Description of two new species of marine gregarine parasites (Apicomplexa) from the intestines of *Lumbrineris inflata* (Annelida)

by

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Abstract

Apicomplexans are diverse single-celled eukaryotes that parasitize animals. The most notorious members include those of particular human interest such as the causative agents of malaria, toxoplasmosis, and cryptosporidiosis. While a subset of apicomplexans has been intensively studied from a medical or veterinary perspective, the diversity of remaining groups is underrepresented in existing literature. This lack of data has left the deep relationships among apicomplexan taxa enigmatic and in turn has hindered the revelation of some major evolutionary processes that sparked the apicomplexan radiation. The dearth of understanding surrounding apicomplexan systematics can be addressed in part through the discovery of novel species and the identification of how morphological and molecular characters are distributed across the apicomplexan phylogeny. Some lineages of marine gregarines have retained plesiomorphic characters that offer unique insight into the earliest stages of apicomplexan evolution. The current thesis describes and establishes two novel marine gregarine species isolated from a polychaete hosts (*Lumbrineris inflata*). Species delimitation and description was based on morphological data acquired using light and electron microscopy and a molecular phylogenetic analysis of 18S small subunit (SSU) rDNA sequences. *Paralecudina anankea* n. sp. possessed an elongated body, an oval nucleus, and gliding motility. The sister relationship of *P. anankea* n. sp. with *P. polymorpha* was robustly supported by molecular phylogenetic analysis (100 MLB, 1.00 BPP) and the SSU rDNA sequences between the two were 12% divergent. In contrast, *L. caspera* n. sp. was morphologically dissimilar to its closest relative *L. longissima* and possessed an acorn-shaped body, a distinct mucron, and gliding motility. Molecular phylogenetic analysis recovered *L. caspera* n. sp. as a sister species to *L. longissima* with strong support (100 MLB, 1.00 BPP)

and their SSU rDNA sequences which were 8% divergent. The generation of additional morphological and molecular traits in gregarines will improve the phylogenetic resolution of the apicomplexan backbone and improve inferences about the evolutionary transition from photosynthetic ancestors to obligate parasites.

Preface

All work presented in the current thesis is original work. I conducted all relevant fieldwork including the collection of organisms and laboratory work including DNA extractions and sequencing, microscopy, and molecular phylogenetic analyses. The identification of the novel species, original primer designs, and amplification protocols were also by my own conception. The funding for the project and final interpretation of the thesis are owed to Dr. Brian S. Leander.

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List of Abbreviations

BPP – Bayesian posterior probability

DNA – Deoxyribonucleic acid

LM – Light microscopy

MLB – Maximum likelihood bootstrap

n. sp. – New species (species novum)

SEM – Scanning electron microcopy

SSU rDNA – Small subunit ribosomal deoxyribonucleic acid

TRAP - Thrombospondin-related anonymous protein

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Chapter 1: Introduction

1.1 Objective

Evolution gives rise to incredible biodiversity and yields an immense range of innovative biological traits. In reconstructing past evolutionary events, there are generally three broad approaches that may be employed. Where fossil evidence is available, it is possible to reconstruct the past by searching for those that help to illustrate the transitions between sets of traits represented in species over time. Fossils, however, are a luxury in biology and most taxa are unrepresented in the fossil record. Controlled evolutionary experiments can take a different approach in that they aim to identify the mechanisms underlying speciation which can in turn be used to predict future events, but also to hypothesize how historical events may have guided the evolution of extant species. Experiments often require model systems and unfortunately cannot be employed where organisms cannot be cultivated. In cases where both fossil evidence and experimental data cannot be found, taxonomy can inform historical evolutionary events. By creating a robust phylogeny and identifying the distribution of characters and character state changes over time, it is possible to create a vista into the past through which ancient evolutionary events are visible.

Phylogenies illustrate the relationships among taxa bound within the framework of common ancestry. The construction of well-supported phylogenies, however, requires a strong dataset which includes a large number of species in addition to the accurate identification of characters that are taxonomically informative. Therefore, a gross underrepresentation of the biodiversity of

a given taxon can blur the relationships among the taxa that have already been described. The lack of taxonomical data is remedied through the discovery of additional species and an accurate cataloguing of their observable traits (e.g., the discovery of *Chromera*; Janouskovec *et al.*, 2010). The current thesis aims to introduce two novel species of marine gregarines within the phylogenetic context of previously described apicomplexans. The description of these two new species helps to highlight the diversity of gregarines as well as the indispensability of using molecular data for gregarine systematics. The discovery of every new species reflects a new combination of biological traits that informs our understanding of biodiversity and evolutionary history. The goal of this project is to more comprehensively characterize the early evolution of apicomplexans and improve the resolution of gregarine phylogeny.

1.2 The Apicomplexa

The Apicomplexa Levine 1980 is a large clade of parasitic, single-celled eukaryotes that infect both aquatic and terrestrial animals. The most infamous members are those of particular medical and veterinary interest and include the causative agents of malaria, toxoplasmosis, and cryptosporidiosis. Although such members have received extensive attention for their often devastating impacts to human society, the bulk of apicomplexan diversity remains undiscovered. The Apicomplexa is immensely speciose with an upwards estimate of 10 million species (Adl *et al.*, 2007). This estimate outnumbers some estimates for the total number of animals suggesting that every animal species on Earth is host to at least one apicomplexan species (Morrison, 2009). Only around 6000 apicomplexan species, however, have been formally described to date (Adl *et al.*, 2007). Despite the overall lack of taxonomical data, several key apicomplexan characters

have been identified. The following section defines the Apicomplexa and its broad relationships with other eukaryotes.

Apicomplexans, dinoflagellates, and ciliates together form a clade known as the Alveolata Cavalier-Smith 1991. Ciliates were the first to diverge within the Alveolata, forming a basal branch, whereas apicomplexans and dinoflagellates share a sister relationship. The name 'Alveolata' derives from a common morphological character among alveolates known as alveoli (Cavalier-Smith, 1999; Leander and Keeling, 2003). Each alveolus consists of a flattened, membrane bound vesicle which supports the plasma membrane in various ways. For example, the alveoli in dinoflagellates form armor plates whereas in many apicomplexans the alveoli form a flexible pellicle and aid in the gliding motility used for host invasion (Dubremetz *et al.*, 1998). The Apicomplexa are set apart from dinoflagellates and ciliates in being obligate parasites that possess an apical complex – their namesake (Leander and Keeling, 2003). Although parasitism is a common strategy employed by numerous dinoflagellate species, apicomplexans are unique in being exclusively parasitic.

The apical complex is a unifying feature of all apicomplexans, although it is found in some other non-apicomplexan taxa. The apical complex is a compound structure consisting of a polar ring and a conoid made of specialized tubulin which provides the scaffolding for dense granules and extrusive organelles known as rhoptries (Paterson *et al.*, 1988; Peterson *et al.*, 1989; Leander and Keeling, 2003; Okamoto and Keeling, 2014). Rhoptries are found near the conoid and often directly aid in host cell invasion through the use of elaborate biochemical mechanisms which have been intensively studied in malarial parasites (Sam-Yellowe *et al.*, 1988; Crewther *et al.*,

1990; Dubremetz *et al.*, 1998; Alexander *et al.*, 2006). The conoid varies morphologically among taxa, but is closed in apicomplexans save some special cases (e.g., *Plasmodium*) in which the conoid has been lost (Leander and Keeling, 2003). The closed character state of the conoid in apicomplexans is derived from the inferred ancestral state in which the conoid is open on one side. The apical complex is primarily used to pierce the plasma membrane of other cells. In predators, such as colpodellids, this piercing enables them to suck the cytoplasm out of the prey cell via myzocytosis (Kuvardina *et al.*, 2002). Some plesiomorphic apicomplexans (i.e., archigregarines) use the apical complex in a similar way to pull cytoplasm from the hosts' intestinal epithelial cells (Leander, 2008b) whereas others use the apical complex for intracellular invasion (Peterson *et al.*, 1989; Dubremetz *et al.*, 1998). Cell invasion is achieved by essentially 'reversing' myzocytosis whereby a parasite pierces the host cell and uses its cellular machinery to forcibly enter the cytoplasm rather than pulling the cytoplasm into itself.

Perkinsids and colpodellids are biflagellated unicells that possess an apical complex with an open conoid (Leander and Keeling, 2003). Perkinsids use their apical complex for parasitism (Norén *et al.*, 1999) whereas colpodellids are a free-living predator which uses its apical complex for myzocytosis (Leander and Keeling, 2003). Myzocytosis was originally observed in dinoflagellates and the intracellular structure involved, the peduncle, may be homologous to the apical complex (Schnepf and Deichgräber, 1984; Norén *et al.*, 1999). Archigregarines are an ancient group of gregarine apicomplexans which also use their apical complexes for myzocytosis (Leander and Keeling, 2003). Perkinsids diverge at the base of dinoflagellates and colpodellids diverge at the base of apicomplexans (Kuvardina *et al.*, 2002; Leander *et al.*, 2003a; Saldarriaga *et al.*, 2003). The unique phylogenetic positions of perkinsids and colpodellids, in combination

with their morphological characters, offers insight into the evolution of the Apicomplexa as a whole. For instance, several traits of the common ancestor to apicomplexans and dinoflagellates can be inferred from perkinsid and colpodellid biology. Current evidence suggests the common ancestor to apicomplexans and dinoflagellates was a free-living, biflagellate with an open conoid used for feeding (Leander and Keeling, 2003). This ancestor was also mixotrophic and employed both photosynthesis and heterotrophy as a means for energy acquisition. The chromealveolate hypothesis (Cavalier-Smith, 1999), which posits that alveolate plastids originated from a single endosymbiosis with a red alga, was among the first efforts to investigate the origin of the apicoplast. The chromealveolate hypothesis, however, has since seen strong counter evidence to its validity and the evolutionary origin of the apicoplast remains inconclusive (Harper *et al.*, 2005; Burki *et al.*, 2008; Bodył *et al.*, 2009; Keeling, 2009). Elucidating the origin of alveolate plastids is important as it casts significant light onto the ancestry of the Apicomplexa in the context of eukaryote evolution.

Apicomplexans possess a relict plastid termed the apicoplast. The discovery of the apicoplast not only manifested in significant advances in the understanding of apicomplexan biology, but also pushed the frontiers of medical research in the apicomplexan field (Waller and McFadden, 2005). The apicoplast was crucial in identifying apicomplexans as the descendants of a photosynthetic ancestor implying its transition from a free-living photosynthesizer to an obligate parasite (Leander and Keeling, 2003; Leander, 2008b). As for medical research, the apicoplast represented a new potential target for anti-apicomplexan drugs (Foth *et al.*, 2003; Ralph *et al.*, 2004; Dahl *et al.*, 2006). Current discussions on the apicoplast largely focus on its origins and role in finding new molecular pathways that can be disrupted for the invention of anti-malarial

and anti-coccidial drugs (e.g., Waller and McFadden, 2005; Sawhney *et al.*, 2015; Wu *et al.*, 2015; McFadden and Yeh, 2016; Milton and Nelson, 2016).

