SPECIES DISCOVERY AND EVOLUTIONARY HISTORY OF MARINE GREGARINE APICOMPLEXANS

by

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Abstract

Gregarine apicomplexans are a diverse but poorly understood group of single-celled parasites infecting a wide range of invertebrates in marine, freshwater and terrestrial environments. My thesis focuses on marine gregarines. Gregarines from marine hosts are unique because some (archigregarines) have maintained a set of pleisiomorphic characteristics from the ancestor of gregarines and apicomplexans alike. Other lineages of marine gregarines (eugregarines) are thought to have been modified from this archigregarine morphotype, and represent a wide-range of diversity with regard to general morphology, motility, and feeding strategies. My work has broadly applied molecular phylogenetics to novel species of marine gregarines from areas around British Columbia, Canada and Okinawa, Japan, with the goal of placing the evolution of gregarines in a molecular phylogenetic context. I amplified mainly SSU rDNA from a distinct life history stage (trophozoites), and coupled that with morphological data I gathered from light, confocal, as well as electron microscopy. Although my work was unable to resolve deep phylogenetic relationships among gregarines (and apicomplexans), this work did improve our understanding of evolution within gregarines. With the discovery of Veloxidium leptosynaptae from the gut of an echinoderm in Bamfield, British Columbia, and Surculinium glossobalanae from a hemichordate in Okinawa, I was able to show the paraphyly of the archigregarine morphotype, and polyphyly of other gregarine lineages, including some groups of neogregarines and eugregarines in terrestrial and freshwater environments. With the description Polyplicarium, my work uncovered and identified an ambiguous environmental sequence clade and, along with other work on Selenidium, was able to show that SSU rDNA can be reliably isolated from single cells as a method for delimiting closely related or morphologically similar species. In my final data chapter, I conducted an in-depth study on the morphology and molecular phylogenetic relationships between two sister species from the same host, Selenidium terebellae, and a newly discovered species, Selenidium melitzanae. Results from this data gave me the first opportunity to compare
character evolution and niche partitioning among closely related gregarines, and provided another example of convergence of the eugregarine morphotype.
Preface

Every photo and figure presented in this thesis is my original work. Some of this data was put together for publication. These details are listed below:

Chapter 2 is based on the following published article:

I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses associated with this project. I also wrote the preliminary draft of this manuscript. Dr. Leander funded the project, and contributed to the writing and interpretation of the final manuscript.

Chapter 3 is based on the following published article:

I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses associated with this project. I also wrote the preliminary draft of this manuscript. Dr. Leander funded the project, and contributed to the writing and interpretation of the final manuscript.
Chapter 4 is based on the following published article:

I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses associated with this project. I also wrote the preliminary draft of this manuscript. Dr. Leander funded the project, and contributed to the writing and interpretation of the final manuscript.

Chapter 5 is based on the following material:

I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses associated with this project. I also wrote the preliminary draft of this manuscript. Dr. Leander funded the project, and contributed to the writing and interpretation of the final manuscript. This project was also funded by myself, in part, by a grant I received through the Professional Association of Diving Instructors (PADI) foundation. Dr. Reimer and Dr. Jenke-Kodama supplied lab space and material during my visit to Okinawa, Japan.

Chapter 6 is based on the following material:
*gen.*, *n.* sp. and *Selenidium terebellae* Ray 1930, highlights niche partitioning and character evolution in marine gregarine apicomplexans.

I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses associated with this project. I also wrote the preliminary draft of this manuscript. Dr. Leander funded the project, and contributed to the writing and interpretation of the final manuscript. Dr. Heintzelman contributed antibodies to an initial stage of the project and interpretation of the data.

Information presented throughout the thesis is based on the following published work:

This work was completed as part of a collaboration with Dr. Sonja Rueckert. I conducted all the fieldwork, microscopy, DNA extraction and sequencing, as well as data analyses for *Trichotokara eunicae*. Projects were funded by grants given to Dr. Sonja Rueckert and Dr. Leander.

