whereby two haploid trophozoites join together to form a gametocyst to initiate nuclear divisions (Desportes and Schrevel, 2013), for sexual association. The majority of known gregarines have lost the apical complex in the trophozoite stage (although the apical complex is retained in the infective sporozoite stage) and demonstrate a wide range of internal and external cellular adaptations to accommodate the various parasitic lifestyles (Desportes and Schrevel, 2013; Diakin, 2018; Leander, 2008) (Fig 2). While gregarine species can greatly vary in their appearance, all gregarines have trophozoites that are distinctly large, ranging from 20 to over 1,000 μm in length, are found exclusively in the extracellular spaces of invertebrate hosts (e.g., intestinal lumen, coelom and reproductive vesicles), and have a modified apical end, called the mucron that functions as an attachment apparatus (Desportes and Schrevel, 2013; Leander, 2008). Recent phylotranscriptomic analyses indicate that gregarine-like parasites are polyphyletic within the Myzozoa (Mathur et al., 2019; Janouskovec et al., 2019; Desportes and Schrevel, 2013; Diakin, 2018; Diakin et al., 2016) (Fig. 1).
Gregarines have traditionally been separated into three different groups, based on their morphology and life cycle stages: neogregarines, eugregarines, and archigregarines (Desportes and Schrevel, 2013; Diakin, 2018; Leander, 2008) (Fig. 2B-F). Neogregarines are generally found in insects, infecting tissues other than the intestines (e.g., Malpighian tubules, fat deposits, hemolymph) (Diakin, 2018; Leander, 2008). The trophozoites of neogregarines tend to be reduced (generally no larger than 50 μm in length) and are often found in syzygy when isolated (Desportes and Schrevel, 2013; Plischuck and Lange, 2009; Yaman et al., 2012).

Eugregarines are found in the intestinal or coelom of marine, freshwater and terrestrial invertebrates, and exhibit great diversity in morphology (Leander, 2008) (Fig 2 D-F). Intestinal eugregarine trophozoites are relatively large, with single trophozoites lengths over 1,000 μm in length, and tend to have hundreds of densely packed longitudinal epicytic folds presumed to function in optimizing surface mediated nutrition in the intestine (e.g. Lecudina tuzetae & Trichotokara nothriae, Fig. 2D-E) (Leander, 2008). Archigregarines — also referred to as Selenidium (Giard, 1884), the only known genus in this lineage — are found exclusively in marine invertebrates and have trophozoites that have retained several ancestral traits, such as sporozoite-like morphology and a myzocytosis-based mode of feeding (Leander, 2008; Leander and Keeling, 2003; Wakeman and Horiguchi, 2018). Whereas the trophozoites of eugregarines have lost the apical complex and myzocytosis-based feeding, and instead rely on surface mediated nutrition absorption (Diakin, 2018; Leander, 2008). The trophozoites of Selenidium tend to have transverse striations on the cell surface and fewer than 50 longitudinal epicytic folds. The number of longitudinal folds has increased steadily over the course of gregarine evolution as a method of increasing surface area for surface-meditated nutrition absorption in the intestine (Diakin, 2018; Leander, 2008) (Figs 2B, C). However, many coelomic eugregarines have completely replaced longitudinal epicytic folds with convoluted cell surfaces of ridges and pits and utilize peristaltic movement to pulsate freely within the coelomic fluid (e.g., Pterospora schizosoma, Fig. 2F; Leander, 2008). Below the epicytic longitudinal folds in Selenidium, there is a dense layer of longitudinally arranged microtubules subtending the plasma membrane that generate dynamic, twisting, and undulating movements (Leander, 2006, 2007 & 2008; Rueckart and Leander, 2009, Wakeman et al., 2014a). Eugregarines do not have a layer of microtubule
bundles below the plasma membrane and have replaced undulating movements with gliding motility (Leander, 2008).

*Platyproteum vivax* is a unicellular parasite that inhabits the intestinal tract of the Pacific peanut worm *Phascolosoma agassizii* (Keferstein, 1967) and was originally described *Selenidium vivax* (Gunderson and Small 1986). When *P. vivax* was first described, there were no light micrographs or molecular phylogenetic data included (Gunderson and Small, 1986). *Platyproteum vivax* was previously considered to be a member of *Selenidium* because the trophozoite was found in the intestinal space of a marine worm, could grow to large lengths (up to 550 μm) and displayed highly dynamic twisting movements (Gunderson and Small, 1986; Leander, 2006). It was noted that *P. vivax* also had relatively few transient longitudinal surface folds that resembled the permanent longitudinal epicytic folds of *Selenidium* species (Gunderson and Small, 1986; Leander, 2006).

The first molecular phylogenetic analyses of *P. vivax* using SSU rDNA sequences showed that this species branched in an unresolved position near the origin of the Myzozoa (Leander *et al.*, 2003). This intriguing phylogenetic position motivated a relatively comprehensive ultrastructural study of *P. vivax* using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and videography of cell movement patterns (Leander, 2006). TEM data revealed that the *P. vivax* possessed a dense layer of longitudinal microtubules, similar to *Selenidium* (Leander, 2006 & 2008). However, it was also noted that the trophozoites of *P. vivax* displayed extreme flattening and folding movements, distinct from the twisting and undulating motion of *Selenidium* (Leander, 2006). Additional SSU rDNA sequences were then obtained from several species of *Selenidium* and another unusual intestinal parasite of sipunculids, namely *Filipodium phascolosomae*. Molecular phylogenetic analyses of these data showed that a distinct clade consisting of *P. vivax* (ex. *Selenidium vivax*) and *F. phascolosoma*, did not branch closely with any of the *Selenidium* lineages in the analysis (Rueckert and Leander, 2009). This molecular phylogenetic context combined with the distinct ultrastructural traits of *P. vivax* justified the removal of *P. vivax* from *Selenidium* and the establishment of a new genus, *Platyproteum* (Rueckert and Leander, 2009). The clade consisting of *Platyproteum* and the co-infecting species *F. phascolosoma* were then reassigned to a higher-level taxon, Order
Squirmida, by an extremely active protistan taxonomist at the time (Cavalier-Smith, 2014). Molecular phylogenetic analyses of multiple gene sequences from transcriptomic data confirmed that squirmids (i.e., *Platyproteum* and *Filipodium*) branch separately from gregarines and form the sister lineage to a clade consisting of apicomplexans and chrompodellids (Mathur et al., 2019) (Fig. 1). Therefore, gregarine-like traits in members of the Squirmida, like *Platyproteum*, unexpectedly reflect convergent evolution and further investigation into the details of their ultrastructural organization is expected to reveal novel differences associated with these independent evolutionary histories.