Other key divergences of the Apicomplexa from their biflagellate ancestor include the loss of flagella except in the gametes of some taxa, a complex life cycle involving infectious sporozoite stages, and the development of cellular machinery allowing for gliding motility (Leander and Keeling, 2003; Baum *et al.*, 2006). An enigmatic species of unknown phylogenetic position named *Acrocoelus glossobalani* raises important questions in terms of apicomplexan parasitism. *Acrocoelus glossobalani* is an intestinal parasite which has organelles that resemble rhoptries, but no conoid. Whether this morphology reflects an ancestral state at the base of both dinoflagellates and apicomplexans or whether it reflects a mode of parasitism following the loss of a conoid remains unknown (Leander and Keeling, 2003). The discovery of new apicomplexan taxa will continue to inform apicomplexan phylogeny and yield a better illustration of the distribution of important character traits. The overall understanding of early apicomplexan evolution will undoubtedly benefit from the continual discovery of new morphological and molecular characters that define the Apicomplexa.

1.3 Major apicomplexan taxa

1.3.1 The Piroplasmida

Piroplasms are generally tick-borne intra-erythrocytic parasites of many vertebrate hosts including mammals and birds (Criado *et al.*, 2006). *Babesia* and *Theileria* are two genera

belonging to this group and are notorious for causing devastating diseases in livestock often involving hemolytic anemia. Currently three piroplasmid genera are recognized: *Babesia*, *Theileria*, and *Cytoxzoon*. New studies on mitochondrial genomes, however, suggest more than five distinct lineages within the Piroplasmida (Schreeg *et al.*, 2016). The overall diversity of within these lineages is potentially quite high (Criado-Fornelio *et al.*, 2004) and piroplasmids are found in many geographical locations including North America and Asia (Gray *et al.*, 2010).

Morphologically, piroplasmids have but a few easily characterized traits. They are generally round and species are delimited mainly on the basis of size and orientation within the host erythrocyte (Laha *et al.*, 2015). Small piroplasmids are defined by size ranging between 1.0 µm to 2.5 µm long whereas large piroplasmids range between 2.5µm to 5.0 µm long. Some characteristic orientations include the Maltese cross formed by *Babesia equi* in which four cells lie perpendicular to one another within the host erythrocyte (Gray *et al.*, 2010; Laha *et al.*, 2015). Inside the host erythrocytes, piroplasmids undergo bouts of asexual reproduction known as merogony (Alvarado-Rybak *et al.*, 2016) which is frequently the cause of severe illness in debilitating apicomplexan infections.

1.3.2 The Coccidia

Since their initial description during the 19th century, coccidians have been continually discovered in virtually every host examined (Tenter *et al.*, 2002). Coccidians parasitize vertebrate hosts including fish (Dykova and Lom, 1981), amphibians (Jirků *et al.*, 2009), avians (Shirley *et al.*, 2007), and humans (Tran Van Nhieu *et al.*, 1996). Infections by coccidians can

cause coccidiosis which is frequently extremely devastating for the host. For instance, *Eimeria* spp. have decimated poultry populations causing severe financial damage to farmers; the cost incurred by coccidiosis in chickens alone was over £35,000,000 for the United Kingdom in 1995 (Williams, 1999).

Species delimitation and characterization of coccidians are mostly based on oocyst morphology, host species, and geography (Tenter *et al.*, 2002). Taxonomic descriptions have traditionally focused on oocyst morphology (Jirků *et al.*, 2009) such as shape, number of sporozoites per oocyst, and the thickness of oocyst walls. Recent taxonomical work has shown that these morphological traits may be insufficient as defining traits and the morphological phylogenies of coccidians are remarkably incongruent with molecular phylogenies (Kvicerová *et al.*, 2008). The difficulty in accurately delimiting coccidian species is further exacerbated by species that infect multiple hosts, but are morphologically identical to each other. Additionally, coccidian diversity is now estimated to be much higher than what is currently represented in the literature. For example, *Eimeria* is one of the most speciose genera of all eukaryotes (Yang *et al.*, 2014) and even in the most extensively studied hosts of coccidians, such as rats, only 8% of the estimated total has been formally described (Tenter *et al.*, 2002). Molecular data, therefore, is necessary to inform existing morphological phylogenies and improve the resolution of coccidian phylogenetics.

1.3.3 The Rhytidocystidae

Rhytidocystids are mysterious apicomplexans belonging to the larger, poorly characterized group known as the Agamococcidiorida Levine 1979 (Cox, 1994; Leander and Ramey, 2006). This group consists of species that infect polychaete hosts and produce cysts resembling those found in coccidians, but do not reproduce by merogony or gamont formation. Rhytidocystids have large bean-shaped trophozoite stages and longitudinal rows of repeating transverse folds. Due to the morphological similarities between rhytidocystids and gregarines, rhytidocystids were originally described as atypical gregarines. Rhytidocystid trophozoites, however, differ from gregarine trophozoites in that they are immotile and embed into the host epithelial cells rather than parasitizing extracellularly (Rueckert and Leander, 2009a). Molecular phylogenetic analyses have also contradicted the suggestion that rhytidocystids are peculiar gregarines and have repeatedly recovered a distinct rhytidocystid clade (Leander and Ramey, 2006; Rueckert and Leander, 2009a). The deeper relationships of the rhytidocystids with the rest of the Apicomplexa remains unresolved.

1.3.4 The Cryptosporidia

Cryptosporidians are parasites of vertebrate hosts including humans (Morgan-Ryan *et al.*, 2002). Infections can cause cryptosporidiosis which often entails gastrointestinal maladies such as gastrocolitis and severe diarrhea (Tzipori, 1983). While cryptosporidiosis is non-lethal for most hosts, the infection is serious for immunocompromised individuals. Human AIDS patients for example suffer from malabsorption, volume depletion, and weight loss due to cryptosporidiosis

which can result in death (Greenberg and Cello, 1996). *Cryptosporidium* outbreaks are also quite common and experimental studies with non-human vertebrates have demonstrated that the ingestion of a single oocyst can be enough to cause disease (Pereira *et al.*, 2002). Transmission is most commonly via the fecal-oral route through contaminated water, but can also occur between people within close contact including family members, sexual partners, and children in daycares (Ramirez *et al.*, 2004). Crops fertilized with manure can also be a source of cryptosporidian infection. Cryptosporidians are now recognized as some of the most difficult waterborne pathogens to control that afflict humans (Ramirez *et al.*, 2004)

More than 22 species of *Cryptosporidium* have been named based on morphology and host occurrence (Ramirez *et al.*, 2004). Morphological traits of cryptosporidians are frequently taxonomically misleading, however, due to the lack of stark visible differences among species. The easily discernible features of a cryptosporidian, without the aid of electron microscopy, include the spherical oocysts ranging between 3 to 6 µm in diameter and location within the gastrointestinal tract of the host. *Cryptosporidium* was originally classified as a coccidian based on morphology and life history (Levine, 1988). This suggestion was challenged as cryptosporidians were more intensively observed following their recognition as debilitating human parasites. Incongruences with the classification of cryptosporidians as coccidians included the confinement of infectious *Cryptosporidium* to the apical, extracytoplasmic surfaces of host cells as well as their unresponsiveness to anti-coccidial drugs (Rosales *et al.*, 2005). Extracellular gamonts in syzygy have also been confirmed by light microscopy (Rosales *et al.*, 2005). Contemporary studies focus on recovering molecular data from cryptosporidians and have been instrumental in refining the inferred phylogenetic position of cryptosporidians in the

apicomplexan tree. Genetic studies have revealed new human *Cryptosporidium* species such as *C. hominis* (Morgan-Ryan *et al.*, 2002) and have also been successful in identifying genotypes of varying virulence to humans (Widmer *et al.*, 1998). Molecular phylogenetic analyses now suggest that cryptosporidians are a descendent of a gregarine lineage (Carreno *et al.*, 1999; Leander and Keeling, 2003), but with distinct divergent characters such as significantly reduced and streamlined metabolic pathways compared to that of gregarines (Templeton *et al.*, 2010).

1.3.5 The Gregarinida

Gregarines are an understudied group of apicomplexan parasites that offer an opportunity to elucidate the earliest stages of apicomplexan evolution. Marine gregarines are of particular interest for apicomplexan systematics because they are uniquely positioned at the base of the apicomplexan tree and have retained key plesiomorphic characters since their divergence from an ancient biflagellate ancestor (Leander, 2008b). For example, marine gregarines are monoxenous and many parasitize host cells via myzocytosis (Leander, 2008b). Marine gregarine parasitism is also extracellular and most commonly confined to the intestinal lumen of an invertebrate host. The more derived apicomplexan relatives of gregarines, in contrast, are typically intracellular and infect multiple host compartments during a dioxenous life cycle. The ubiquity of gregarines across oceanic environments is also reflective of their early descent from a free-living marine ancestor. The prevalence of gregarines across marine habitats has been revealed through environmental sequencing surveys (Rueckert et al., 2011) and gregarines have been found in the most extreme of habitats such as deep sea methane cold seeps (Wakeman and Leander, 2013a). This wide geographical distribution reflects the biodiversity of gregarine

species, but most remain undiscovered (Leander, 2008b). Where formal taxonomic descriptions do exist, the original sources are often badly scattered across obscure journals in various languages. Furthermore, traditional approaches to species delimitation of gregarines relied on morphology and the comparison of line drawings. Gregarine morphology, however, is at times taxonomically uninformative due to the challenge of accurately identifying the range of variation of any given morphological trait without first knowing the degree of intraspecific morphological plasticity. There are also relatively few morphological traits of gregarines that are easily studied and interspecific variation in these few morphological traits is often large. A more comprehensive catalogue of gregarine diversity can alleviate some of these taxonomical challenges by introducing more data that can be used to construct increasingly robust gregarine phylogenies. The discovery and description of additional marine gregarine species can improve the understanding of fundamental gregarine biology as well as provide insight into two major evolutionary events: 1) the radiation of apicomplexans from basal gregarine groups; and 2) the biological innovations required to transition from a free-living predatory lifestyle to that of an obligate parasite.

Gregarines are characterized by numerous traits including their particularly large extracellular feeding stages known as trophozoites (Fig. 1), monoxenous life cycles, and infection of invertebrate hosts (Leander, 2008b). Host species, location within the host body, and cytoskeletal morphology vary among gregarine species. In most cases, however, gregarine infections have been documented from marine annelids and terrestrial insects (Zuk, 1987; Clopton *et al.*, 1992; Schilder and Marden, 2006; Leander, 2008b; Rueckert and Leander, 2009b; Wakeman and Leander, 2013a; b). The host compartment targeted by gregarines is generally confined to the

intestinal lumen, but some infect coelomic spaces (e.g., urosporidians; Leander *et al.*, 2006) or reproductive organs (e.g., *Monocystis agilis*; Field and Michiels, 2005). These types of morphological and life history traits were traditionally employed to divide the gregarines into three major categories: archigregarines, neogregarines and eugregarines (Grasse, 1953; Leander, 2008b; Adl *et al.*, 2012).