Some of the information presented in the conclusion is based on the following work currently be prepared for publication:
Wakeman, K.C., Rueckert, S., Malik-Pightling, S., Slamovits, C.H. and Leander, B.S. (in prep) *Evolutionary history of gregarine apicomplexans as inferred from HSP90, Actin and SSU rDNA sequences.*

I conducted all the fieldwork, RNA extraction, cDNA synthesis/amplification, RT-PCR, cloning and sequencing of HSP90 and Actin for 10 of the 14 species in this study. Other species were collected as part of collaboration. These include: *Lankesteria abbotti* (Collected by KCW, SR and SBMP), *Pterospora schizosoma*
(Collected by SR and BSL), *Platyproteum vivax* (Collected by SR and BSL), and *Monocystis agilis* (Collected by SR and BSL). I made the alignments, and conducted all phylogenetic analyses, and wrote the initial manuscript. All authors contributed to the final version of the manuscript and interpretation of the data. This project was funded by Dr. Slamovits and Dr. Leander.
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1 Introduction

1.1 The Apicomplexa

Apicomplexans are a diverse group of single-celled eukaryotes, recognized not only as prolific parasites of vertebrates, but for their significant impacts on human health, economics and ecology around the world. Apicomplexans have been traditionally divided into four groups: haemosporidians (Plasmodium spp.), coccidians (Toxoplasma gondii and Eimeria spp.), piroplasmids (Babesia spp. and Theileria spp.) and gregarines (Selenidium spp. and Lecudina spp.) (Adl et al. 2005; Levine 1970). The diversity of this group is rich; however, the relationships, especially among lineages that are distantly related, is still uncertain (Adl et al. 2005, 2012; Barta et al. 2006). It’s thought that every animal species on the planet plays host to at least one apicomplexan parasite (Morrison 2009). Some of these apicomplexans (e.g., Toxoplasma gondii and Plasmodium spp.) have been well established as human parasites, carry a vast economic importance, and have garnered much of the attention given to apicomplexans (Tonkin et al. 2011). Other groups like gregarine apicomplexans, while diverse, remain poorly explored (Leander 2008). Consequently, it’s likely that less than 1% of the diversity within Apicomplexa has been revealed (Morrison 2009).

A contemporary view unites the apicomplexans based on parasitism, and the presence of an apical complex, an elaborate tool for cell invasion, supported by a closed conoid, and located in the anterior region of the cell (Adl et al. 2005, 2012; Hu et al. 2006; Leander and Keeling 2003). The more well-studied apicomplexans are renowned for manipulating host-cell function to allow for invasion into the host cell (Bargieri et al. 2012; Plattner and Soldati-Favre 2008). Similar motors and complexities appear to be present in the movement and feeding/attachment of apicomplexans in the lesser-known groups (e.g.,
gregarines) (Heintzelman 2004; Valigurová 2012). In general, apicomplexans are highly specific and specialized for a specific host, or group of hosts. For example, some behavioral studies have shown that apicomplexans can alter the behavior of intermediate hosts, increasing the likelihood of being passed to the determinate host (Berdoy et al. 2000; Webster 2007; Webster and McConkey 2010). These relationships with hosts are very unique, and have prompted researchers to speculate on the long, and intertwined history (coevolution) between early metazoans and early-branching alveolates over 600 million years ago (Okamoto and McFadden 2008).

1.2 Significance of the ‘less important’ groups of apicomplexans: gregarine apicomplexans

Relatively little attention has been given to what is probably the most abundant and diverse groups of apicomplexans, namely gregarines. Some research has shown interest in gregarines as biological control agents for mosquitos or beetles (Lipa et al. 1996; Tsent 2007). Indeed, very little work beyond taxonomy and morphological data has been gathered, especially on those gregarines infecting marine hosts (archigregarines and eugregarines). Still, a more recent molecular interest in marine gregarines (Leander et al. 2003; Leander 2007), as well as other studies looking at Cryptosporidium (Barta et al. 2006), particularly the phylogenetic position of Cryptosporidium as an early-branch among apicomplexans (Kuo et al. 2008; Zu et al. 2000), or its phylogenetic proximity to gregarines (Carreno et al. 1999), have suggested that the study of gregarines, could reveal more about apicomplexans and their evolution and diversity than might have been previously assumed.
1.3 Marine gregarines: archigregarines

Archigregarines are unique among gregarines because they have retained many characteristics such as an apical complex, and are the only apicomplexans known to feed via myzocytosis (Dyson et al. 1993, 1994; Schrével 1968; Simdyanov and Kuvardina 2007; Vivier and Schrével 1964). These character traits are common with the ancestor of gregarines and relatives of apicomplexans, namely colpodellids and perkinsids (Kuvardina et al. 2002; Leander et al. 2002, 2003b; Leander and Keeling 2003). Only about 60 species of archigregarines have been described (Levine 1971; Rueckert and Leander 2009). This is relatively few compared to other gregarine lineages. Even fewer of these lineages have molecular data associated with them, and so their phylogenetic position among gregarine and other apicomplexans is not completely understood (Leander et al. 2003a, b; Leander 2007; Rueckert and Leander 2009; Wakeman and Leander 2012).