1.3 Ultrastructure of myzozoans

1.3.1 Overview

Myzozoan species are enveloped by alveolar vesicles that sit just below the plasma membrane. In apicomplexans, this system of alveoli is referred to as the inner membrane complex (Frenal et al., 2017). Many dinoflagellates, especially photosynthetic species, have developed hardened cellulosic material within the alveoli called thecal plates, that form a protective layer to encase the cell (Blackman and Bannister, 2001; Janouskovec et al., 2017). Another major trait present in many myzozoans is a pair of flagella, which functions in swimming (Alberts et al., 2014). Free-living dinoflagellates and colpodellids have flagella which are equal to or longer than their cell length and allow for propulsion through liquid media (Brugerolle, 2002; Moestrup, 2000; Myl’nikov, 2009; Okamoto and Keeling, 2014; Okamoto and Keeling, 2014;). Flagella are also present in some parasitic dinoflagellates and coral-associating chromerids (Moore et al. 2008; Portman et al., 2014; Fussy et al., 2017; Obornik et al., 2012). However, it has been established that apicomplexan parasites do not possess flagella in their life history stages, apart from some highly modified versions in some microgametes (Adl et al., 2019; Cavalier-Smith, 2004; Moore et al., 2008; Simpson and Patterson, 1996; Nichols and Chiappino, 1987). Several different traits have evolved independently in different lineages of myzozoans, so improved knowledge of early diverging species like *P. vivax*, will provide important context for understanding character evolution throughout the group.
1.3.2 Cytoskeletal components and mitochondria

The eukaryotic cytoskeleton is made up of two main structural components: microtubules, which are composed of cylindrical assortments of alpha and beta tubulin heterodimers, and filamentous actin, composed of elongated double helix-form polymers of globular actin coupled together with additional motor proteins (mainly myosin) just below the plasma membrane (Alberts et al., 2014; Matis, 2020; Ghoshdastider et al., 2015; Huber et al., 2013; Luders and Stearns, 2007; Preisner et al., 2018; Warner and Mitchell, 1980; Yubuki et al., 2016). Cell shape, stiffness and motion are mediated by the displacement of actin and microtubule filaments coupled to otherwise stationary structures; displacement of these filaments is achieved with mechanochemical coupling ATPase proteins myosin and dynein (specific to actin and tubulin, respectively) (Warner and Mitchell, 1980; Soldati-Favre, 2008).

Components of the cytoskeleton form some of the most important structures in myzozoans, such as the apical complex in apicomplexans, and variations in their organization reflect phylogenetic relationships. For instance, intracellular apicomplexan parasites, like *Toxoplasma gondii* and *Plasmodium*, have a specific number of subpellicular microtubules that organize the cell shape and radiate down from the apical complex, tapering off just before the posterior end (Francia et al., 2015; Morissette et al., 1997). This arrangement allows for alterations of the cell shape for host cell penetration. Residing between the plasma membrane and the inner membrane complex, is a system composed of myosin, associated myosin-gliding proteins and multiple-membrane spanning proteins, collectively known as the glideosome system (Bullen et al., 2009; Frenal et al., 2017; Heintzelman, 2015). The myosin-gliding proteins and multiple-membrane spanning proteins are inferred to play a role in anchoring the subpellicular microtubules to myosin to generate traction resulting in manipulation of the subpellicular microtubules, producing the dynamics involved in cell invasion (Bullen et al., 2009; Frenal et al., 2017; Heintzelman, 2015). Species of eugregarines, which do not have dense layers of cortical microtubules, rely more heavily on the actin-myosin system of the cytoskeleton to generate gliding movements, while *Selenidium* and *Platyproteum* rely more heavily on the dense layer of cortical microtubules for movement (Heintzelman, 2004; Leander, 2006, 2008; Yokouchi et al., 2022). Examination of these components with confocal laser scanning microscopy (CLSM) will allow a three-dimensional reconstruction of cytoskeletal mechanisms.
to better determine how they are involved in motility. These data will also allow for comparisons of the cytoskeletal systems in *Platyproteum*, gregarine apicomplexans, and other lineages of myzozoans to reconstruct patterns of convergent evolution that reflect specific parasitic lifestyles (e.g., intestinal parasitism).

Mitochondria are membrane-bound organelles that carry out oxidative phosphorylation and produce the majority of the ATP within a cell (Alberts *et al.*, 2014). Mitochondria are not technically considered to be a cytoskeletal component, however the ATP produced by mitochondria are vital for functionality of microtubule-bound ATPases such as dynein and myosin. Variation in the position, structure, and abundance of mitochondria within different kinds of cells reflect different and potentially novel processes that occur within those cells.

### 1.3.3 The flagellar apparatus = the microtubule organizing center

The microtubular cytoskeleton of eukaryotic cells is organized by a system of components usually consisting of two basal bodies, interconnected microtubular roots and a surrounding matrix of associated proteins (e.g., gamma tubulin and centrin), collectively called the ‘flagellar apparatus’ or the ‘microtubule organizing center’ (MTOC) (Yubuki and Leander, 2013; Moestrup, 2000; Luders and Stearns, 2007). Although the fundamental components of MTOCs are conserved, variations in these structures are phylogenetically informative at the deepest levels in the tree of eukaryotes (Yubuki and Leander, 2013; Yubuki *et al.*, 2016; Moestrup, 2000). Basal bodies (and the homologous centrioles in animals) are built around nine sets of short length microtubule bundles arranged in an open-ended cylinder (Alberts *et al.*, 2014). In free-living single-celled eukaryotes, basal bodies are usually arranged in pairs, positioned near the cell surface, and anchor the ‘9 + 2’ arrangement of microtubules within the axoneme of a flagellum (syn., cilium) (Kilburn and Winey, 2008; Beisson and Wright, 2003; Marshall, 2008). In animal cells, centrioles are arranged in orthogonal pairs within a matrix of proteins positioned near the nucleus, collectively called the centrosome, that functions to organize the microtubules of the mitotic spindle (Alberts *et al.*, 2014). Although not technically relevant to myzozoans, the term centriole is occasionally used to describe the role of basal bodies when apicomplexans, which lack flagella, are in the invasive life cycle stage (Okamoto and
Keeling, 2014). Basal bodies are a crucial structure of the cytoskeleton as they mediate the organization and coordination of the cytoskeletal elements utilized for motility and mitosis. Investigation of these structures in early-diverging myzozoans will undoubtedly reveal important insights regarding the evolution of cytoskeletal organization.

A calcium binding protein known as centrin is essential for the structure and function of the MTOC (Beisson and Wright, 2003). Although centrin was originally described as a protein that was associated with the basal bodies of the unicellular alga, *Chlamydomonas reinhardtii* (Salisbury et al., 1984), it has since been associated with function of MTOCs across the tree of eukaryotes (Klotz et al., 1997; Ruiz et al., 2005; Salisbury, 1995). Studies of single-celled eukaryotes, (e.g., *Paramecium, Chlamydomonas*, and yeast) have used centrin localization as a way to study the basal bodies (Beisson et al., 2001; Salisbury, 1995; etc.). However, only a few studies of species within the Myzozoa have investigated the involvement and localization of centrin in the MTOC, and these are limited to dinoflagellates. In these species, centrin is associated with ‘collars’ located at the proximal end of the basal bodies and with fibers that connect the basal bodies together to form the MTOC (Hohfield et al., 1994; Roberts and Roberts, 1991; Melkonian et al., 1991). Visualization of centrin localization in other lineages of myzozoans will provide context for understanding how centrin-based structures have evolved over time.

### 1.4 Project justification and specific goals

The trophozoites of *P. vivax* are similar to the trophozoites of *Selenidium*, so much so that this species was originally classified within *Selenidium*. However, molecular phylogenetic analysis has demonstrated that *P. vivax* represents an early-diverging lineage of myzozoans that forms the sister lineage to a clade consisting of chrompodellids and apicomplexans. The goal of this study is to characterize the cytoskeletal organization of squirmids, specifically *P. vivax*, using CLSM, differential interference contrast (DIC) and SEM in order to identify novel traits and fundamental differences among gregarine apicomplexans. These traits are expected to reflect the independent origins (i.e., convergent evolution) of traits associated with intestinal parasitism in molecular phylogenetic trees of myzozoan diversity. This study is expected shed light onto the early evolutionary history of myzozoans.
Project outline:

1. Collection and dissection of Pacific peanut worms from the west coast of BC.

2. High resolution light microscopy to investigate the morphology and behaviour of living trophozoites isolated from host intestines.

3. Scanning electron microscopy of trophozoites to obtain surface details.

4. Immunohistochemistry and confocal laser scanning microscopy to investigate the organization of microtubules, centrin and F-actin in the trophozoites.