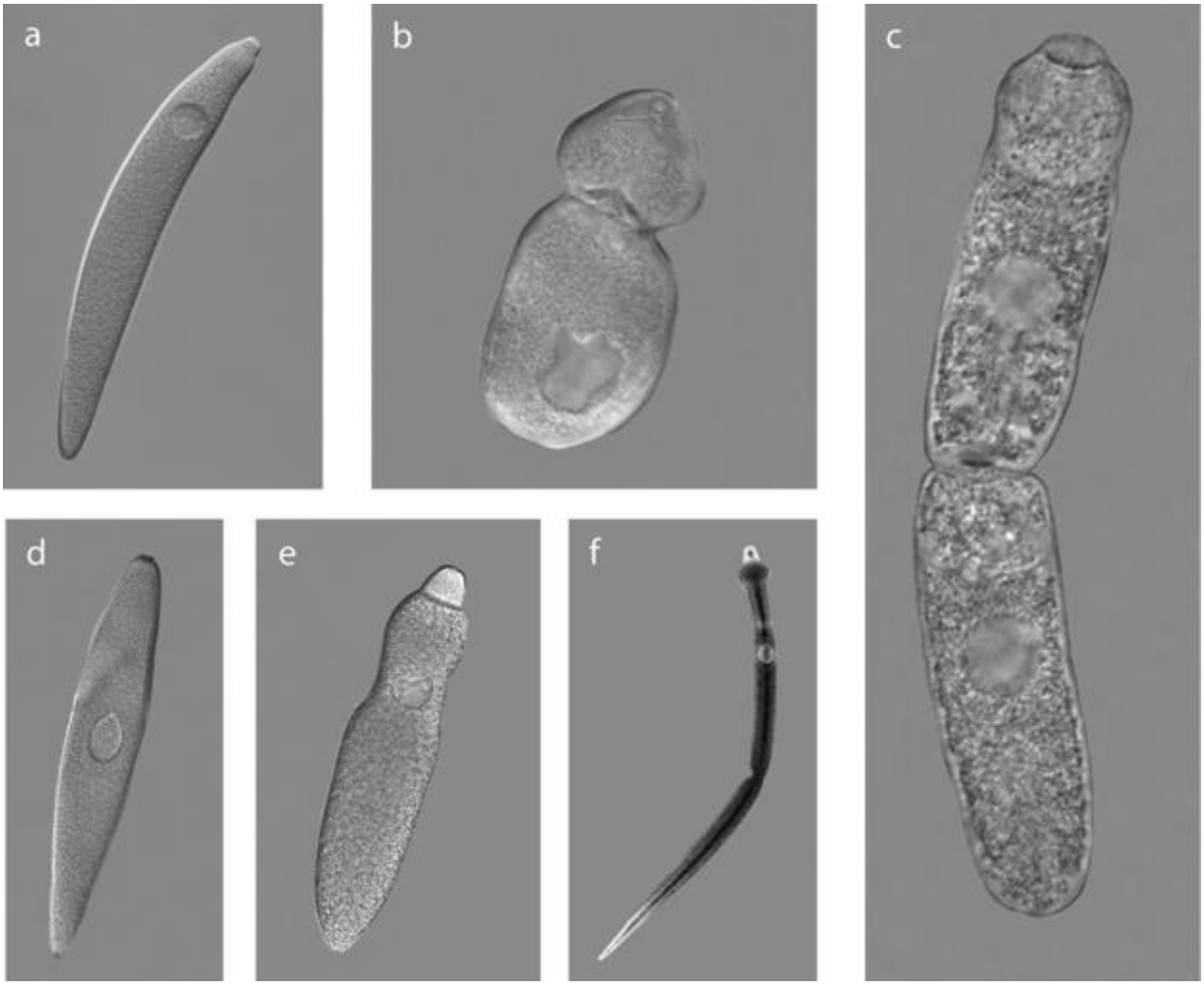

Figure 1 – Various morphologies of marine gregarine trophozoites isolated from annelid and crustacean hosts. **a** Unidentified *Selenidium*-like species from a polychaete host. **b** Unidentified marine lecudinid isolated from *Balanus glandula*. **c** A pair of unidentified marine lecudinid gamonts from a subtidal barnacle host. **d** Unidentified archigregarines-like trophozoite from a polychaete host. **e** Unidentified marine lecudinid from a polychaete host **f** *Paralecudina polymorpha* morphotype 2.

Archigregarines are a paraphyletic group constituted by gregarine species possessing plesiomorphic traits including trophozoites that closely resemble sporozoites, an exclusively marine distribution, and intestinal parasitism via myzocytosis. Phylogenies generated through comparisons of small subunit ribosomal DNA (SSU rDNA) show that other apicomplexans arose from archigregarines lineages (Leander, 2007, 2008b; Rueckert and Leander, 2009b; Wakeman and Leander, 2012). In contrast, neogregarines are parasites of terrestrial insects (Golemansky, 2015) and have diverged from their marine roots in many ways including the exploitation of host tissue outside of the intestinal lumen and reproduction by merogony (Leander, 2008b). Molecular phylogenetic evidence suggests that terrestrial gregarines arose twice independently from separate marine gregarine lineages (Wakeman *et al.*, 2014). Eugregarines are another paraphyletic group of gregarines and include members that parasitize a variety of marine, freshwater, and terrestrial hosts. The Eugregarinorida Léger 1900 is further divided into two groups based on a transverse groove that separates the trophozoite stage into two cellular compartments: the aseptate and septate gregarines. Septate eugregarines are commonly found in marine crustaceans (Figure 1; b and c) and terrestrial insects (Levine, 1979; Valigurová and Koudela, 2006; Simdyanov *et al.*, 2015). The taxonomic resolution of the eugregarines is currently poor and most of its backbone is composed of unsupported branches. The paraphyly of archigregarines and eugregarines are taxonomically problematic and the details of neogregarine divergence from marine lineages remains enigmatic. Revisiting historical morphological

phylogenies with molecular data in addition to the construction of new molecular phylogenies will further clarify the deep relationships among archigregarines, eugregarines, and neogregarines.

The mode of movement is another trait that broadly divides gregarines and can help to classify archigregarines, eugregarines, and neogregarines. Gregarines move in one of two distinct ways depending on the taxon. Archigregarines use longitudinal arrays of microtubules to bend and are capable of movements that closely resemble that of nematodes; convergent evolution has been proposed as an explanation for the similarity in movement between some archigregarines and nematodes (Leander, 2008a). Eugregarines and neogregarines, on the other hand, are capable of folding, but mainly move by gliding motility. Gliding in gregarines is not fully understood, but is likely a product of actin-myosin systems concentrated at the periphery of the cell near the inner membrane complex and longitudinal epicytic folds (Valigurová *et al.*, 2013). Two classes of myosins (GpMyoA and GpMyoB) have been cloned from *Gregarina polymorpha* and were found to concentrate along epicytic folds (Heintzelman, 2004). The current model for apicomplexan gliding motility is largely based on studies of *Toxoplasma* and *Plasmodium* which have revealed the gliding mechanism and associated cellular machinery collectively termed the glideosome (Opitz and Soldati, 2002). In apicomplexan parasites that glide along the surface host cells, the first stage of gliding motility involves the release of membrane-bound adhesins from the micronemes (Baum *et al.*, 2006). These adhesins belong to a family of proteins known as thrombospondin-related anonymous proteins (TRAP). The TRAP molecules protrude through the parasite plasma membrane and attach to specific receptors on the host plasma membrane. Inside the parasite cytoplasm, the TRAP molecules are connected to short actin filaments that are

continuously nucleated at one end and denucleated at the other. The apicomplexan myosins involved in gliding motility are anchored into the inner membrane complex which is formed by alveoli. These anchored myosins walk along the aforementioned nucleating short actin filaments. The overall motion can be likened to walking on a treadmill. The net movement is a forwards gliding of the parasite as the adhesins connected to the host cell receptors are pushed backward. The adhesins eventually cleave from these host receptors and prepare to engage with the next adhesive point. Gregarines glide freely in the absence of host cells and how the gregarine glideosome interacts with the environment is poorly understood.

Energy storage in gregarines occurs in the form of a polysaccharide known as paraglycogen or amylopectin (Daniels, 1938; Mercier *et al.*, 1972). The debranched form of paraglycogen has an intermediate profile between amylopectin found in plants and glycogen found in animals. Paraglycogen granules vary in appearance among taxa, but is typically oval and brown. Analysis of the chemical structure of gregarine paraglycogen has revealed similarities to coccidian storage molecules (Mercier *et al.*, 1972). Paraglycogen granules are typically visible in gregarines at the trophozoite stage of their life cycle.

1.4 Gregarine life cycles

Gregarine infections are generally monoxenous although a single host is often simultaneously infected with multiple gregarine species (Leander *et al.*, 2003b, 2006). The monoxeny of gregarines is taxonomically convenient in that the discovery of new gregarine species is often predictable when investigating new hosts. The typical gregarine life cycle begins with the

ingestion of a gregarine oocyst by the host from its immediate environment (Fig. 2). In some exceptional cases, oocysts are transmitted between hosts during copulation (e.g., *Monocystis agilis;* Vivier and Desportes., 1990). The cysts, upon reaching a suitable location within the host, break and release the infectious stages of gregarines known as sporozoites. These sporozoites then use their apical complex to penetrate the appropriate tissue. For marine gregarines, the most common location for sporozoites to infect is the intestinal lining. However, some species will continue to burrow through the intestinal wall to become coelomic parasites. The sporozoites then progress to the next stage of the life cycle by growing into mature feeding stages known as trophozoites.