Archigregarines are found only in intestines of marine hosts, most commonly polychaete worms (Leander et al. 2003b; Ray 1930; Rueckert and Leander 2009; Schrével 1971b). However, they are also found in other groups like echinoderms and sipunculids (Levine 1971; Rueckert and Leander 2009; Wakeman and Leander 2012). Still, most described archigregarines belong to a single genus, *Selenidium*. Gregarines belonging to the genus *Selenidium* are defined by their vermiform shape and nematode-like movement (Schrével 1971a, b; Ray 1930; Rueckert and Leander 2009). *Selenidium*, and other archigregarines, have relatively few epicytic folds on the surface of their cell (typically 4 to 50). In some species like *Platyproteum*, only transverse folds are present on the surface (Leander 2006; Rueckert and Leander 2009).

Schrével (1971b) imaged a conspicuous structure resembling the four-membrane apicoplast found in all other apicomplexan groups (McFadden et al. 1996; Köhler et al. 1997), with the exception of *Cryptosporidium* (Zhu et al. 2000). It is unclear
whether these structures imaged by Schrével (1971b) are homologous the remnant of the photosynthetic ancestor of apicomplexans. Toso and Omoto (2007) was unable to find any evidence of a plastid genome or organelle in the terrestrial gregarine, *Gregarina nephandrodes*. However, it does seem possible that the early-branching and pleisiomorphic archigregarines have maintained these structures, as well as the plastid genome.

**Figure 1.1 General morphology of archigregarine trophozoites.** A. Differential interference contrast (DIC) of *Selenidium terebellae*. The nucleus (N) is in the center of the cell. B, C. DIC light micrographs of *Siedleckia* sp. Caullery and Mesnil, 1898. The trophozoite of *Siedleckia* has a multinucleated stage (arrows). D. Scanning electron micrograph of the trophozoite stage from a novel species of *Selenidium*. The mucron (arrow) narrows to a point. Eight to ten epicytic folds (arrowhead) inscribe the surface of the trophozoite. The posterior end of the cell is oriented toward the bottom of the figure, and spirals to a fine point. Scale bars: A. = 15 µm; B, C. = 5 µm; D = 10 µm.

### 1.4 Marine gregarines: eugregarines

There are conflicting views on the classification of eugregarines and archigregarines (Levine 1971; Perkins et al. 2002; Rueckert and Leander 2009; Schrével 1971a). Probably the most pragmatic view splits eugregarines and archigregarines based on visible morphology of the trophozoite stage. In this
view, eugregarines have a limited ability to bend, twist or pulsate, compared to archigregarines (Grassé 1953, Leander 2008; Schrével 1971a). This morphotype that unites eugregarines has essentially become a dumping ground for the vast majority of the nearly 1,700 described gregarine species (Lankester 1866; Levine 1976; 1977a, b; Rueckert et al. 2010). Eugregarine trophozoites lack a microtubular cytoskeleton beneath the surface of the cell. Instead, many employ gliding motility, using an actin and myosin-based system associated with hundreds of longitudinal epicytic folds (Heintzelman 2004; Leander et al. 2003b; Leander 2008; Vivier 1968). Still others, like Pterospora spp. and Lankesteria spp. are capable dynamic pulsating movements, or have an array of bumps on the surface of the trophozoite stage (Ciaincio et al. 2001; Landers and Leander 2005; Leander et al. 2006). Eugregarine trophozoites also lack an apical complex and instead probably feed by utilizing an increased surface area to facilitate surface-mediate nutrient uptake. Like archigregarines, most marine eugregarines are parasites of the intestinal system, however, exceptions can also be found (Landers 2002; Landers and Leander 2005; Leander et al. 2006). The morphology of trophozoite stages of many species has become highly modified and exhibits a wide range of variation that seems to reflect the host lineage and the specific compartment within which the gregarines live (e.g., coelom or the intestines) (Landers 2002; Landers and Leander 2005, Leander et al. 2003a).
Figure 1.2 General morphology and motility of marine eugregarines. A – F. Time-series Differential Interference Contrast (DIC) micrographs taken every 2 seconds showing the general cell morphology and gliding motility of a lecudinid marine eugregarine. G. Scanning electron micrograph highlighting the surface morphology of the species shown in time-series frames in A-F. The mucron (arrow) is bulbous, and a number of epicytic folds (arrowhead) are visible on the surface. Scale bars: A – F. = 50 µm; G. = 10 µm.
Figure 1.3 Diversity of gregarine trophozoites. Differential interference contrast (DIC) micrographs highlighting the wide diversity of trophozoites isolated from invertebrate hosts in British Columbia, Canada. A. Pyxinoides sp. isolated from a goose-neck barnacle. B. Lecudina longissima isolated from Lumbrineries