5. Fluorescence and confocal laser scanning microscopy to investigate the distribution of mitochondria in living cells.

6. Synthesis of results within the context of existing data from transmission electron microscopy.
Chapter 2: Novel cytoskeletal traits in the intestinal parasites of peanut worms (Myzozoa, *Platyproteum vivax*)

2.1 Synopsis

*Platyproteum* is an early-diverging lineage of parasitic myzozoans with trophozoites (i.e., feeding stages) that inhabit the intestines of peanut worms (Annelida, Sipunculida) and that resemble those in marine gregarine apicomplexans, particularly *Selenidium*, in habitat, morphology and behavior. A detailed analysis of the ultrastructure of *Platyproteum* using CLSM was expected to reveal novel cytoskeletal traits that would help demonstrate convergent evolution between different lineages of intestinal parasites within the Myzozoa and beyond. In this study, I isolated *P. vivax* from Pacific peanut worms (*Phascolosoma agassizii*) collected from Grappler Inlet (Bamfield) and Whiffin Spit (Sooke). I used DIC, SEM, CLSM and immunofluorescence labeling to examine the 3-dimesnionsal organization of microtubules, basal bodies and mitochondria in the trophozoites of *P. vivax*. These data demonstrated the presence of a flagellar apparatus and a novel system of dorsoventral microtubule bundles. Analysis of the data led to the establishment of new anatomical terminology for *Platyproteum*, such as a distinction between dorsal and ventral sides of a highly flattened cell. The novel traits in *P. vivax* are consistent with recent phylogenomic data showing *P. vivax* as only a distant relative of *Selenidium* within the Myzozoa (Marthur et al., 2019) and some recent ultrastructural data from *P. noduliferae* (Yokouchi et al., 2022). The data also confirm the presence of transient transverse surface folds, a dense layer of longitudinal microtubule bundles just below the inner-membrane complex, and a superficial layer of mitochondria, which are all traits shared with *Selenidium* (Leander, 2006). Therefore, the morphological and behavioural similarities between the trophozoites of *Platyproteum* and *Selenidium* reflect convergence of evolution in myzozoan trophozoites that inhabit the intestines of marine invertebrates.
2.2 Materials and Methods

2.2.1 Collection of hosts and isolation of *Platyproteum vivax*

Individuals of the sipunculid *Phascolosoma agassizii* (Keferstein, 1967) were collected at low tide (0.8–1.0 m above Canadian Chart Datum) from the rocky intertidal zone of Grappler Inlet near the Bamfield Marine Sciences Centre in Bamfield, BC (48.838239, -125.133946) in April of 2022, and from the Whiffin Spit intertidal zone in Sooke, BC (48.356404, -123.726090), in August and October of 2022 (DFO Licence Numbers: XR 224 2021 & XR 195 2022). Hosts were transported to the University of British Columbia (UBC) in containers with seawater aerated with portable air pumps (Zyyini cat# B07T6ZMFLG) in a cooler. In the laboratory, *P. agassizii* were stored in a recirculating sea table kept at 10 °C and a salinity of 34 ppt. The host worms were dissected using fine tip scissors to cut through the thick epidermis, along with pins and forceps to help isolate their intestinal tract. Depression microscope slides filled with cold filtered seawater and fine tip forceps were used to dissect the intestinal tract to release the gut contents onto the slide. Depression slides were then placed on an inverted microscope to locate trophozoites. Trophozoites conforming to the description of *P. vivax* were isolated from the gut contents using glass pipette micromanipulation and were then washed twice in filtered seawater. The isolated trophozoites were then prepared for DIC, LM, SEM, and CLSM.

2.2.2 Light Microscopy

Differential Interference Contrast images were taken using a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera. Live trophozoites were isolated and placed on a microscope slide with a drop of cold filtered seawater. A 1.5 mm thick, 20 x 20 mm coverslip with a Vaseline coating around the edges was gently placed on top of the specimens. Images of the trophozoites were then taken using the DIC setting on the Zeiss Axioplan 2 microscope.

2.2.3 Scanning Electron Microscopy

Using a glass micropipette, isolated trophozoites were placed into the threaded hole of a Swinnex filter holder, containing a 10 µm polycarbonate membrane filter (Milipore Sigma, cat #:
TCTP04700), submerged in seawater within a cylindrical film canister (5 cm tall, 3 cm diameter). The base of a 50 mL glass beaker was covered with Whatman filter paper and saturated with 4% Osmium tetroxide (OsO₄). The trophozoites were fixed with OsO₄ vapors for 10 min by placing the beaker over the canister. Following vapor fixation, six drops of 4% OsO₄ were added directly to the seawater in the Swinnex filter holder and the trophozoites were fixed for an additional 10 min. A 10 mL syringe filled with distilled water was screwed to the Swinnex filter holder while in the canister. The entire apparatus (filter holder + syringe) was then removed from the film canister containing seawater and fixative and the trophozoites were washed with distilled water two times to remove host debris and salt granules from seawater, using a syringe and plunger which screwed to the filter holder. Following distilled water wash, the trophozoites were dehydrated with a graded series of ethyl alcohol beginning at 30% ethanol, with 10% incremental increases. To change the solution in the syringe (for washes/dehydration), the syringe was first unscrewed from the filter holder, and the piston was removed, the syringe was then reattached (screwed) to the filter holder, the next solution was added, and then the piston was used to push the solution through the filter holder. This method was crucial to ensure that the trophozoites were not sucked up into the syringe in between washes. Additional sample preparation was done in the bioimaging facility, where specimens were critical point dried with carbon dioxide using the Tousimis Autosamdri 815B Critical Point Dryer. Filters were then mounted on stubs using carbon double sided tape, and sputter coated with gold palladium using the Cressington 208HR Resolution Sputter Coater and visualized using the Hitachi S2600 Scanning Electron Microscope.

2.2.4 Immunofluorescence

2.2.4.1 Tubulin staining

The tubulin staining protocol used is derived from Angel et al. (2021). Isolated trophozoites were placed in the chambers of 8-well removable silicon chamber slides (ibidi, cat# 80841) coated with 0.1% poly-l-lysine (Ted Pella inc. cat# 18026) and contained 4% paraformaldehyde (PFA) and filtered seawater. The trophozoites were fixed in PFA in a fume hood for 20 minutes. After fixation, each chamber was washed three times with 10% phosphate buffered solution (PBS) for ten minutes each at room temperature (hereafter, washed for 30
After the final PBS wash, the specimens were incubated in a blocking solution containing 10 % PBS Triton™ X-100 0.2% (Millipore Sigma cat # T9284, hereafter, PBT) and 5% bovine serum albumin (BSA, Sigma-Aldrich, cat # A9418, hereafter, BSA) at room temperature for 30 minutes. The specimens were then treated with mouse anti-acetylated alpha tubulin (Sigma-Aldrich, cat# T6793) at a concentration of 1:100 in blocking solution (same as described above) overnight in a 4 °C chamber. After incubation, the primary antibodies were removed with three 10-minute washes of 10% PBT at room temperature. The cells were then treated with a fluorescently tagged secondary antibody mix consisting of donkey-antimouse Alexa fluor 647 (Invitrogen, cat# A31571) at a concentration of 1:100 at room temperature for three hours. Secondary antibodies were removed with three washes of 10 % PBT at room temperature.