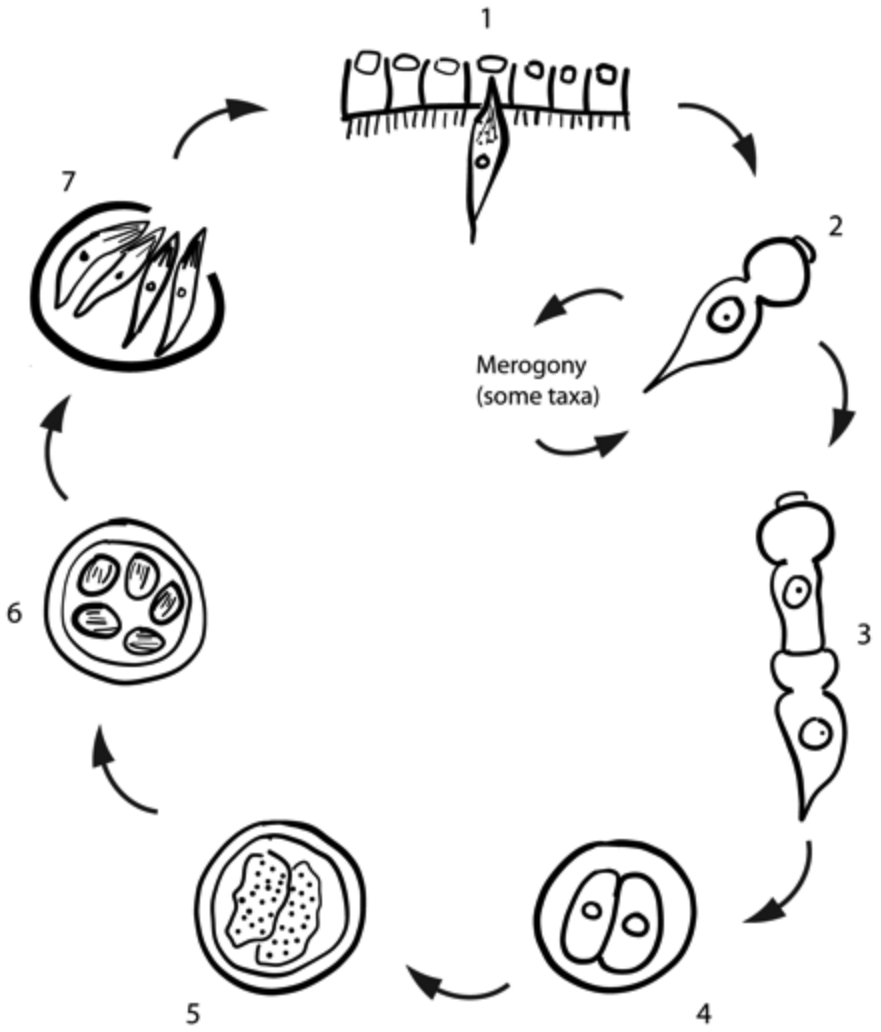

Figure 2 – Lifecycle of a hypothetical gregarine. **1** Gregarine sporozoite utilizes its apical complex to infect host intestinal cell. **2** Sporozoites grow into large feeding stages (trophozoites) and loses apical complex. Some taxa (e.g., neogregarines) may undergo bouts of asexual reproduction known as merogony. **3** Trophozoites pair in preparation for sexual reproduction (syzygy) **4** A gametocyst wall forms around the pair of trophozoites; each trophozoite is a gamont **5** The gamonts become multinucleated and each nucleus becomes a gamete **6** An oocyst wall forms around each zygote which undergoes meiosis to yield four haploid sporozoites. Some gregarines will undergo additional rounds of mitosis to increase the number of sporozoites per oocyst. **7** The oocysts are shed back into the environment, typically through host defecation, and ruptures to release the sporozoites upon ingestion by the next host.

Trophozoites can feed directly on host cells (e.g., *Selenidium* spp. via myzocytosis; Leander and Keeling, 2003) while others are inferred to use their large surface areas created by arrays of longitudinal folds to absorb pre-digested nutrients (e.g., *Platyproteum vivax*; Leander, 2008a). Trophozoite morphology is highly variable and differs taxonomically. In archigregarines, the sporozoites and trophozoite stages closely resemble one another, whereas, eugregarine trophozoites lose the apical complex and use a mucron (aseptate) or epimerite (septate) for attachment to the host. Parasitism by gregarine trophozoites is historically held to be quite benign unless the host suffers from other factors contributing to lowered immunity or malnourishment. In some cases, however, gregarine infections have been shown to lower host fecundity (Zuk, 1987), territoriality (Marden and Cobb, 2004), and cause metabolic syndrome (Schilder and Marden, 2006). The trophozoites continue to feed and grow in size until they have acquired enough energy to reproduce.

Asexual reproduction during the haploid trophozoite life stage is known as merogony, but for most gregarine species this process has never been observed. Merogony is, however, quite common in other apicomplexans (e.g., malaria) and is a major factor in how debilitating the infection is on the host. Trophozoites prepare for sexual reproduction by conjoining in pairs during a process known as syzygy. Each trophozoite in the pair is referred to as a gamont. The alignment of gamonts is variable across species with some species aligning head to head while others will align head to tail, tail to tail, or side by side. Some gregarine species spend prolonged periods of time in syzygy before reproducing (e.g., *Pterospora*; Leander *et al.*, 2006). A gametocyst wall forms around the gamont pair and each gamont becomes multinucleated. The multinucleated gamonts eventually break and form membranes around each nucleus to form

gametes during gametogeny. Gametes from one gamont will find compatible gametes from the other and form diploid zygotes. Oocyst walls form to protect each zygote. Within the oocysts, the zygotes undergo meiosis to produce four haploid sporozoites. Depending on the species, these sporozoites undergo rounds of mitosis to increase the number contained within a single oocyst. The oocysts containing sporozoites are shed to the environment either through host feces or through host death and decay where it will passively be transmitted to the next host.

1.5 Marine gregarine systematics

The diversity of marine gregarines is high and environmental DNA sequencing surveys have consistently recovered sequences of gregarine origin (Leander, 2008b). However, these gregarine sequences have been difficult to identify past broad taxonomic ranks due to the lack of any similar sequences with proper accompanying species descriptions. The 18S SSU rDNA sequence has been the primary target for gregarine molecular phylogenetic analyses and has been incredibly informative in giving the environmental sequences appropriate identities. Furthermore, SSU rDNA sequence data support the several distinct groups of marine gregarines.

Archigregarines are consistently found at the base of the apicomplexan tree and consist of three main genera: *Selenidium*, *Platyproteum*, and *Filipodium* (Rueckert and Leander, 2009b). All three genera are capable of bending movements characteristic to archigregarines and have no gliding motility. *Selenidium* spp. also have trophozoites that use their apical complex to parasitize host intestinal epithelium via myzocytosis. All three genera have been found in the intestinal lumen of their invertebrate hosts except *S. melongena* which was isolated from the

coelomic space of a marine worm (Wakeman *et al.*, 2014). The discovery of *S. melongena* suggests that a switch in the host compartment can drive speciation in gregarines. In the case of *Selenidium*, it is conceivable that some sporozoites may have burrowed too far into the host intestine and, rather than attaching to the epithelial lining of the intestinal lumen, found themselves travelling through the intestinal wall into the coelomic space beyond.

Urosporidians are a clade of aseptate marine eugregarines that also inhabit coelomic spaces (Coulon and Jangoux, 1987; Landers and Leander, 2005; Leander *et al.*, 2006). *Pterospora* spp. are particularly peculiar in that they do not glide in the typical eugregarine fashion, but instead, move by peristaltic pulsations. The trophozoites of *Pterospora* are usually found as gamonts in the coelom of bamboo worms and roam through the coelomic spaces in a prolonged state of syzygy. In place of typical epicytic folds, *Pterospora* has distinct regions that are either smooth or covered in clusters of pellicular ridges. *Lithocystis* is another urosporidian genus which lacks epicytic folds and is instead covered by pits and ridges. Both *Pterospora* and *Lithocystis* also lack a mucron or epimerite which are attachment structures commonly employed by other eugregarine taxa. Although function has not been assigned to the divergent morphology of urosporidians, their peculiarities are inferred to be adaptations to parasitism in the coelom.

Polyplicarium is another genus of aseptate marine eugregarines that forms its own clade (Wakeman and Leander, 2013a) and conforms more to the typical set of eugregarine traits. *Polyplicarium* spp. have been described from capitellid polychaetes and form a sister clade to the terrestrial gregarine clade II. The general morphology for the genus consists of trophozoites with a blunt mucron and ranging in overall length between 50 μm and 200 μm. Some species have an

elongated body that tapers at the posterior end whereas others have a round posterior end. The genus derives its name from the dense array (4–5/μm) of longitudinal epicytic folds found on the surface of trophozoites. The depth of the epicytic folds are wider and shallower on some distinct regions of the cell surface. Consistent with the presence of the epicytic folds, *Polyplicarium* trophozoites are capable of gliding motility.

Trichotokara is a clade of aseptate marine eugregarines first discovered in polychaete hosts (Rueckert *et al.*, 2013). *Trichotokara* species have divergent SSU rDNA sequences when compared even to derived non-gregarine apicomplexans and display high morphological variation. Some species are giants reaching almost 600 μm while others remain close to the average marine gregarine length of around 150 μm. *Trichotokara eunicae* trophozoites are shaped like tadpole larvae and possess epicytic folds. Their mucron is free of paraglycogen granules and protrudes slightly. In contrast, *T. japonica* is rhomboidal and possesses a mucron cover in hair-like projections. The apex of the mucron consists of a unique antler-like projections similar to the apical protrusion seen in its sister species *T. nothriae*. Function is again difficult to discern for the highly modified morphologies of *Trichotokara*. The genus shares a sister relationship with the lecudinids which represents the bulk of described marine gregarines.

The Lecudinidae Kamm 1922 is a poorly defined family of aseptate eugregarines to which most aseptate marine eugregarines belong (Levine, 1977). There are 25 genera of lecudinids recognized within the Lecudinidae representing roughly 90 named species. About 40 of these named species belong to the single genus *Lecudina*. Although improved resolution of lecudinid phylogeny has been achieved through the integration of molecular data with detailed

morphological data, deepest relationships within the family are still unresolved. The discovery of additional taxa, however, have continually revealed subclades within the Lecudinidae and indicate progress towards taxonomic clarity (e.g., Rueckert *et al.*, 2010, 2013).

Paralecudina, for instance, was a monotypic genus that was initially formed by moving *L. polymorpha* from the Lecudinidae after molecular phylogenetic analyses of SSU rDNA sequences revealed that this species did not fall within the *Lecudina* clade (Rueckert *et al.*, 2013). This was corroborated each time new lecudinid taxa such as *Difficilina* spp. grouped cleanly into subclades within the Lecudinidae (Rueckert *et al.*, 2010), but *L. polymorpha* always associated with unidentified environmental sequences. With the discovery of the *Trichotokara* clade, the sister relationship between *Trichotokara* and *L. polymorpha* became even clearer and as such was renamed *Paralecudina polymorpha*.

Two new species of aseptate marine eugregarines are described in the current thesis with accompanying formal taxonomic descriptions. One is a new *Paralecudina* species and is the only one found since the inception of the genus. The other is a new *Lecudina* species which forms a subclade within the marine lecudinids together with one other *Lecudina* species. The morphologies of the two species are incongruent with the divergences of their 18S SSU rDNA sequences when compared to major representative groups of apicomplexans. The establishment of the two new species, *P. anankea* n. sp. and *L. caspera* n. sp., was based on strongly supported molecular phylogenies. The discovery of these two species also helps to illustrate the challenges associated with assigning taxonomical sense to gregarine morphology and the indispensability of molecular data in apicomplexan systematics.

Chapter 2: Material and methods

2.1 Host and parasite collection

The annelid host *Lumbrineris inflata* Moore 1911 was collected at low tides during Fall of 2015 and Summer of 2016 from Clover Point (48°24’14.18”N 123°21’00.91”W), British Columbia, Canada. The geography of the beach consists of a sheltered, rocky topography interspersed with patches of eelgrass beds. The hosts are infaunal and often burrow in the sediment between the roots of eelgrass, thus could be haphazardly collected by pulling tufts of eelgrass by the roots. The hosts were isolated from the sediments by vigorously rinsing the eelgrass in a bucket of seawater. The collected hosts were placed in plastic bags, stored on ice, and transported to a holding tank at the University of British Columbia. All hosts were dissected within the first week of collection.

Individual trophozoites from *P. anankea* n. sp. and *L. caspera* n. sp. were collected from the hosts via dissection. A single host worm was placed in a Petri dish filled with filtered seawater and split longitudinally with fine forceps to spill their gut content. Gregarine trophozoites were located among food particles and digestive debris using a Leica (Wetzlar, Germany) DM IL inverted microscope. Each trophozoite was isolated using hand-drawn glass pipettes**.** Every trophozoite was washed three times with filtered seawater in a well slide and set aside to be prepared for light microscopy, scanning electron microscopy, and DNA extraction.