1.5 The gregarine lifecycle

Gregarine life cycles include distinctive extracellular trophozoite stages that inhabit the body cavities of invertebrates. The trophozoites of most gregarines attach to epithelial tissues and occupy the gut lumen of their hosts. Many other gregarines, however, can be encountered in coelomic cavities and spaces associated with host reproductive systems. When two trophozoites join together just before sex, a process called syzygy, the two cells are referred to as gamonts. A robust wall forms around the gamonts forming a gametocyst, within which several rounds of mitosis produce hundreds of gametes (gametogony). Zygotes (the fleeting diploid stage) are formed after one gamete from each gamont fuse together. A sporocyst wall forms around each zygote, which rapidly undergoes meiosis producing 4 or more infective sporozoites within each sporocyst (note: ‘oocyst’ has been used synonymously with ‘sporocyst’ in gregarine literature). Gametocysts filled with sporocysts are released into the environment either by way of host feces (for intestinal gregarines), host gametes (for gonadal gregarines) or host disintegration (coelomic gregarines). New host organisms consume sporocysts in their environment. After entering the intestinal tract, the infective sporozoites are released from sporocysts and begin to feed, grow and eventually develop into trophozoites within the intestinal lumen. The sporozoites of coelomic and gonadal gregarines make their way into the appropriate host body cavity before developing into trophozoites.
1.6 Using molecular phylogenetics for understanding gregarine diversity and evolution

Molecular phylogenetic analyses of small subunit (SSU) rDNA sequences from marine gregarines do not resolve the deeper relationships in the group. To date, fewer than 25 SSU rDNA sequences are available from identified marine gregarines (Leander 2007, Leander et al. 2003b; Rueckert and Leander 2009, 2010, Rueckert et al.; Rueckert et al. 2010, 2011a). Still, results from the emerging molecular data are still able to reinforce or challenge some of the previous hypotheses regarding the evolutionary history of gregarines (Grassé 1953; Schrével 1971a; Théodoridès 1984). Among those supported by molecular data is the position of archigregarines as short-branches on the tree of apicomplexans, and as potential early-branching groups to terrestrial and freshwater eugregarines and neogregarines (albeit with low statistical support) (Leander et al. 2003a, b; Leander 2007, Rueckert and Leander 2009).

Although exploration into the diversity of the SSU rDNA sequences in marine gregarines is still in its infancy, studies in this area have provided new insights into the relationships between morphological and molecular variation in this group of parasites (Leander et al. 2003a, b Leander 2007, Rueckert and Leander 2008, 2009; Rueckert et al. 2010; Rueckert et al. 2011a; Rueckert et al. 2011b). For instance, two species of Lankesteria isolated from two different species of tunicates were separated on the basis of morphological variation (e.g., L. chelyosomae can be over 10 times larger than L. cystodytæ), host specificity, and a discontinuity in the molecular variation as inferred from phylogenetic analyses; the SSU rDNA sequence divergence between these two species of Lankesteria, however, only ranged from 2.1% – 3.1% (Rueckert and Leander 2008). By contrast, comparative analyses of SSU rDNA sequences isolated from three different morphotypes of Lecudina cf. tuzetae found in two closely related polychaete hosts from two different locations (Bamfield, British Columbia, Canada and Vancouver, British Columbia, Canada) demonstrated that there
were no molecular discontinuities (i.e., clades) that correlated with morphotype, host affiliation, and geographic region (Rueckert et al. 2011b). In some cases, clones from a single-cell PCR product from one morphotype of *L. cf. tuzetae* isolated from Bamfield were identical to clones from a different morphotype of *L. cf. tuzetae* isolated in Vancouver (Rueckert et al 2011b). Nonetheless, the intraspecific variation of *L. cf. tuzetae* ranged from 0.0 – 3.9%, which is comparable to the range of molecular variation used to help discriminate different species in other gregarine genera (e.g., *Lankesteria*). As discussed by Rueckert et al. (2011b), the delimitation of species boundaries in marine gregarines should incorporate more than just one criterion. In other words, distinguishing species on the basis of combined morphological variation, sequence variation, host affiliation and geographic distribution, offers the most complete understanding of species diversity in gregarine apicomplexans. Ultimately, robust clades of molecular variation as inferred from phylogenetic analyses provide the most compelling evidence for (1) species boundaries in marine gregarines and (2) how these boundaries correlated with other levels of variation, such as morphology, motility, host specificity and geographic distribution.