2.2.4.2 Centrin staining

The centrin staining protocol used is derived from the protocol used in Wakeman et al. (2014a). Although the protocol was very similar to the tubulin staining, the key differences are highlighted here. For centrin labeling, specimens were fixed in 4% PFA in 10 % PBS containing 50 mM of egtazic acid (EGTA) and washed for 30 minutes with 10 % PBT containing 50 mM of EGTA (hereafter, PBT-EGTA). This protocol utilized a more aggressive permeabilization step. A permeabilization buffer containing 15 % PBT, 63 mM EGTA and 5 % BSA, was prepared and trophozoites were permeabilized for 1 hour after the PFA washes were completed. Earlier trials of the protocol tested various concentrations of EGTA and PBT (ranging from 30-100 mM EGTA and 10-30 % PBT), the concentrations described here provided the highest quality of images. Following permeabilization, specimens were incubated with the primary antibodies rabbit anti-centrin (Sigma-Aldrich, cat# ABE480) at a concentration of 1:100 in a blocking solution containing 20% PBT, 50 mM of EGTA and 5 % BSA overnight at 4 °C. After incubation, the primary antibodies were removed with multiple washes of PBT-EGTA at room temperature for 30 minutes. The cells were then incubated in the dark with a fluorescently tagged secondary antibody mix consisting of goat anti-rabbit Alexa Fluor 488 (Invitrogen, cat# A11070) at a concentration of 1:100 at room temperature for three hours. Stained specimens were washed with three exchanges of 10 % PBT-EGTA at room temperatures.
2.2.4.3 Slide Mounting

Following the secondary antibody incubation, the silicon chambers were removed by peeling them off using fine forceps. Cityfluor mounting media (Electron Microscopy Science, cat# 17970-25) was then placed on both the slide and on a 1.5 mm thick 20 x 20 mm coverslip. The slide containing the specimens was carefully covered with the coverslip using fine forceps. Excess moisture was removed using strips of Whatman filter paper which were placed at the edge of the coverslip to collect moisture. The coverslip was then sealed onto the slide using nail polish. The slides were kept in a dark, dry space to avoid photobleaching until imaging.

2.2.4.4 Controls

For each centrin and tubulin staining trial, a control slide containing specimens only fixed in PFA (PFA with EGTA for centrin protocol) and a negative control slide containing fixed specimens only treated with the fluorescently tagged secondary antibodies (to ensure that non-specific binding was not occurring) were visualized using the same settings on the FV1000 confocal scope. The control and negative control slides were also verified for autofluorescence using the Olympus BX53 light/fluorescence microscope at the UBC Bioimaging Facility. Fluorescence was verified under filter cubes for: DAPI, Hoechst (Ex: BP375/28, 415DC, Em: LP435); FITC, eGFP (Ex: BP470/40, 495DC, Em: BP525/50); eCFP, Alexa430 (Ex: BP436/20, 455DC, Em: BP480/40) and eYFP (Ex: BP500/20, 515DC, Em: BP535/30). No significant patterns of autofluorescence were detected.

2.2.4.5 CLSM image capture

CLSM data for all slides (control, negative control, and treatment groups) were captured with the Olympus FV1000 CLSM scope with 20x, and 40x magnification lens (40x with water immersion, Numerical Aperture (NA): 1.10) at the UBC Bioimaging Facility. Visualization was performed with excitation lasers of wavelength 473 nm and 635 nm to excite Alexa 488 (centrin, recommended excitation wavelength: 498/520 nm) and Alexa 647 (tubulin, recommended excitation wavelength: 648/670 nm) fluorescent molecules (tagged onto secondary antibodies), respectively. Images were taken using Olympus Fluoview software (FV10-ASW2) operated by Windows 7.
2.2.5 MitoTracker procedure

To visualize mitochondria in *P. vivax*, a protocol was derived to treat live cells with a fluorescently labeled MitoTracker dye which functions by using the mitochondrial membrane potential. Live trophozoites were placed in removable silicon chamber slides containing chilled, filtered seawater. A 1 mM stock solution of MitoTracker™ Green (Thermo Fisher, cat# M7514) was prepared. Chamber slides were then filled with MitoTracker treatment solution containing 400 nM MitoTracker™ Green (earlier trials attempted to use lower concentrations (ex: 250 nM) to match protocols recommended by the manufacturer and by previous researchers, however, 400 nM resulted in the highest quality of images), 10 % PBS and 2.5 % BSA. Cells were incubated in solution for 30 minutes in a 4 °C refrigerator. Following incubation, two ten-minute washes with chilled, filtered seawater were performed. After washing, the silicon chambers of the slide were gently removed using fine forceps and 5 µL of SlowFade™ Diamond Antifade Mountant (Thermo Fisher, cat # S36968) was placed on the slide containing the live trophozoites, and on a 1.5 mm thick 60 x 20 mm coverslip. The coverslip was gently placed on top of the remaining liquid containing the trophozoites. A small amount of excess moisture was gently removed using Whatman filter paper. The slides were kept in a small Styrofoam cooler with ice while being carefully transported to the UBC Bioimaging Facility for visualization on the Olympus FV1000 CLSM scope with a 20X magnification lens with water immersion.

Visualization was performed with an excitation laser of 473 nm to excite MitoTracker Green (recommended excitation wavelength: 490/516). Images were taken using Olympus Fluoview software (FV10-ASW2) operated by Windows 7. A control slide with live specimens which were not treated with MitoTracker was also visualized under the same settings as the treatment slide and was verified for autofluorescence under the BX53 Fluorescence Microscope; no significant patterns of autofluorescence were detected.

2.2.6 Actin/phalloidin staining procedure

Phalloidin staining was attempted on the trophozoites of *P. vivax*. Phalloidin functions by binding to and stabilizing filamentous actin, staining with phalloidin can thus reveal the F-actin cytoskeleton of a cell. Two main protocols were tested with several attempted trials and various alterations of the concentrations. Isolated trophozoites were placed in a chamber slide and fixed.
using 4 % PFA in seawater buffer for 20 minutes at room temperature. Following fixation, a 30-
minute PBS wash (with three changes) was conducted. Attempts were made with two different
phalloidin products one was Alexa Fluor 405 Phalloidin (Thermo Fisher cat # A30104) and the
other was the Alexa Fluor 647 Phalloidin (Thermo Fisher cat# A22287). After fixation, a 100
nM phalloidin solution in PBS buffer was added to the chamber slide and cells were incubated
for 30 minutes. Several different trials were conducted where the concentration of phalloidin and
the incubation times were altered (200, 250, 300, 400 and 500 nM concentrations were
attempted, and incubation times ranging from one hour to overnight were tested). A different
protocol was also tested whereby a permeabilization step was included, using the same
permeabilization buffer as described for the centrin staining above. This protocol also utilized
EGTA in the fixation and washing buffers, at the same concentrations as described above.

After phalloidin incubation, the phalloidin solution was removed and two 20-minute PBS
washes were conducted at room temperature for both protocols. Silicon chambers were then
removed, and slides were mounted with cityfluor media and sealed using nail polish. Imaging
was performed at the UBC Bioimaging Facility using the Olympus FV1000 confocal laser
scanning microscope with a 40X magnification lens with water immersion (NA: 1.10).
Visualization was performed with an excitation laser of wavelength 405 nm (phalloidin,
recommended excitation wavelength: 405/450 nm) and 635 nm (phalloidin, recommended
excitation wavelength: 650/668 nm) to excite Alexa 405 and 647, respectively. Images were
taken using Olympus Fluoview software (FV10-ASW2) operated by Windows 7. A control slide
with specimens fixed in PFA (and not treated with MitoTracker) was also visualized under the
same settings as the treatment slide and was verified for autofluorescence under the BX53
Fluorescence Microscope, no significant patterns of autofluorescence were detected.