2.2 Light and scanning electron microscopy

Light micrographs of both *P. anankea* n. sp. and *L. caspera* n. sp. were taken in differential interference contrast (DIC) with a Zeiss Axioplan 2 microscope (Carl-Zeiss, Göttingen, Germany) paired to a Zeiss Axiocam 503 color camera (Carl-Zeiss, Göttingen, Germany). Trophozoites were placed in a drop of filtered seawater on a glass slide and held down with a cover slip. To ensure that the trophozoites did not burst under the weight of the glass cover slip, the cover slips were heightened off of the slide by applying a small amount of Vaseline to the edges. Each light micrograph was edited on Adobe Photoshop 11 to be desaturated of color and presented on the same grey background.

Trophozoites from each new gregarine species were isolated from *L. inflata* and fixed separately for SEM using 24-well tissue culture plates and plastic capsules to hold and move the trophozoites between fixation steps. The plastic capsules were made with TEM embedding capsules by cutting off the bottoms, creating a hollow cylinder, and adding 50 μm mesh to cover one of the open ends. The mesh was held in place by pinching it between the capsule and an appropriately sized TEM embedding capsule cap. The customized capsules were submerged in the wells of the tissue culture plates filled with filtered seawater plus a drop of 2.5% glutaraldehyde. Trophozoites were transferred to these capsules using hand-drawn glass pipettes and the remaining open end was closed with more mesh and a cap. The trophozoites were left to fix in the 2.5% glutaraldehyde for 30 minutes in the dark, on ice. Each capsule holding trophozoites was then moved to an adjacent well and was rinsed with 0.1M sodium cacodylate ($(CH_3)_2AsO_2Na$) and left to soak for several minutes. The capsules were moved to the next well

filled with filtered seawater plus three drops of 1% OsO_4 and left to soak for 30 minutes in the dark, on ice. Each capsule was rinsed and soaked again with 0.1M sodium cacodylate. The trophozoites were then dehydrated with serial ethanol baths by submerging the capsules for three minutes at 30%, 50%, 75%, 85%, 95%, and 100% dilutions. Following the ethanol baths, the capsules were placed in a Tousimis Autosamdri® 815B critical point dryer for CO_2 dehydration. Individual trophozoites were transferred from the 50μm mesh onto SEM stubs using an eyelash glued to a glass pipette and then sputter coated with 6nm of gold/palladium alloy. SEM images were taken on a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA) and edited on Adobe Photoshop 11 to be presented on a black background.

2.3 DNA extraction, amplification, and sequencing

Trophozoites of *P. anankea* n. sp. were pooled into four samples on the basis of individual hosts. The number of trophozoites pooled from a single host often ranged between six to ten cells. All trophozoites collected from a single host, were washed three times with filtered, autoclaved seawater and placed in 1.5 ml Eppendorf tubes with 4μl of molecular grade distilled water. Trophozoites of *L. caspera* n. sp. were less numerous per host than *P. anankea* n. sp. and separating them by individual hosts posed challenges to steps after DNA extraction due to the low concentration of DNA. Therefore, approximately six *L. caspera* n. sp. trophozoites from multiple hosts were pooled into two separate samples in 1.5 ml Eppendorf tubes filled with 4 μl of molecular grade distilled water after three washes with filtered and autoclaved seawater. Genomic DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Manufacturer protocols were followed except for the

final step in which the DNA was eluted in 8µl of EB buffer, in an effort to concentrate the DNA, instead of the suggested 40 µl.

The 18S (small subunit) ribosomal DNA sequences were targeted for this study to be used in subsequent species delimitation and phylogenetic analysis. The 18S regions from *P. anankea* n. sp. and *L. caspera* n. sp. were amplified differently due to the aforementioned challenges with amplifying and sequencing the latter. Extracted *P. anankea* n. sp. DNA was initially amplified by a polymerase chain reaction (PCR) targeting the entire 18S region using the universal eukaryote primers PF1 5' – CGCTACCTGGTTGATCCTGCC – 3' and SSUR4 5' – GATCCTTCTGCAGGTTCACCTAC – 3' primers (Leander *et al.*, 2003a) with PCR beads (Illustra, PuReTaq Ready-To-Go PCR beads, GE Healthcare, Quebec, Canada). The following thermal cycle was used for the PF1 and SSUR4 PCR: initial denaturation at 95^{o}C for 5:00mins followed by 35 cycles of denaturation at 95^{o}C for 30s, annealing at 51^{o}C for 30s, extension at 72^{o}C for 2:00mins, and a final extension at 72^{o}C for 5:00mins. The product from this initial amplification was used for a second round of nested PCRs using internal primers.

The nested PCR design was used to reduce the possibility of sequencing contaminant host material amplified by the general PF1 and SSUR4 primers. The primers used for *P. anankea* n. sp. were LecuF 5' – GTDAATCGGCGTGTTCYACG – 3' and LecuR 5' – GAATGCCCTCARCCGTTC – 3' (Rueckert *et al.*, 2015). The thermal cycles employed were as follows: initial denaturation at 95^{o}C for 5:00mins followed by 25 cycles of denaturation at 95^{o}C for 30s, annealing at 54^{o}C for 30s, extension at 72^{o}C for 1:30mins, and final extension at 72^{o}C for 5:00mins. The products were screened on an 1% agarose gel to confirm that the reactions

yielded expected sequence lengths. Bands were cut from the gels using a sterile razor and cleaned using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Although it is technically possible at this step to sequence the products from the nested PCRs, prior attempts to do so were hampered by noisy or ambiguous peaks on resultant chromatograms. Therefore, the nested PCR products were further amplified and purified for this study by cloning with a TOPO TA cloning kit (Invitrogen, Frederick, MD) according to manufacturer protocols. Eight clone colonies were isolated for each species and amplified with TOPO vector primers in a 25-µl reaction with EconoTaq 17" Master Mix (Lucigen Corp. Middleton, WI). The thermal cycles used in this reaction were as follows: initial denaturation at 94ºC for 2:00mins followed by 25 cycles of denaturation at 94ºC for 30s, annealing at 52ºC for 30s, and extension 72.0ºC for 1:30 min, and final extension at 72.0ºC for 5:00mins. The clone colonies were then fingerprinted using an HAE III digestion reaction and clone variants were sequenced using the cloning vector primers in an ABI BigDye® reaction mix.

The 18S region of *L. caspera* n. sp. could not be amplified with commonly used universal eukaryote primers including PF1, SSUR4, 1050MRD (Chantangsi and Leander, 2010), and 1391R (Turner *et al.*, 1999). Therefore, a universal gregarine forward primer and a universal apicomplexan reverse primer were designed for this study to target the 18S rDNA sequence. The forward primer (65FDeGr) sequence was 5' – YDAARCTGCGRAKRGCTCAT -3' and was paired with the reverse primer (1958RAp) 5' – TGTGTACAAAGGGCAGGGAC -3'. The thermal cycle employed was as follows: initial denaturation at 95ºC for 5:00mins followed by 45 cycles of denaturation at 95ºC for 30s, annealing at 54.9ºC for 30s, extension at 72ºC for

2:00mins, and a final extension at 72°C for 5:00mins. The nested PCRs and cloning steps were not performed for the amplification of the 18S rDNA sequence in *L. caspera* n. sp. Instead, the following six internal primers were used for the sequencing reactions of the 18S amplicon in an ABI BigDye®: 648F: 5'– CGCGGTAATTCCAGCTTCA -3'; 648R: 5'-TGGAGCTGGAATTACCGCG-3'; 1300F: 5'-ATGGTTGCAAGACTGAAACT-3'; 1300R: 5'-AGTTTCAGTCTTGCAACCAT-3'; 1321F: 5'-AAAGGAATTGACGGAAGGGCA-3'; and 1321R: 5'-TGCCCTTCCGTCAATTCCTTT-3'. Because Sanger sequencing with a dye-terminator experiences a drop in base calling accuracy past ~800 bp from the primer binding site, the internal sequencing primers yield more reliable regions of an amplicon. These regions can subsequently be assembled into one higher quality contig.

2.4 Molecular phylogenetic analysis

The phylogenetic positions of *P. anankea* n. sp. and *L. caspera* n. sp. were determined using a 74-taxon alignment of 18S rDNA sequences, including three dinoflagellate sequences (outgroup) and representatives from the major clades of apicomplexans. The 18S rDNA sequences for *P. anankea* n. sp. and *L. caspera* n. sp. were aligned using the MAFFT algorithm (Katoh *et al.*, 2002) on Geneious version 10.0.2 (Kearse *et al.*, 2012). The MAFFT algorithm was chosen over others for its ability to account for the secondary structure of ribosomal subunits. Ambiguously aligned regions and gaps were cut from the final alignment using Aliscore version 2.0 (Mlsof and Katharina, 2009; Kück *et al.*, 2010) and Alicut version 2.3. The resulting alignment included 1,512 unambiguously aligned sites.

The GTR+I+Γ model (proportion of invariable sites = 0.2020, gamma shape = 0.7330) was selected by jModelTest version 2.1.10 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) for maximum likelihood and Bayesian analyses under Aikaike Information Criterion (AIC). The maximum likelihood (ML) tree and ML bootstrap values were inferred using RAxML version 8.2.9 (Stamatakis, 2014) through the Cipres Science Gateway version 3.3 (Miller *et al.*, 2010). Bayesian posterior probabilities were calculated using Mr. Bayes version 3.2.6 (Ronquist *et al.*, 2012) using the GTR substitution model with invariable sites over a gamma distribution (lset nst = 6, rates = invgamma) and Monte Carlo Markov Chains (MCMC) run with the following parameters: 10,000,000 generations (ngen = 10000000), 2 runs (nruns = 2), 4 chains (nchains = 4), temperature parameter at 0.2 (temp = 0.200), sample frequency of 100, prior burn-in of 0.25 of sampled trees, and a stop rule of 0.01 to terminate the program when the split deviation fell below 0.01.

Chapter 3: Results

3.1 Morphology of *Paralecudina anankea* n. sp.

The trophozoites of *P. anankea* n. sp. were spindle-shaped with the anterior end forming an elongated compartment about 67 μm long; the morphological measurements are averages between the two representatives trophozoites shown in the LM and SEM (Fig.1). The cell was approximately 234 μm in length and 38 μm in width at the broadest point. Trophozoites were light brown overall, but translucent at the edges all around. The oval, centrally located nucleus was entirely translucent and ran approximately 18 μm along the major axis and 13 μm along the minor axis. The trophozoites displayed gliding motility in a single direction and were capable of folding lengthwise multiple times. A permanent, superficial fold ran transversely which separated the cell into a posterior region comprising the nucleus and an anterior region that forms the mucron. Epicytic folds ran longitudinally down the entire length of the trophozoite at a density of six folds per micron. The depth of each epicytic fold appeared uniform throughout the cell. Some trophozoites were covered in host sperm and other organic material.