### 1.7 Approaches and goals

My thesis research will focus on species discovery and patterns of morphological diversity in marine gregarines using high-resolution microscopic techniques such as scanning electron microscopy, light microscopy, and fluorescence confocal microscopy. I will also extract DNA/RNA from individually isolated gregarine cells. Using single cell PCR approaches, I will target small subunit (SSU) rDNA sequences with the goals of (1) establishing unambiguous markers for delimiting different species of gregarines from one another, (2) establishing the cellular identities of ambiguous sequences derived from environmental DNA surveys and (3) placing the morphological diversity of marine gregarines within a molecular phylogenetic context.
2 Molecular phylogeny of Pacific archigregarines (Apicomplexa), including descriptions of *Veloxidium leptosynaptae* n. gen., n. sp., from the sea cucumber *Leptosynapta clarki* (Echinodermata) and two new species of *Selenidium*

2.1 Synopsis

Archigregarines are single-celled, intestinal parasites of marine invertebrates and are inferred to have retained many characteristics found in the most recent common ancestor of all apicomplexans (Cavalier-Smith and Chao 2004; Cox 1994; Grassé 1953; Leander 2008; Leander and Keeling 2003b). For instance, the extracellular trophozoites of archigregarines are similar in morphology and behavior to their infective sporozoite stage and to the sporozoites of other apicomplexans (Cox 1994; Dyson et al. 1993, 1994; Kuvardina & Simdyanov 2002; Leander 2006; Ray 1930; Schrével 1968). Moreover, the oocysts of archigregarines contain just four infective sporozoites, and the life cycle of archigregarines is completed within a single host species (Grassé 1953; Schrével 1970, Théodoridés 1984). The nearest free-living sister lineages to the Apicomplexa are predatory and biflagellated colpodellids that, like some archigregarine trophozoites, possess an apical complex and utilize a vampire-like mode of feeding called myzocytosis (Kuvardina et al. 2002; Leander et al. 2003c). This insight, along with molecular phylogenetic analyses of small subunit (SSU) rDNA, has led to the hypothesis that archigregarines branched early within the Apicomplexa, and represent a paraphyletic stem group from which all other gregarines, and possibly the Apicomplexa as a whole, evolved (Grassé 1953, Leander 2008; Rueckert and Leander 2009; Théodoridés 1984).

There are several differences that distinguish archigregarine trophozoites from those of so-called ‘marine eugregarines’ (e.g., lecudinids and urosporids). The
trophozoites of archigregarines are generally spindle-shaped and are capable of
nematode-like bending and coiling motility (e.g. *Selenidium* spp.) (Rueckert and
Leander 2009; Schrével 1970). The trophozoites of marine eugregarines, by
contrast, are generally rigid and exhibit gliding motility (Leander et al. 2003b;
Leander 2008; Rueckert et al. 2010). Moreover, trophozoites of marine
eugregarines differ significantly from their infective sporozoite stages. The
trophozoite surface of most eugregarines has hundreds of epicytic folds that run
along the longitudinal axis of the cell (Leander et al. 2003b, Rueckert et al. 2010;
Rueckert and Leander 2010). This stands in contrast to archigregarines that
typically have much fewer longitudinal folds (i.e., 0-50) on the trophozoite surface
(Leander et al. 2003b; Leander 2007, Leander 2008; Rueckert and Leander
2009; Schrével 1971). The trophozoite surface of archigregarines is supported
by microtubules, each surrounded by a transparent sheath, which are organized
in one or more layers beneath the trilayered inner membrane complex (Leander
2007; Mellor and Stebbings 1980; Schrével 1971b.; Stebbings et al. 1974; Vivier
and Schrével 1964). This subcellular organization of microtubules is not present
in the trophozoites of eugregarines (Leander 2008).

Of the approximate 65 species of archigregarines that have been described,
roughly 56 belong to the genus *Selenidium* (Leander 2007; Levine 1971;
Rueckert and Leander 2009). Levine (1971) proposed that several species of
*Selenidium* should be considered eugregarines rather than archigregarines if
there is no report of merogony/schizogony (i.e. asexual reproduction of
trophozoites) at any point during the life cycle. As a result, the genus
*Selenidioides* was erected to accommodate the 11 members of *Selenidium* with
reports of merogony, and the remaining members of *Selenidium* were moved into
the order Eugregarinorida (Levine 1971). As pointed out previously, the presence
or absence of merogony in the lifecycle of any gregarine is difficult to
substantiate, which makes this feature a poor indicator of phylogenetic
relationships (Leander 2006, 2007, Rueckert and Leaner 2009). Therefore, this
study will consider *Selenidium* the main genus of archigregarines, which is
consistent with most other contemporary work on this subject (Gunderson and Small 1986; Kuvardina and Simdyanov 2002, Leander 2006, 2007; Rueckert and Leander 2009; Schrével 1971a.; Simdyanov and Kuvardina 2007; Théodoridés 1984).