2.2.7 Image processing and editing

Images were processed and edited using cyberduck, cellSens and Fiji, version 2.00
(Wayne Rasband, National Institute of Health). Fiji was used to take measurements of cells and
organelles. Adobe Photoshop 2023 was used to add scale bars and to compile images into
figures. Microsoft PowerPoint was used to create Figure 1 and 2, and Adobe Illustrator 2023 was
used to create Figure 9.
2.3 Results

2.3.1 General morphology

Trophozoite shape and movement was consistent with descriptions provided by Leander (2006). The trophozoites are flattened and display multiple shapes; the elongated cell is tape-like (Fig. 3A, D), the semi-contracted cell is peanut shell-shaped (Figs 1B, 2C), and the contracted cell is disk-shaped (Figs 4A-B, 8B). The trophozoites display highly dynamic peristaltic movement with the cell shape changing constantly. The size of the trophozoites observed varied considerably, with some contracted cells as small as 40 μm in the length (Fig. 4A) to fully elongated cells as long as 510 μm (Fig. 3A). The relaxed cell shape (not completely contracted or completely elongated) averaged 230.5 ± 26.9 μm (mean ± SE; n = 15) in length (Figs 3-8, S1-S2). DIC images revealed a relatively large nucleus ranging from 13 μm to 40 μm in diameter and located in the middle of the cell (n = 13, Figs 3, 5-7, 8); the nucleus was shaped like a flattened oval and often contained a conspicuous nucleolus (Figs 6, 7). SEM revealed surface patterns consisting of transient longitudinal waves and arrays of transverse (i.e., circumferential) striations arranged in clusters across the entire trophozoite (Fig. 4A-C). Transverse striations were present in higher numbers in areas of the cell where the trophozoite was more contracted (less elongated) (Fig. 4A vs 4C).

The anterior end of the trophozoites took on the form of an oblique straight-edge, termed the ‘anterior apparatus’ in this study, that is analogous to the host attachment end (i.e., mucron) in gregarines apicomplexans (Figs 3, 4D-E, 5-8). Like the rest of the trophozoite, the anterior apparatus took on a range of various shapes during continuous peristaltic-like movements. Nonetheless, the angle of the oblique straight-edge allowed for distinguishing between the two different sides of the flattened trophozoites, where the ‘dorsal side’ is visible when the anterior apparatus is oriented to the right, and the ‘ventral side’ is visible when the anterior apparatus is oriented to the left. DIC and SEM revealed the presence of two flagella protruding from the anterior apparatus (Figs 3 C, D, 4E, F). The flagella were 6-9 μm long with a mean length of 7.4 ± 0.4 μm (mean ± SE; n = 4) and with the flagella bases approximately 2 μm apart (n = 2). Flagella were constantly beating while the trophozoite was in motion.
Figure 3. Differential interference contrast (DIC) light micrographs of living trophozoites of *Platyproteum vivax* showing general morphology. (A) An elongated trophozoite viewed from the lateral edge showing the flatness of the cell, the anterior apparatus (bracket) and the flattened nucleus (N). (B) A semi- contracted trophozoite showing the anterior apparatus oriented to the right (bracket), the dorsal surface of the cell and the central position of the oval nucleus (N). (C, D) High magnification views of the anterior end of a cell showing the presence of two short flagella (double arrowheads). Scale bars: A, B = 50 μm; C, D = 10 μm.
Figure 4. Scanning electron micrographs of trophozoites in *Platyproteum vivax*. (A-C) Trophozoites represent different stages of movement showing the anterior end oriented to the left, longitudinal surface folds (triple overhead arrows), transverse surface folds (arrowheads) and two short flagella (double arrowheads). (D, E) High magnification views of the anterior apparatus showing the emergence of an anterior flagellum (AF) and a posterior flagellum (PF). Scale bars: A-C = 10 μm B, D-E = 2 μm.
2.3.2 Localization of centrin

Centrin staining revealed two distinct bodies positioned below the plasma membrane along the elongated edge of the anterior apparatus (Fig. 5). The distance between the two centrin-stained bodies was approximately 10 μm. The exact positions of the two centrin-stained bodies along the anterior apparatus varied slightly between the trophozoites observed; however, there was always one body that was closer to the anterior-most tip of the anterior apparatus. The relative positions of the centrin-stained bodies along the anterior apparatus were consistent with the positions of the two flagella along the anterior apparatus observed with LM and SEM (Figs 3C-D, 4E-F).
Figure 5. Differential interference contrast (DIC) and confocal laser scanning micrographs (CLSM) showing two different trophozoites of *Platyproteum vivax* stained for centrin (green). (A) DIC image showing the central nucleus (N) and the dorsal side of the cell as indicated by the anterior apparatus (bracket) oriented upwards and to the right. (B) Centrin staining showing two distinct bodies (arrows) positioned at the anterior end of the cell. (C) Superimposed DIC and centrin-stained CLSM images showing the position of the two distinct bodies (arrows) along the edge of the anterior apparatus and just below the plasma membrane. (D) DIC image showing the central nucleus (N) and the ventral side of the cell as indicated by the anterior apparatus (bracket) oriented upwards and to the left. (E) Centrin staining showing two distinct bodies (arrows) positioned at the anterior end of the cell. (F) Superimposed DIC and centrin-stained CLSM images showing the position of the two distinct bodies (arrows) along the edge of the anterior apparatus and just below the plasma membrane. Scale bars: 20 μm.

2.3.3 Localization of tubulin

Fluorescent visualization of alpha tubulin staining on the cell surface focal plane revealed longitudinal striations of microtubules running along the entire length of the trophozoites, termed the longitudinal microtubule bundles (LMBs) (Fig. 6). Fluorescence was consistently pronounced along the periphery of the trophozoites regardless of the focal plane (Figs 6, 7). Images taken at deeper focal planes of the trophozoites did not show longitudinal striations of microtubules other than the tubulin localized to the exterior edge of the cell (Fig. 7). Comparison of the images from the superficial focal planes and deeper focal planes confirm that the tubulin was localized in longitudinal bundles positioned just below the entire cell’s outer membrane (Figs 6, 7). Tubulin staining also revealed an unexpected, curved row of evenly spaced points, which extended along the mid-line of the trophozoites from the anterior apparatus to the posterior end (Fig. 7). This pattern was evident in all focal planes of the observed trophozoites (Fig. 7), with the exception of the very ‘top’ surface focal plane (e.g. Fig 6), demonstrating that each point represents a bundle of tubulin running through the cell from the ventral side to the dorsal side (i.e., perpendicular to the longitudinal bundles of microtubules) (Fig 7). These tubulin-based structures have therefore been termed ‘dorsal ventral microtubule bundles’ (DVMBs) (Fig. 7). Some of the DVMBs in some focal planes were elongated, indicating areas where the cell has been flattened in a way that slightly angles the DVMBs relative to the dorsoventral axis of the cell (Fig. 7). Each trophozoite had between 15 and 20 DVMBs (n = 5), and the distance between the DVMBs (found in a curved row along the midline) ranged from 5 to 30 μm with an average distance of 10.9 ± 2.1 μm (mean ± SE; n = 20, 4 random measurements...
per trophozoite) between DVMBs. The trophozoites also had some scattered DVMBs located on the opposite side of the nucleus from the curved row of DVMBs; the number of these additional scattered DVMBs ranged from 1-4 (Fig. 7 B, C, F, G).