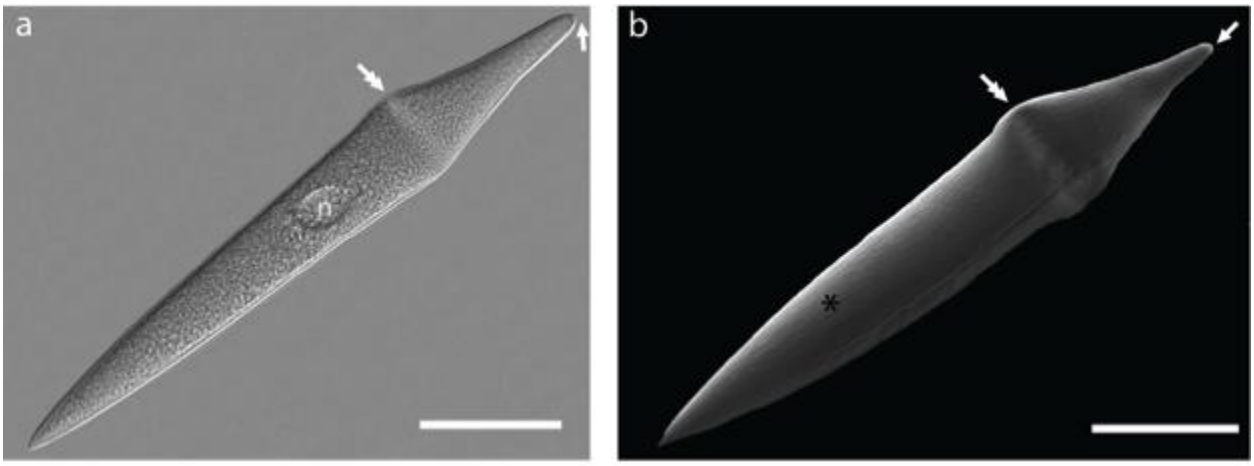

Figure 3 – Light micrograph (LM) and scanning electron micrographs (SEM) of *Paralecudina anankea* n. sp. trophozoite morphology and ultrastructure. **a** LM of spindle-shaped trophozoite taken in differential interference contrast (DIC). An oval nucleus (n) is visible located centrally within the cell. A superficial fold (double arrow) running transversally separates the posterior region including the nucleus with the anterior region. **b** SEM of the trophozoite showing the transverse fold. Epicytic folds (*) run down the entire length of the cell longitudinally. *Scale bars*: a, b = 50 μm.

3.2 Morphology of *Lecudina caspera* n. sp.

Lecudina caspera n. sp. trophozoites were generally large and acorn-shaped measuring 236 μm in length and 66 μm at the narrowest point; the morphological measurements are averages between the two representatives trophozoites shown in the LM and SEM (Fig. 2). The cells were brown and coloration appeared denser compared to *P. anankea* n. sp. A circular nucleus with a ~28 μm diameter was situated at a transverse constriction at the base of the mucron. The cells were capable of gliding forward and were rigid; they were never observed to contract, elongate, or fold. A permanent constriction was visible around the nucleus which demarcated the posterior region from a round, stub-nosed mucron. The anterior-most portion of the mucron ended with a

nipple-like protrusion which was the only translucent structure on the trophozoite. Epicytic folds lined the entire length of the trophozoite at a density of four folds per micron. The depth of each epicytic fold were uniform across the cell.

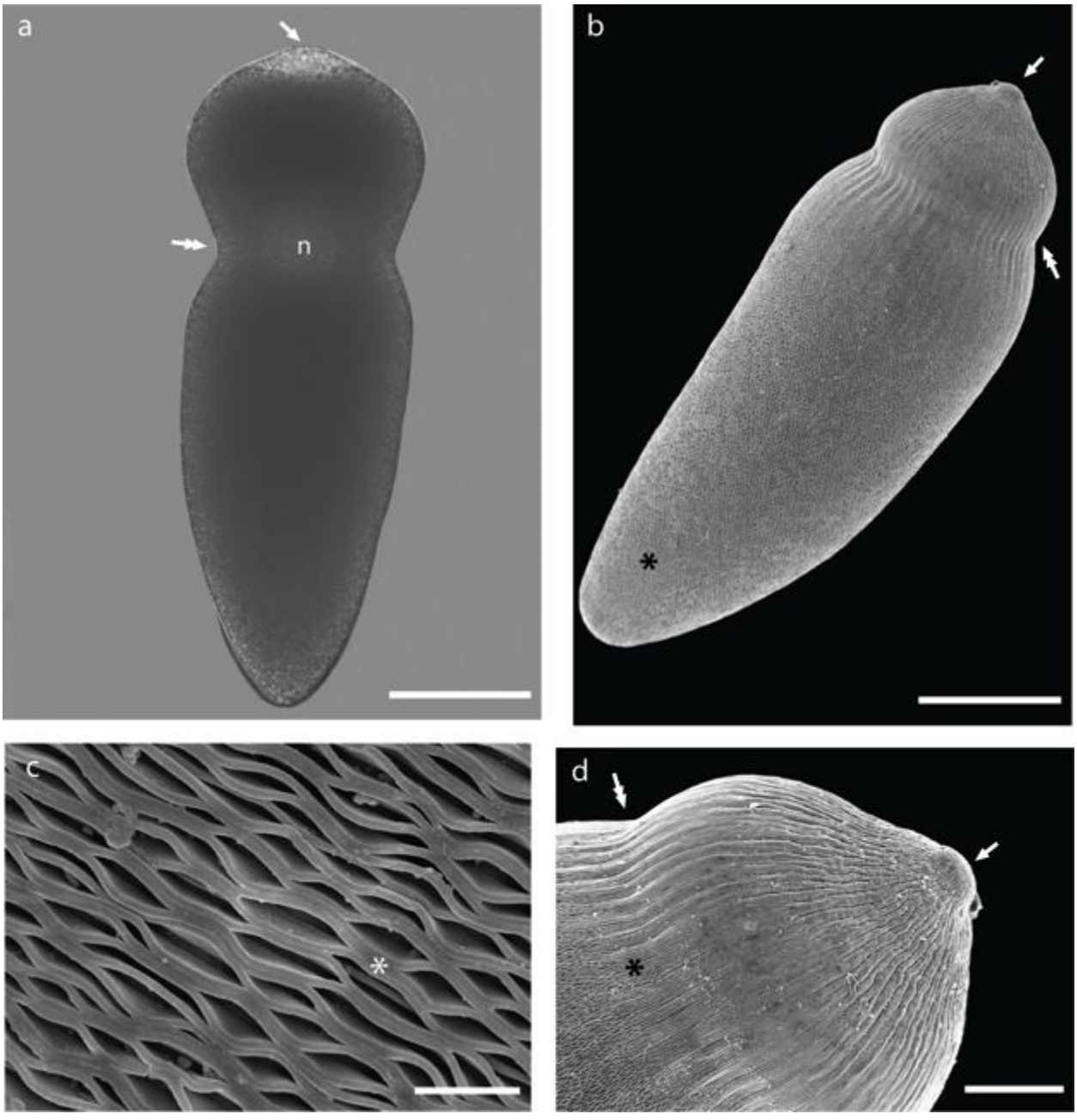

Figure 4 – Light micrograph (LM) and scanning electron micrographs (SEM) of *Lecudina caspera* n. sp. trophozoite morphology and ultrastructure. **a** LM of the acorn-shaped trophozoite in differential interference contrast (DIC). A circular nucleus (n) is visible located at a transverse constriction (double arrow) of the cell. The constriction divides the cell into two regions. The mucron ends anteriorly with a translucent, nipple-like protrusion (single arrow). **b** SEM of the trophozoite showing the constriction, mucron, and anterior protrusion. Epicytic folds (*) are also seen running along the entire length of the trophozoite. **c** SEM of epicytic folds close up. **d** SEM of the anterior end (arrow) of a trophozoite. *Scale bars*: a, b = 50 μm; c = 2 μm; e = 30 μm.

3.3 Molecular phylogenetic analysis of SSU rDNA sequences

The 74-taxon alignment of SSU rDNA sequences yielded a strongly supported outgroup of dinoflagellates (95 MLB, 0.99 BPP) and an ingroup of apicomplexans with a poorly resolved backbone (Fig. 3). Both maximum likelihood and Bayesian analyses recovered identical tree topologies. The apicomplexan backbone gave rise to piroplasmid, coccidian, rhytidocystid, cryptosporidian, and gregarine clades. The "archigregarines" were paraphyletic with *Platyproteum vivax* and *Filipodium phascolosomae* forming the most basal apicomplexan branch while *Selenidium* spp. form a second group. Two terrestrial gregarine clades were also recovered. Terrestrial gregarine clade I was well supported (92 MLB, 1.00 BPP), but included genera comprised of species with extremely short branches. The clade also included environmental sequences (AF372779 and AY179975) acquired from marine environmental PCR surveys of biodiversity. The support for terrestrial gregarine clade II was robust (100 MLB, 1.00 BPP) and included only gregarines described from terrestrial hosts. The marine gregarines formed clades composed of members that infect similar hosts. For example, the capitellid gregarines, urosporids, and lecudinid subgroups recovered from tunicates all grouped separately. The resolution amongst members within each clade, however, was quite poor.

The paralecudinid clade was highly supported (100 MLB, 1.00 BPP) within which there were two species, *P. anankea* n. sp. and *P. polymorpha*, and an unidentified environmental sequence (AB252765). *P. anankea* n. sp. and *P. polymorpha* were recovered as sister species (64 MLB, 0.99 BPP). The two sister species were divergent by 12% in SSU rDNA sequences. *L. caspera* n. sp. had robust support for forming the sister to *L. longissima* (100 MLB, 1.00 BPP). The two sequences were 8% divergent.