Although our knowledge of archigregarines is based primarily on studies of species within the genus *Selenidium*, archigregarines are much more diverse. Close relatives of *Selenidium* show bizarre twisting and peristaltic movements (e.g., *Platyproteum vivax*) and highly modified morphology, such as matted hair-like projections on the trophozoite surface (e.g., *Filipodium* spp.) (Gunderson and Small 1986; Hoshide and Todd 1996; Leander 2006; Rueckert and Leander 2009). Ultrastructural information has been reported on fewer than 20 species of archigregarines, mostly *Selenidium* spp., and molecular phylogenetic data of SSU rDNA is available for only six species (e.g., *Selenidium terebellae*, *Selenidium pisinnus*, *Selenidium orientale*, *Selenidium serpulae*, *Filipodium phascolosomae*, and *Platyproteum vivax*); none of these DNA sequences have a well-supported phylogenetic position within the context of all major lineages of apicomplexans (Leander et al. 2003b; Leander 2007; Rueckert and Leander 2009; Rueckert et al. 2011a). Improved understanding of archigregarine diversity, especially from a molecular phylogenetic perspective, is expected to lead to the discovery of novel gregarine lineages and shed considerable light onto the earliest stages in apicomplexan evolution.

Here, we characterize the surface morphology and molecular phylogeny of three species of *Selenidium* isolated from polychaete hosts, namely *Selenidium idanthysae* n. sp. and *Selenidium boccardiellae* n. sp. and the previously reported *Selenidium cf. mesnili*. We also describe and establish a novel genus of archigregarine, namely *Veloxidium leptosynaptae* n. gen., n. sp., from the intestines of a Pacific sea cucumber. Comparative morphology and molecular phylogenetic analyses of these new data helped demonstrate that
archigregarines form a paraphyletic stem group from which several different eugregarine lineages evolved.

2.2 Materials and methods

2.2.1 Collection of organisms.

*Selenidium* cf. *mesnili* (Basil 1909) and *Selenidium boccardiellae* n. sp. were isolated from the Pacific polychaetes *Myxicola infundibulum* Montagu, 1808 and *Boccardiella ligerica* Ferronnière, 1898, respectively. Both host animals were collected by SCUBA diving in August 2010 at Ogden Point (48° 25' 43" N 123° 21' 56" W), near Victoria, British Columbia, Canada. *Selenidium idanthyrsae* n. sp. was isolated from the polychaete *Idanthyrsus saxicavus* Baird, 1863 collected in June 2010 in a dredge haul at 20 m depth near Wizard Islet Bamfield, British Columbia, Canada (48° 50' 17" N, 125° 08' 02" W). *Veloxidium leptosynaptae* n. gen., n. sp. was isolated from the sea cucumber, *Leptosynapta clarki* Heding, 1928, collected in May 2011 from intertidal mud at low tide, approximately 1.5 km inward from the mouth of Grappler Inlet, Bamfield, British Columbia, Canada (48° 49' 49" N 125° 8'15" W).

The intestines of all host animals were isolated using fine-tipped forceps and placed in a Petri dish filled with autoclaved filtered seawater. Intestinal contents were transferred to a well slide and viewed under a Leica (Wetzlar, Germany) DM IL inverted microscope. Individual trophozoites were manually isolated from the intestinal contents using a micropipette and washed at least three times in autoclaved filtered seawater prior to examination with light microscopy and preparation for DNA extraction.
2.2.2 Light and scanning electron microscopy.

Individual trophozoites were placed into a drop of seawater on a glass slide, secured with a coverslip and Vaseline, and viewed with differential interference contrast (DIC) using a Zeiss Axioplan 2 microscope (Carl-Zeiss, Göttingen, Germany) connected to a Leica DC500 color digital camera. Cell motility was recorded using an inverted Zeiss Axiosvert 200 microscope connected to a PixeLink Megapixel color digital camera (PL-A662-KIT, Ottawa, Canada).