Figure 6. Confocal laser scanning micrographs (CLSM) of a trophozoite of *Platyproteum vivax* stained for alpha tubulin (red). (A) Differential interference contrast (DIC) light micrograph showing the dorsal surface of a trophozoite, nucleus (N), nucleolus (arrow), and anterior apparatus oriented upward and to the right (bracket). (B) Tubulin staining at a superficial focal plane showing the bundles of microtubules (red striations) running longitudinally along the entire length of the cell. (C) Superimposed DIC and tubulin-stained CLSM images showing the longitudinal microtubule bundles positioned beneath the dorsal surface of the cell. Scale bar: 20 μm.
Figure 7. Confocal laser scanning micrographs (CLSM) of two different trophozoites of *Platyproteum vivax* stained for alpha tubulin (red). (A) Differential interference contrast (DIC) light micrograph showing a ventral view of a trophozoite, the nucleus (N), the nucleolus (arrow), and the anterior apparatus oriented upward and to the left. (B) Tubulin staining at a deep focal plane showing a row of ‘dorsal ventral microtubule bundles’ (DVMBs, white arrowheads) running along the midline from the anterior apparatus to the posterior end of the cell. Some DVMBs (white arrowheads) are positioned outside of the distinct row of DVMBs on the opposite side of the cell. (C) Tubulin staining at a more superficial focal plane showing the same row of DVMBs (white single arrowheads). (D) Superimposed DIC and tubulin-stained CLSM images showing the same row of DVMBs passing to the left side of the nucleus when viewing the ventral side of the trophozoite. (E) DIC light micrograph showing a dorsal view of a trophozoite, the nucleus (N), the nucleolus (arrow), and the anterior apparatus oriented upward and to the right. (F) Tubulin staining at a deep focal plane showing a row of DVMBs (white arrowheads) running along the midline from the anterior apparatus to the posterior end of the cell. Some DVMBs (white arrowheads) are positioned outside of the distinct row of DVMBs on the opposite side of the cell. (G) Tubulin staining at a more superficial focal plane showing the same row of DVMBs (white arrowheads). (H) Superimposed DIC and tubulin-stained CLSM images showing the same row of DVMBs passing to the right side of the nucleus when viewing the dorsal side of the trophozoite. Scale bars: 20 μm.
2.3.4 Localization of mitochondria

Mitotracker staining on living trophozoites showed a continuous superficial layer of fluorescence that was 3-6 μm thick, with an average thickness of 4.2 ± 0.5 μm (mean ± SE, n = 4) and localized below the plasma membrane (Fig. 8). Mitochondrial staining was absent in deeper parts of the cell and within the inner-membrane complex that envelops the cell.

![Figure 8](image)

**Figure 8.** Confocal laser scanning micrographs (CLSM) of two different live trophozoites of *Platyproteum vivax* stained for mitochondria (green). (A, C) DIC images of each cell showing the anterior apparatus (bracket) and the nucleus (N). (B, E) Mitochondria staining shows a superficial layer of fluorescence that envelops the entire cell and the absence of fluorescence in the deeper regions of the cell. (C, F) Superimposed DIC and mitochondria-stained CLSM images showing the dense layer of fluorescence sitting below the inner-membrane complex and plasma membrane. Scale bars: 20 μm.
2.3.5 Localization of actin

Phalloidin staining for F-actin did not reveal any patterns of fluorescence or localization in *Platyproteum vivax*.

2.4 Discussion

2.4.1 General morphology of the trophozoites

Previous observations of the trophozoites in *P. vivax* have reported lengths ranging from 120–550 µm (Gunderson and Small, 1986; Leander and Keeling, 2003; Leander, 2006), which is consistent with the range reported in this study. The large size, flattened shape and transverse surface striations increase the surface area of the trophozoites in *P. vivax*. These morphological traits combined with the dynamic peristaltic movements are inferred to facilitate surface-mediated nutrient uptake via endocytosis within the intestinal lumen of their host (Leander, 2006). Transmission electron micrographs (TEM) of *P. vivax* demonstrated that the transverse surface striations are folded regions of the plasma membrane above the inner membrane complex that form during the dynamic changes (i.e., contractions) in trophozoite shape (Leander, 2006). The trophozoites of some gregarines, especially species within *Selenidium*, also undergo dynamic undulating movements and produce similar transverse surface striations in contracted regions of the cell (Leander, 2006; Paskerova *et al.*, 2018; 2007; Schrevel, 1970). These transverse striations occur over broad epicytic folds that run along the longitudinal axis of the gregarine trophozoites (Leander, 2007). The number of longitudinal epicytic folds in different lineages of gregarines varies considerably (e.g., from 4 to over 200), and higher numbers of epicytic folds dramatically increase surface area for surface-mediated nutrition and gliding motility (Leander, 2007 & 2008; MacGregor and Thomas, 1965; Rueckart and Leander, 2009; Schreval, 1970; Simdyanov and Kuvardina, 2007; Yokouchi *et al.*, 2022). However, molecular phylogenetic evidence demonstrates that *Platyproteum* and *Selenidium* are only distantly related within the Myzozoa (Mathur *et al*. 2019) (Fig. 1). Therefore, the presence of dynamic undulating movements and transverse surface striations in the trophozoites of these two lineages clearly reflect the convergent evolution of traits associated with acquiring nutrients within a similar habitat: the intestines of marine annelids and sipunculids.
The pair of flagella reported here on the trophozoites of *P. vivax* was also recently reported in another *Platyproteum* species collected off the coast of Japan, *P. noduliferae* (Yokouchi *et al.*, 2022). These concordant findings explain the previous report of two unidentified ‘thread-like structures’ emerging from pores on the anterior apparatus of the trophozoites in *P. vivax* (ex., *Selenidium vivax*) (Leander, 2006). This previous report was unexpected because flagella were inferred to have been lost in the most recent ancestor of all apicomplexans, except in the male microgametes of some taxa (Adl *et al.*, 2019; Cavalier-Smith, 2004; Moore *et al.*, 2008; Nichols and Chiappino., 1987). Therefore, the presence of two flagella in *Platyproteum* corroborates the molecular phylogenetic data that shows lineage branching (near the myzozoan ancestor) as the sister group of a clade consisting of chrompodellids, and apicomplexan parasites (Mathur *et al.*, 2019) (Fig. 1).

Even though *P. vivax* can drastically change its cell shape, there is one feature that remains fairly constant: the presence of a straight edge at the anterior end of the cell that reflects the extremely flattened shape of the trophozoites. A mucron is the anterior attachment apparatus in the trophozoites of gregarine apicomplexans. Although the shape and ornamentations of the mucron varies in different lineages of gregarines, it is distinctively radially symmetrical (Mita *et al.*, 2012; Rueckart *et al.*, 2012, 2015 & 2018; Wakeman and Leander, 2012; Wakeman *et al.*, 2014a & 2014b). This fundamental difference in the anterior end of the trophozoites of *Platyproteum* spp. and gregarines reflects the independent evolutionary origins of these two lineages in molecular phylogenetic analyses (Figs 1 & 2). Therefore, the term ‘mucron’ is no longer applicable to describe the anterior end of *Platyproteum* and their relatives within the Squirmida which is why the term ‘anterior apparatus’ has been introduced in this study. The function of the two flagella and the anterior apparatus as a whole in *Platyproteum* remains unclear, but it was previously inferred to be an attachment apparatus like the mucron in gregarine apicomplexans (Leander, 2006). It is also possible, however, that the surface area created by the highly flattened trophozoites allows them to adhere to the intestinal lining of their hosts by some unknown mechanism which does not involve the anterior apparatus.

The anterior apparatus of the intestinal parasite *Filipodium*, which is a close relative of *Platyproteum* found in the same host species, also takes on the form of a straight edge (Hoshide
and Todd, 1992 & 1996; Hukui, 1939; Rueckert and Leander, 2009; Tuzet and Ormieres, 1965). *Platyproteum noduliferae*, however, was reported to have a ‘hooked’ attachment apparatus (Yokouchi *et al.*, 2022). These reports show that even though there are variations in the form of the anterior apparatus within the Squirmida, the anterior end of the trophozoites in members of this clade are distinct from the radially symmetrical and often pointed mucrons of *Selenidium* species: *S. pisinnus* (co-infects *Phascolosoma agassizii* intestine), *S. oriental* and *S. pendula* (Levine, 1971; Rueckert and Leander, 2009; Schrevel, 1970; Simdyanov and Kuvardina, 2007).