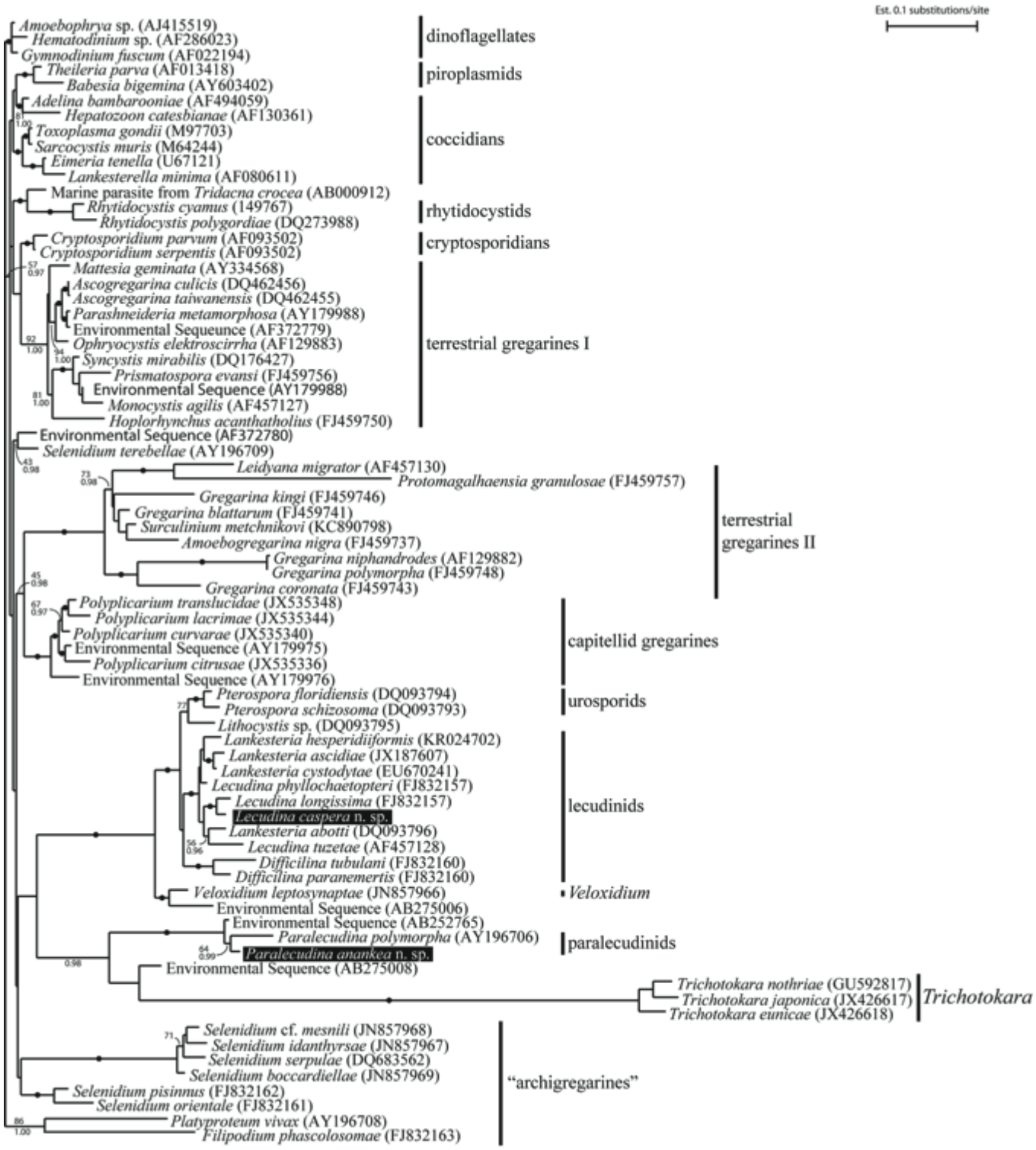
Est. 0.1 substitutions/site
Amoebophrya sp. (AJ415519)
Hematodinium sp. (AF286023)
Gymnodinium fuscum (AF022194)
dinoflagellates
Theileria parva (AF013418)
Babesia bigemina (AY603402)
piroplasmids
Adelina bambarooniae (AF494059)
Hepatozoon catesbianae (AF130361)
Toxoplasma gondii (M97703)
Sarcocystis muris (M64244)
Eimeria tenella (U67121)
Lankesterella minima (AF080611)
coccidians
Marine parasite from Tridacna crocea (AB000912)
Rhytidocystis cyamus (149767)
Rhytidocystis polygordiae (DQ273988)
rhytidocystids
Cryptosporidium parvum (AF093502)
Cryptosporidium serpentis (AF093502)
cryptosporidians
Mattesia geminata (AY334568)
Ascogregarina culicis (DQ462456)
Ascogregarina taiwanensis (DQ462455)
Parashneideria metamorphosa (AY179988)
Environmental Sequeunce (AF372779)
Ophryocystis elektroscirrha (AF129883)
Syncystis mirabilis (DQ176427)
Prismatospora evansi (FJ459756)
Environmental Sequence (AY179988)
Monocystis agilis (AF457127)
Hoplorhynchus acanthatholius (FJ459750)
terrestrial gregarines I
Environmental Sequence (AF372780)
Selenidium terebellae (AY196709)
Leidyana migrator (AF457130)
Protomagalhaensia granulosae (FJ459757)
Gregarina kingi (FJ459746)
Gregarina blattarum (FJ459741)
Surculinium metchnikovi (KC890798)
Amoebogregarina nigra (FJ459737)
Gregarina niphandrodes (AF129882)
Gregarina polymorpha (FJ459748)
Gregarina coronata (FJ459743)
terrestrial gregarines II
Polyplicarium translucidae (JX535348)
Polyplicarium lacrimae (JX535344)
Polyplicarium curvarae (JX535340)
Environmental Sequence (AY179975)
Polyplicarium citrusae (JX535336)
Environmental Sequence (AY179976)
capitellid gregarines
Pterospora floridiensis (DQ093794)
Pterospora schizosoma (DQ093793)
urosporids
Lithocystis sp. (DQ093795)
Lankesteria hesperidiiformis (KR024702)
Lankesteria ascidiae (JX187607)
Lankesteria cystodytae (EU670241)
Lecudina phyllochaetopteri (FJ832157)
Lecudina longissima (FJ832157)
Lecudina caspera n. sp.
Lankesteria abotti (DQ093796)
Lecudina tuzetae (AF457128)
Difficilina tubulani (FJ832160)
Difficilina paranemertis (FJ832160)
lecudinids
Veloxidium leptosynaptae (JN857966)
Environmental Sequence (AB275006)
Veloxidium
Environmental Sequence (AB252765)
Paralecudina polymorpha (AY196706)
Paralecudina anankea n. sp.
paralecudinids
Environmental Sequence (AB275008)
Trichotokara nothriae (GU592817)
Trichotokara japonica (JX426617)
Trichotokara eunicae (JX426618)
Trichotokara
Selenidium cf. mesnili (JN857968)
Selenidium idanthyrsae (JN857967)
Selenidium serpulae (DQ683562)
Selenidium boccardiellae (JN857969)
Selenidium pisinnus (FJ832162)
Selenidium orientale (FJ832161)
Platyproteum vivax (AY196708)
Filipodium phascolosomae (FJ832163)
"archigregarines"

Figure 5 – Maximum likelihood tree inferred from a 74 taxon dataset of SSU rDNA sequences with 1,512 unambiguously aligned sites using the GTR+I+ Γ model of substitution (-ln L = 35245.7137, gamma shape = 0.7330, proportion of invariable sites = 0.2020). Numbers denote support values with the top values indicating bootstrap support and the bottom indicating Bayesian posterior probabilities. The black dots were used on branches when both bootstrap support and Bayesian posterior probabilities were equal to or greater than 95 and 99 respectively. Support values were excluded from this tree when both bootstrap support and Bayesian posterior probabilities fell below 55 and 0.95 respectively for any given branch. The new species described in the current study is highlighted with black boxes.

3.4 Formal taxonomic descriptions

Phylum Apicomplexa Levine, 1970

Order Eugregarinorida Léger, 1900

Family Lecudinidae Kamm, 1922

Paralecudina anankea n. sp. Iritani, Wakeman, and Leander

Diagnosis Trophozoites are spindle shaped with an elongated anterior compartment compared to typical marine aseptate eugregarines. Cells approximately 230 μm in length and 34 μm in width at the broadest region. Cells are light brown except around the edges that are transparent. Both anterior and posterior ends taper to a point. Nucleus positioned centrally and is oval (major axis = 18 μm, minor axis = 13 μm). Permanent superficial runs transversally at anterior third of trophozoite. Gliding motility. Longitudinal epicytic folds with a density of 6/μm. Epicytic folds of even depth throughout the trophozoite.

DNA sequence SSU rDNA gene sequence (GenBank XXXXXXXXX).

Type locality Clover Point (48°24’14.18”N 123°21’00.91”W), British Columbia, Canada. Host in sand between roots of seagrass; lower intertidal; 0.30 m above mean sea level.

Type habitat Marine.

Type host *Lumbrineris inflata* Moore, 1911 (Annelida, Polychaeta, Eunicida, Lumbrineridae). The host was barcoded with the 18S rDNA sequence and tissue has been preserved and deposited with the parasite hapantotypes in the Beaty Biodiversity Museum.

Location in host Intestinal lumen.

Iconotype Figure 3

Hapantotype Trophozoites on SEM stubs with 6 nm of gold/palladium alloy sputter coat have been deposited in the Beaty Biodiversity Museum.

Etymology The species name, *anankea*, refers to the female figure Ananke from Greek mythology who is often depicted holding a spindle which resembles the shape of the trophozoites found in this species.

Lecudina caspera n. sp. Iritani and Leander

Diagnosis Trophozoites acorn shaped with a nipple-like mucron. Cells approximately 232 μm in length and 62 μm in width at the narrowest region. Cells are brown to dark brown. Anterior compartment is bulbous and circular, whereas posterior compartment tapers to a blunt end. Circular nucleus (diameter = 28 μm) positioned at the anterior third where trophozoite constricts transversally. Gliding motility. Longitudinal epicytic folds with a density of 4/μm. Epicytic folds of even depth throughout the trophozoite.

DNA sequence SSU rDNA gene sequence (GenBank XXXXXXXXX).

Type locality Clover Point (48°24’14.18”N 123°21’00.91”W), British Columbia, Canada. Host in sand between roots of seagrass; lower intertidal; 0.30 m above mean sea level.

Type habitat Marine.

Type host *Lumbrineris inflata* Moore, 1911 (Annelida, Polychaeta, Eunicida, Lumbrineridae).

Location in host Intestinal lumen.

Iconotype Figure 4

Hapantotype Trophozoites on SEM stubs with 6 nm of gold/palladium alloy sputter coat have been deposited in the Beaty Biodiversity Museum. The host was barcoded with the 18S rDNA sequence and tissue has been preserved and deposited with the parasite hapantotypes in the Beaty Biodiversity Museum.

Etymology The species name, *caspera*, refers to the resemblance of the trophozoite to the body shape of Casper the Friendly Ghost from the 1995 film. The suffix -a assigns a Greek, female form to the species name for consistency with *Lecudina*.

Chapter 4: Discussion

Comparative morphology and molecular analyses of SSU rDNA indicate that both gregarines found in this study are novel species. Furthermore, the host (*Lumbrineris inflata*) in which both *P. anankea* n. sp. and *L. caspera* n. sp. were found has not been previously investigated for gregarines. Both *P. polymorpha* and *L. longissima***,** the sister species to *P. anankea* n. sp. and *L. caspera* n. sp., were interestingly discovered from a similar host: *Lumbrineris japonica* (Rueckert *et al.*, 2010). The literature for gregarine taxonomy, however, is badly scattered and original descriptions of many species are difficult to find. Many of these original descriptions when found only include line drawings and are lacking in molecular data. Comparisons between historical and contemporary literature were made possible with the revival of gregarine taxonomy in recent years and efforts have been made to pair morphology with molecular data to increase taxonomic resolution and improve species delimitation.

Morphology alone is often insufficient for species identification of gregarines. Although it can serve, with experience, as a useful tool for making initial taxonomic inferences, DNA data remains indispensable. Trophozoites can be morphologically plastic (e.g., the morphotypes of *P. polymorpha*; Leander *et al.*, 2003a) depending on various factors. Trophozoite shape in many cases are also dynamic (e.g., *Pterospora schizosoma*; Leander *et al.*, 2006) and size ranges are large depending on growth conditions within the host. There are only several morphological characters that can be easily compared amongst gregarine taxa, especially without the use of high-resolution microscopy, and these several traits often vary widely enough to render visual comparisons of trophozoites taxonomically uninformative. The advantage of using SSU rDNA

sequence data lies in this plasticity not being directly represented within the sequences, thus, alleviating the challenge associated with visually identifying morphotypes.