Twelve, eight, ten, and seven individual trophozoites of *S. cf. mesnili*, *S. boccardiellae* n. sp., *S. idanthyrsae* n. sp., and *V. leptosynaptae* n. gen., respectively, were prepared for scanning electron microscopy. Very few trophozoites are present in some hosts; for instance, only nine trophozoites of *V. leptosynaptae* were recovered from *L. clarki*. For each species, a 10-µm polycarbonate membrane filter was placed within a Swinnex filter holder (Millipore Corp., Billerica, MA, USA); the filter holder was then placed within a small beaker (4 cm diam. and 5 cm tall) that was filled with 2.5% (v/v) glutaraldehyde in seawater. The manually isolated trophozoites were placed directly into the Swinnex filter holder and fixed on ice for 15 min. Ten drops of 1% (w/v) OsO₄ were then added directly to the opening of the Swinnex filter holder, and the samples were post-fixed on ice for 30 min. A syringe was attached to the Swinnex filter holder, and distilled water was slowly washed over the samples. A graded series of ethanol (30%, 50%, 75%, 85%, 95%, and 100%) was then used to dehydrate the fixed cells using the syringe system. Following dehydration, the polycarbonate membrane filters containing the trophozoites were transferred from the Swinnex filter holders into an aluminum basket submerged in 100% ethanol in preparation for critical point drying with CO₂. The dried polycarbonate membrane filters containing the trophozoites were mounted on aluminum stubs, sputter coated with 5 nm gold, and viewed under a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe
Systems, San Jose, CA). The SEM stubs containing holotype and paratype material were deposited in the Beaty Biodiversity Research Centre Marine Invertebrate Collection, University of British Columbia, Vancouver, Canada.

2.2.3 DNA isolation, gene amplification, cloning and sequencing of the *Selenidium* species.

Twelve to 20 individual trophozoites were isolated from host gut contents, washed until clean, at least three times, in autoclaved filtered seawater and transferred into 1.5 ml microfuge tubes. DNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). A region of the small subunit (SSU) SSU rDNA (~1,800 bp) was targeted for DNA amplification and sequencing. Initial PCR was performed using a PCR bead (Illustra, PuReTaq Ready-To-Go PCR beads, GE Healthcare, Quebec, Canada), the extracted trophozoite DNA in 24 µl of H₂O, and 1 µl the following primer pair (total reaction volume of 25 µl): F1 5'-GCGCTACCTGGTTGATCCTGCC-3' and R1 5'-GATCCTTCTGCAGGTTCACCTAC-3'(Leander et al. 2003a). The thermocycler was set for the following conditions: initial denaturization of 96.0 °C for 5:00 min; 40 cycles of 96.0 °C for 30 sec, 52.0 °C for 30 sec (annealing temperature), and 72.0 °C for 2:00 min; and a final extension of 72.0 °C for 9:00 min. One µl of the initial PCR product using primers F1-R1 was used as a template for two semi-nested PCR reactions using a higher annealing temperature of 58 °C in 25 cycles (all other PCR and thermocycler conditions as listed above) and the following primer pairs: F2 5'-GATCCCGGAGAGGGAGCTTGAG-3' and R1 producing a 1,200-bp product; F1 and R2 5'-GCCTYGCGACCATACTTC-3' producing a 1,000-bp product. PCR products corresponding to the expected sizes were gel isolated and purified using UltraClean 15 DNA Purification Kit (MO Bio, Carlsbad, CA). Purified DNA was cloned into a pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Four clones from each species were PCR amplified and screened using vector primers in a 25-µl reaction with EconoTaq
Master Mix (Lucigen Corp. Middleton, WI) with the following thermocycler program: initial denaturization of 94.0 °C for 2:00 min; 30 cycles of 94.0 °C for 2:00 min., 50.0 °C for 30 sec, and 72.0 °C for 1:30 min; and final extension of 72.0 °C for 9:00 min. Cloned products corresponding to the expected sizes were sequenced using vector primers and ABI Big Dye reaction mix. Novel SSU rDNA gene sequences were initially identified using BLAST and confirmed with molecular phylogenetic analyses. New SSU rDNA sequences from S. cf. mesnili, S. boccardiellae n. sp., and S. idanthrysae n. sp. were deposited into GenBank as JN857968, JN857969, and JN857967, respectively.

### 2.2.4 Single-cell PCR and sequencing of *Veloxidium leptosynaptae* n. gen., n. sp.

Three individual trophozoites of the nine recovered of *V. leptosynaptae* n. gen., n. sp. were manually isolated, photographed, video recorded, and placed into two separate 0.2-ml PCR reaction tubes containing 10.0 µl FFPE DNA QuickExtract (DNA Extraction Kit, Epicentre Biotechnologies, Madison, WI). ‘*V. leptosynaptae* Isolate 1’ consisted of a single trophozoite, and ‘*V. leptosynaptae* Isolate 2’ consisted of two attached trophozoites (gamonts) undergoing head-to-head syzygy (i.e., the onset of sexual reproduction). Both tubes were then placed in a thermocycler and incubated at 56.0 °C for 60:00 min and 98.0 °C for 2:00 min.