### 2.4.2 Centrin, basal bodies and the flagellar apparatus

The trophozoites of both *P. vivax* and *P. noduliferae* have similar features that reflect their parasitic lifestyles within the intestines of sipunculids, such as highly flattened cells with dense arrays of transverse surface striations that increase surface area, presumably for surface-mediated nutrition via endocytosis. This mode of nutrition is presumably enhanced by the rhythmic peristaltic movements displayed by these relatively large cells. However, the function of the two flagella in both species of *Platyproteum* is unclear (Yokouchi *et al.*, 2022). The flagella are tiny relative to the size of the trophozoites, so it is unlikely that the flagella are used to propel the cells through a liquid medium. Perhaps the flagella have a sensory function to help the trophozoites identify a suitable attachment site within the host intestine. Moreover, pores previously observed along the edge of the anterior apparatus in *P. vivax* could be involved in either the endocytosis of nutrients or the exocytosis of adhesive molecules for attachment (Leander, 2006). Nonetheless, it is clear that the presence of two flagella in the trophozoites of *Platyproteum* spp. reflects an ancestral trait within the Myzozoa and distinguishes this lineage from all known apicomplexan parasites, especially gregarines.

Two centrin-stained bodies were localized just below the plasma membrane around the anterior apparatus in the trophozoites of *P. vivax*. The position of these bodies within the anterior apparatus corresponded to the location of the two flagella in *P. vivax* and to the two basal bodies identified in the trophozoites of *P. noduliferae* (Yokouchi *et al.*, 2022); this indicates that the centrin-stained bodies in *P. vivax* are also basal bodies. The two basal bodies of the flagellar apparatus of *P. noduliferae* were each associated with microtubule roots that were connected to fibrous material known as the root connective (Yokouchi *et al.*, 2022). Previous studies of
single-celled eukaryotes, such as green algae, ciliates, dinoflagellates and retortamonads, have
found that centrin is associated with connective fibers around the basal bodies (Beisson et al.,
2001; Hohfield, 1988; Klotz et al., 1997; Levy et al., 1996; Melkonian et al., 1991; Okamoto and
Keeling, 2014; Vaughan and Gull, 2015; Weerakoon et al., 1999). It should be mentioned that
the distance between the centrin-stained bodies in P. vivax is greater than the relative distance
between both the flagella bases (of P. vivax, measured in LM and SEMs) and the basal bodies in
P. noduliferae, roughly 10 µm and 1-2 µm, respectively. This difference might be due in part to
artifacts (e.g., cell squashing resulting in basal bodies being pushed apart) introduced during the
preparation of the cells for confocal laser scanning microscopy. The difference between the
species could also reflect the much larger cell size of the trophozoites in P. vivax (e.g., over 500
µm long) than in P. noduliferae (e.g., ~100 µm long) or. Altogether, the presence, location, and
some of the contents of the MTOC (flagellar apparatus) in the P. vivax have been identified in
this study.

2.4.3 Organization of the tubulin-based cytoskeleton

The tubulin staining confirmed that loosely arranged bundles of (10–15) microtubules
subtend the plasma membrane and run longitudinally from the anterior to the posterior end of the
trophozoites in P. vivax (Leander, 2006). This dense layer of superficial microtubules is inferred
to generate the dynamic peristaltic movements observed in these trophozoites. Movement of
microtubules in a cell occurs by dynamic instability and through the forces generated by tubulin-
associated ATP-driven motor proteins, such as dynein and kinesin (Alberts et al., 2014; Guha et
al., 2021; Tanenbaum et al., 2013). Dyneins and kinesins physically crosslink bundles of
adjacent microtubules, resulting in sliding of the microtubules relative to each other (Guha et al.,
2021; Tanenbaum et al., 2013). This action was first discovered as the mechanism responsible
for generating the undulating movements of flagella (Summers and Gibbons, 1971). Studies of
Drosophila cell embryos also show that microtubule sliding drives changes in cell shape (Jolly et
al., 2010; Takeda et al., 2018). It has been inferred that the same mechanism is used to drive the
undulating movements of trophozoites in Selenidium (Leander, 2007; Schrevel, 1970) and the
peristaltic movements in Platyproteum (Leander, 2006). The transverse striations on the surface
of Selenidium and Platyproteum are indicative of the plasma membrane folding over on itself
during the sliding of microtubules. This is consistent with the observation that there is a higher density of transverse surface striations on the more rounded, contracted cells than on elongated, relaxed cells. The dense superficial layer of mitochondria positioned just below the LMBs observed in both *P. vivax* and *P. noduliferae* likely supplies ATP to the motor proteins associated with the LMBs and therefore fuels the rhythmic cellular deformations (Leander, 2006; Yokouchi *et al.*, 2022).

Tubulin staining also revealed a novel pattern of dorsoventral microtubule bundles (DVMBs) arranged in a curved row beginning at the anterior end of the cell and ending at the posterior end of the cell. This row of DVMBs was always situated to the right of the nucleus in dorsal view and to the left of the nucleus in ventral view. A few isolated DVMBs are scattered on the side of the nucleus opposite of the curved row of DVMBs. Because the DVMBs connect the LMBs on the dorsal side of the cell to the LMBs on the ventral side of the cell, they are likely involved in maintaining the flattened shape of the trophozoites during peristaltic movements. The DVMBs have the same orientation as the dorsal ventral muscles in flatworms, which are involved in body flattening and in coordinating body shape changes (Brusca *et al.*, 2022). If the DVMBs were also functioning to coordinate cell shape changes, then we would expect more of them distributed throughout the cell, and likely mitochondria scattered around them as is around the LMBs. Nonetheless, previous TEM data reported an ‘unidentified linear thread’ that runs from the dorsal side of the cell to the ventral side of the cell, which corresponds exactly to the orientation of DVMBs when viewed with CLSM (see Fig. 7D in Leander, 2006). Higher-magnification TEMs show a uniform row of small electron-dense granules that runs adjacent to the linear thread (see Fig. 7D & F in Leander, 2006). A synthesis of the previous TEM data with the new CLSM data suggests that this previously unidentified linear thread is actually a tubulin-based DVMB. Currently, *P. vivax* is the only myzozoan known to have DVMBs, and the functional significance of this ultrastructural system appears to be a derived trait for this lineage.
**Figure 9.** Illustrations summarizing the overall organization of microtubules, basal bodies and mitochondria in the trophozoites of *Platyproteum vivax*. (A) Dorsal and ventral views of a trophozoite showing the anterior apparatus with two flagella oriented to the right and left, respectively. Green denotes the position of the basal bodies from which the flagella are derived. Red denotes microtubules. ‘Longitudinal microtubule bundles’ (LMBs) are arranged in parallel along the entire anteroposterior axis of the cell, and ‘dorsoventral microtubule bundles’ (DVMBs), indicated by red dots, are arranged perpendicular to the anteroposterior axis of the cell. Most of the DVMBs are aligned in a distinctive curved row that runs from the anterior to the posterior end of the trophozoite. The row of DVMBs is positioned to the right of the nucleus in dorsal view and to the left of the nucleus in ventral view. A few isolated DVMBs are scattered on the side of the nucleus opposite of the curved row of DVMBs. (B) Sagittal section showing the LMBs subtending the inner membrane complex and the perpendicular DVMBs running from the dorsal side to the ventral side of the trophozoite. Brown denotes the superficial layer of mitochondria located just above the LMB layer. (C) Cross sections through the nucleus of the trophozoite showing the LMBs subtending the inner membrane complex and a perpendicular DVMB running from the dorsal to ventral side of the cell.
2.4.4 Actin cytoskeletal elements

Actin dynamics are associated with generating the forces necessary to produce dynamic cell shape changes and movements in a wide variety of eukaryotes (Etienne-Manneville, 2004). For instance, quick polymerization-depolymerization cycles between filamentous actin (F-actin) and globular actin (G-actin) combined with the actions of myosin motor proteins drive gliding motility in many apicomplexans (Soldati-Favre, 2008; Woo et al., 2015). Eugregarines (e.g., Gregarina spp.) have F-actin networks below the longitudinal epicytic folds, which facilitate gliding movements and control cell shape (Heintzelman, 2004; Valigurova et al., 2013). Therefore, the presence of F-actin and its possible role in cell movement in the trophozoites of P. vivax was investigated using the widely used fluorescently-marked cyclic peptide ‘phalloidin’.