4.1 Paralecudina anankea n. sp.

Paralecudina was originally established with the discovery of *Trichotokara* (Rueckert *et al.*, 2013). Molecular phylogenies which included SSU rDNA sequences from various *Lecudina* spp. and *Trichotokara* spp. revealed a clade composed of environmental sequences, *Trichotokara*, and what is now *Paralecudina*. This split of *Paralecudina* from *Lecudina* left the remaining lecudinids and urosporids together in a separate clade. *Paralecudina* consists of one other species with two known morphotypes referred to simply as morphotype 1 and morphotype 2 (Leander *et al.*, 2003b; Rueckert *et al.*, 2010). Trophozoites of both morphotypes are described as rigid and capable of gliding motility. The posterior end tapers to a distinct point whereas the anterior is constituted by an elongated mucron. An oval nucleus is situated within the anterior half of the trophozoite. Morphotype 2 displays long, tapering bodies with a distinct bulge around the anterior third of the trophozoite. With the exception of nucleus position, *P. anankea* n. sp. shares all of the above morphological characters with *P. polymorpha* and is especially similar to morphotype 2. These morphological similarities support the placement of *P. anankea* n. sp. within the genus.

Several key morphological differences between *P. polymorpha* and *P. anankea* n. sp., however, indicate that *P. anankea* n. sp. is not simply a third morphotype of *P. polymorpha*. A stark contrast in morphology lies in the distinct transverse fold seen in the anterior region of *P.*

anankea n. sp. trophozoites. The fold has not been observed in either *P. polymorpha* morphotypes and is perhaps a synapomorphy for *P. anankea* n. sp. *Paralecudina anankea* n. sp. trophozoites are also flexible and folding along the transverse axis was often observed in addition to gliding motility. Additionally, the nucleus is located more centrally in the body. *P. anankea* n. sp. differs also in size with trophozoites measuring ~230 μm whereas *P. polymorpha* ranges in size between 175 to 300 μm (morphotype 1) and 475 to 575 μm (morphotype 2). The epicytic folds on *P. anankea* n. sp. trophozoite are arranged at a density of six folds per micron in comparison to *P. polymorpha* which has three folds per micron (morphotype 1) or five folds per micron (morphotype 2). The contrasting morphologies between *P. anankea* n. sp. and *P. polymorpha* trophozoites suggests the necessity of establishing a new species within the *Paralecudina* genus.

The molecular data is also in strong support of *P. anankea* n. sp. as a new species and gives robust support to the whole paralecudinid clade (100 MLB, 1.00 BPP) which includes *P. anankea* n. sp., *P. polymorpha*, and an unidentified environmental sequence (AB252765). The molecular phylogenetic analysis recovered the sister relationship between *P. anankea* n. sp. and *P. polymorpha* (64 MLB, 0.99 BPP) and the SSU rDNA sequences were 12% divergent between the two. Marine gregarine SSU rDNA sequences that are more than 10% is considered highly divergent (Rueckert *et al.*, 2013). These molecular data, in combination with the aforementioned morphological characters, justify the establishment of *P. anankea* n. sp. as a distinct, new sister species to *P. polymorpha*.

4.2 Lecudina caspera n. sp.

Lecudina is a taxonomically problematic genus inferred to be paraphyletic and giving rise to many other marine gregarines (Leander *et al.*, 2003a; Rueckert *et al.*, 2013). As such, identifying morphological synapormorphies for *Lecudina* spp. is difficult. In general, *Lecudina* trophozoites are rigid, capable of gliding motility, and have epicytic folds (Levine, 1976). To this end, *L. caspera* n. sp. satisfies the morphological characters for *Lecudina*.

Lecudina caspera n. sp. forms a sister species to *L. longissima*. Trophozoites of *L. longissima* are linearly ellipsoid (Rueckert *et al.*, 2010) and are morphologically dissimilar to *L. caspera* n. sp. Both species, however, have a circular nucleus that is situated in the anterior third of the trophozoite.

The molecular phylogenetic analysis of *L. caspera* n. sp. SSU rDNA sequences is congruent with the morphological data and strongly supports the establishment of *L. caspera* n. sp. as a new species. Although the deeper relationships of taxa within the lecudinid clade remains poorly resolved, sister relationships at the species level is relatively well supported in its constituent genera. The sister relationship of *L. caspera* n. sp. with *L. longissima* is robustly supported (100 MLB, 1.00 BPP) the two sequences were 8% divergent. The divergent SSU rDNA sequences in combination with the strikingly different morphologies between *L. caspera* n. sp, and *L. longissima* justify the establishment of *L. caspera* n. sp. as distinct, new species.

Comparison between *P. anankea* n. sp. and *L. caspera* n. sp. with their sister taxa highlight the necessity of molecular data for gregarine taxonomy. Although some studies have attempted to rely solely on morphometric data for species delimitation (e.g., Clopton *et al.*, 1992) molecular phylogenies repeatedly reveal that morphology alone is often misleading. For instance, *P. anankea* n. sp. is morphologically similar to *P. polymorpha* morphotype 2, yet the SSU rDNA sequences between the two are 12% divergent. On the other hand, *L. caspera* n. sp. is highly dissimilar to *L. longissima* in morphology, but their SSU rDNA sequences are only 8% divergent. Morphology, therefore, can be taxonomically misleading and fine resolution of taxonomic relationships within gregarines must be inferred using molecular data. Despite the ubiquity of gregarine species, very few have been formally described. The discovery and description of additional gregarine taxa is necessary in resolving deep taxonomic relationships and will bring new insight into the evolutionary events that shaped the apicomplexans.

Chapter 5: Conclusion

Over a century and half has passed since the first publication of The Origin of Species by Charles Darwin and the process of evolution is still viewed in awe by contemporary biologists. Evolution has produced a great host of biodiversity and the small fraction of species that are known has been immensely informative in shaping the understanding of the biological world. The evolutionary history of these extant species, however, has inevitably been left in the past. In order to better understand the mechanisms that drive speciation and to appreciate a more accurate sense of the true biodiversity of the world, the past must somehow by reconstructed. Taxonomy and systematics, in the broadest sense, are a means to reveal historical events by cataloguing species – each of which contribute to a comprehensive understanding of biodiversity and biologically innovative traits. With enough taxonomic data, the relationships amongst taxa and the characters that define them become increasingly clear. By investigating the distribution of informative characters across a tree space we can see into the past and infer how species evolved.

The prerequisite for such reconstructions of the past through taxonomy and systematics is a robust dataset of species and characters. In the case of apicomplexan diversity and evolution, such a dataset is currently lacking. Although some species of particular human interest have been the subject of extensive study, most apicomplexan species remain undiscovered. The remedy for this dearth of knowledge is the discovery and description of additional species using methods that employ a marriage of comparative morphology and robust molecular analysis.

Improving the taxonomic resolution of the apicomplexan tree offers an opportunity to reconstruct the earliest stages of apicomplexan evolution and also to more accurately illustrate the constituent species of modern ecosystems.

The current thesis contributes two new species of marine gregarine apicomplexans to the existing apicomplexan phylogeny. The discovery of marine gregarine species is important for apicomplexan systematics in that marine gregarines as a whole represent the earliest stages of adaptive radiation of the Apicomplexa. Many marine gregarines possess key plesiomorphic traits that are inferred to have served as the template character state that more derived apicomplexans modified in response to new hosts and environments. The description of *P. anankea* n. sp. reinforces the existence of a *Trichotokara* and *Paralecudina* clade as well as emphasizes the need for refinement of the Lecudinidae. *Paralecudina anankea* n. sp. is also the first species to be added since the formation of *Paralecudina* as a monotypic genus with *P. polymorpha*. The description of *L. caspera* n. sp. highlights the morphological variation that makes gregarine systematics challenging. Although it shares a sister relationship to *L. longissima* the morphological differences between the two species are stark. Morphology alone is not always taxonomically informative and *L. caspera* n. sp. is an example of where molecular data is necessary to infer phylogenetic relationships. The discovery, characterization, and phylogenetic analysis of *P. anankea* n. sp. and *L. caspera* n. sp. contributes to the collective understanding of marine gregarine traits and to the improvement in resolution of the gregarine phylogeny.

Through discovering and describing *P. anankea* n. sp. and *L. caspera* n. sp. I acquired many valuable skills that I will continue to employ in future studies. The techniques used for formal

species descriptions are often quite involved and the use of such techniques underscored the importance of independently learning difficult methods and subsequently troubleshooting them in creative ways. I collected and dissected a large number of gregarine hosts that not only illustrated the diversity of gregarines, but also of the invertebrate animals. The characterization of various gregarine trophozoites involved deeply familiarizing myself with light and electron microscopy that entails isolation of the trophozoites from their hosts, appropriate fixations for SEM and TEM, and the operation of different microscopes and accompanying software. Furthermore, no single fixation protocol works perfectly for a given gregarine species and existing methods had to be modified for the current thesis. Acquisition of molecular data required that I learn DNA extraction protocols, DNA amplification, and screening amplicons on agarose gels. As trophozoite abundance varies between seasons and among hosts, DNA extraction was often done on single trophozoites. Existing primers also frequently failed to amplify target sequences and new primers had to be designed for divergent gregarine SSU rDNA sequences where relatively few existing sequences were available for comparison. Amplification by PCR also was not foolproof and I learned to use cloning kits as an alternate amplification method. The sequencing of DNA is first preceded by cleaning DNA product via chemical or enzymatic methods and raising the concentration of DNA to levels that can be detected by Sanger sequencing. I learned to read chromatograms and identify ambiguities as well as assembling contigs on numerous different software. Through creating DNA sequence alignments, I also acquired experience using four separate alignment programs and multiple algorithms for aligning and deleting ambiguous sites. Finally, I gained the skillset to analyze and present these data in the form of edited micrographs and a molecular phylogeny.

Future research will continue to focus on apicomplexan systematics and evolution through the use of aforementioned techniques. It is my hope to learn additional skillsets such as transcriptomics, transmission electron microscopy, and advanced used of SEM such as focused ion beam SEM. By employing more advanced technique I think gregarine morphology and genetics can be better characterized ultimately leading to more resolved apicomplexan phylogeny. Many marine gregarines have been described from the Pacific Northwest, but many other regions of the world remain unexplored. Japan, in particular, is a great candidate for gregarine research due to its geographical diversity ranging across many latitudes in addition to the lack of historical gregarine research. Furthermore, Japan takes an encouraging stance on valuing both basic and frontier science with a strong background in protistology.

The ultimate goal of my next research program is to contribute to a deeper understanding of the Apicomplexa through continued work on gregarine systematics and expanding to other apicomplexan taxa. In particular, I wish to taxonomically investigate the diversity of marine gregarines in Japan, how apicomplexan infections affect hosts, and to search for an apicomplexan-host system that can serve as an exemplar for understanding early apicomplexan parasitism. In learning to use new imagining and molecular techniques, it will be my ambition to further describe the basic parameters of apicomplexan biology to better understand character evolution in the Apicomplexa. In this vein, future studies will include an emphasis on: 1) gregarine systematics; 2) apicomplexan co-evolution with their hosts; 3) biogeography of marine gregarines; and 4) a search for model apicomplexan-host systems that are informative to early apicomplexan parasitism.

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