Filamentous actin in P. vivax failed to bind to phalloidin, so the organization of the actin-based cytoskeleton, if present, remains unclear. Interestingly, previous studies have shown that phalloidin also failed to bind to F-actin in several other apicomplexan genera, including Gregarina spp., Toxoplasma spp., Tetrahymena spp. and Plasmodium spp. (Heintzelman, 2004; Hirono et al., 1989; Shaw and Tilney, 1999). F-actin visualization in these groups was done by generating antibodies specific to the species being studied for immunolocalization or with immunogold labeling using TEM, which were techniques beyond the scope of this master's project. However, phalloidin binding has been successful in some eugregarine species, such as Urospora travisiae and U. ovalis (Valigurova et al., 2013; Valigurova et al., 2017), indicating that different lineages of apicomplexans express different families of F-actin. Molecular phylogenetic analyses suggests that apicomplexans have developed distinct properties in the highly-conserved actin binding proteins and have lost many of the actin-associated genes that chrompodellids possess (Morrissette, 2015; Woo et al., 2015). Considering that P. vivax is more distantly related to apicomplexans than are chrompodellids, there appear to have been several independent losses of actin-associated genes within myzozoans. Nonetheless, P. vivax is similar to several other apicomplexan genera (e.g., Gregarina) in having F-actin populations that do not bind to phalloidin.
Chapter 3: Conclusions

By examining the ultrastructure of *P. vivax*, I was able to identify novel traits that support recent phylogenomic data showing *P. vivax* as a distant relative of *Selenidium* and that provide morphological context for comparing new species within the Squirmida. In this chapter, I summarize the convergence of traits in intestinal parasites within the Myzozoa and outline future directions for this area of research.

3.1 Convergent evolution of traits in *Platyproteum* and *Selenidium*

A more detailed investigation of the ultrastructure of squirmid trophozoites has revealed novel traits that reflect the independent origins of *Platyproteum* spp. and *Selenidium* spp. within the intestines of marine invertebrates; this is consistent with phylogenomic data indicating that ultrastructural and behavioural similarities in the trophozoites of these two lineages arose by convergent evolution. Intestinal eugregarines (e.g., *Lecudina, Pterospora* and *Lankesteria*) have trophozoites that do not dynamically change shape and lack conspicuous longitudinal subpellicular microtubules. Instead, eugregarine trophozoites have a dense array of epicytic folds (> 50) and actin-myosin based gliding motility. In contrast, members of *Selenidium* have trophozoites with relatively few epicytic folds (< 50), vermiform cell shapes, dynamic undulating behaviour and robust layers of subpellicular microtubules originating from within the mucron (Leander, 2007; Schrevel, 1971a, 1971b; Schrevel *et al.*, 2016; Valigurova *et al.*, 2017; Vivier and Schrevel, 1964 & 1966; Wakeman *et al.*, 2014a). These traits are similar to those in *Platyproteum*. Both *Platyproteum* and *Selenidium* appear to utilize surface-mediated nutrient absorption through similar mechanisms. *Platyproteum* is flattened with transverse surface folds that increase surface area, while *Selenidium* has both longitudinal epicytic folds and transverse surface folds that increase the surface area for nutrient absorption.
Table 1: Morphological comparison of the trophozoites in *Platyproteum* and *Selenidium*.

<table>
<thead>
<tr>
<th>Trait</th>
<th><em>Platyproteum</em></th>
<th><em>Selenidium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host type/environment</strong></td>
<td>Invertebrate, marine</td>
<td>Invertebrate, marine</td>
</tr>
<tr>
<td><strong>Host tissue</strong></td>
<td>Intestine</td>
<td>Intestine</td>
</tr>
<tr>
<td><strong>Cell shape</strong></td>
<td>Tape-like, flattened</td>
<td>Spindle-shaped, vermiform</td>
</tr>
<tr>
<td><strong>Anterior end</strong></td>
<td>Straight-edge, hooked</td>
<td>Pointed, radially symmetrical</td>
</tr>
<tr>
<td><strong>Longitudinal folds</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Transverse folds</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Movement</strong></td>
<td>Twisting, undulating, folding</td>
<td>Twisting, undulating</td>
</tr>
<tr>
<td><strong>Longitudinal microtubules</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Dorsoventral microtubules</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Flagella</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
The flattened trophozoites in *Platyproteum* are similar in shape, peristaltic behaviour, and ultrastructure to the dorsoventrally flattened proglottids of tapeworms (Cestoda) (Leander, 2008). Tapeworms have lost the gut of their ancestors and instead acquire nutrients within the host intestine using surface-mediated absorption (Arme, 1983; Brusca *et al*., 2022; Leander, 2008; Rueckart and Leander, 2009). The intestinal trophozoites of *Platyproteum* are inferred to acquire nutrients in the same way. Both tapeworms and the trophozoites of *Platyproteum* have a superficial layer of mitochondria that reflects the low-oxygen conditions within the intestinal lumen of their host and that generates the ATP required to fuel endocytosis and the contraction of muscles and the sliding of LMBs, respectively. Moreover, the arrangement of LMBs and DVMBs in *P. vivax* is similar to the arrangement of longitudinal and dorsoventral muscles in flatworms (Platyhelminthes), which help maintain the surface area created by the extremely flattened shape of *Platyproteum* trophozoites and proglottids.

Future studies using CLSM to investigate the ultrastructural systems of undescribed species of squirmids (e.g., *Filipodium, Platyproteum* and novel genera) will determine whether any of the novel traits described in this study, such as a pair of inconspicuous flagella and a system of dorsoventral microtubule bundles, are synapomorphic for the group. The presence of two flagella protruding from the anterior end of the trophozoites was corroborated by centrin staining showing basal bodies located just below the membrane along the anterior edge. This is the first observation of flagella in *P. vivax* and corresponds with the recent report of flagella in *P. noduliferae* from Japan. The dorsoventral microtubule bundles had a punctate distribution in dorsoventral view and were mostly organized along a curved line that extended from the anterior end to the posterior end of the trophozoites. This system of microtubules might be synapomorphic for the Squirmida as a whole and probably functions to maintain the flattened trophozoite shape and facilitate dynamic folding movements via microtubule sliding.

The uniform layer of longitudinal microtubule bundles that subtends the entire inner membrane complex of *P. vivax* is analogous to the cortical microtubules found in the trophozoites of *Selenidium*. The layer of mitochondria positioned just below the layer of longitudinal microtubule bundles probably supplies the ATP needed to fuel microtubule sliding and ultimately the dynamic undulating movements of the trophozoites. The presence of
transverse surface folds on the trophozoites of both *Platyproteum* and *Selenidium* during undulations reflects convergent evolution at both the behavioural and morphological levels. This study also demonstrated that the population of F-actin present in *P. vivax* does not bind to phalloidin, a trait that is shared with several genera of apicomplexans (e.g., *Gregarina*; Heintzelman, 2004). Overall, this study has provided ultrastructural evidence that corroborates phylogenomic data showing the Squirmida branching independently as the sister lineage to a clade consisting of chrompodellids and apicomplexans (Mathur *et al.*, 2019).
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