A PCR bead (illustra, PuReTaq Ready-To-Go PCR beads, GE Healthcare, Quebec, Canada), 1 µl of the F1-R1 primer pair, and 14 µl of autoclaved distilled water were added to each 0.2 ml tube containing 10 µl of the DNA extract in order to bring the total PCR reaction volume to 25.0 µl. The same primers, semi-nested PCR protocols, thermocycler programs, and sequencing protocols described above were used to sequence the SSU rDNA sequence from *V. leptosynaptae* n. gen., n. sp. The two new SSU rDNA gene sequences from the two isolates of this species were identical; the sequence was initially identified.
using BLAST, confirmed with phylogenetic analyses, and deposited in GenBank as JN857966.

2.2.5 Molecular phylogenetic analyses.

The four new SSU rDNA sequences generated in this study were aligned with three sequences from dinoflagellates as the outgroup and 80 additional sequences representing the major subgroups of apicomplexans, forming an 87-taxon dataset. The alignment was then visually fine-tuned using MacClade 4 (Maddison and Maddison 2000); gaps and ambiguously aligned regions were excluded resulting in 1,008 unambiguously aligned sites. JModeltest (Guindon and Gascuel 2003; Posada and Crandall 1998) selected a GTR+I+Γ model of evolution under AIC and AICc (proportion of invariable sites = 0.1770, gamma shape = 0.6170). Garli-GUI (Zwickl 2006) was used to generate a maximum likelihood (ML) tree and ML bootstrap analysis (100 pseudoreplicates, one heuristic search per pseudoreplicate). A distance analysis between the three *Selenidium* species isolated in this study was completed using PAUP* 4.0 (Swofford 1999).

Bayesian posterior probabilities were calculated using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We set our program for four Monte Carlo Markov chains starting from a random tree (MCMC; default temperature = 0.2), a gamma distribution, and a stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate). A sum of 7,400,000 generations was calculated. Trees were sampled every 100 generations, with a prior burn-in of 500,000 generations. Burn-in was confirmed manually, and a majority-rule consensus tree was constructed. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.
2.3 Results

2.3.1 *Selenidium boccardiellae* n. sp.

Trophozoites were vermiform, 158 µm long (range = 87-250 µm) and 10 µm wide (range = 10-12 µm; n = 13) (Figure 2.1). An ellipsoidal nucleus was located in the middle anterior half of the cell and was 11 µm long (range = 10-12) and 5 µm wide (range = 4-6 µm). The trophozoites were compressed, especially near the anterior region. The mucron was dome-shaped and terminated with a conspicuous nipple (Figure 2.1). Two pronounced ridges, one on each side of the compressed cell, formed near the base of the mucron and extended to about the middle of the cell. Ten-to-twelve shallow longitudinal folds extended nearly the entire length of the trophozoites; the folds were absent on the extreme anterior, in the mucron region, and posterior ends. The trophozoites tapered at each end, forming a narrow tip at the posterior end and the anterior end; the epicytic folds became helically arranged at the posterior end of the cell (Figure 2.1). The trophozoites were capable of nematode-like coiling movements. Individual trophozoites were opaque-silver under light microscopy; there were no conspicuous accumulations of brown amylopectin granules within the cytoplasm.

2.3.2 *Selenidium cf. mesnili*.

Trophozoites were conspicuously brown from an accumulation of amylopectin granules within the cytoplasm and were capable of a high degree of bending and coiling. The trophozoites were vermiform with tapered, but relatively rounded, anterior and posterior ends. The cells were 127 µm long (range = 85-157 µm; N = 16) and 20 µm wide (range= 18-24 µm) (Figure 2.2). An ellipsoidal nucleus was 8 µm long (range = 7-9 µm) and 10 µm wide (range= 10-11 µm). The trophozoites underwent rapid changes in shape and were capable of shortening when agitated. The cell surface was covered with 22-24 deeply carved longitudinal folds that bifurcated near the anterior and posterior ends of the cell.
The longitudinal folds were covered with densely packed transverse striations at ~3-4 striations / µm, except near the extreme anterior and posterior ends of the cell. The mucron was capable of being protracted and retracted (Figure 2.2).

2.3.3 *Selenidium idanthyrsae* n. sp.

The trophozoites were relatively long, skinny, and flattened; the cells were 438 µm long (range = 450-543 µm; N = 22) and 15 µm (range = 14-16 µm) wide (Figure 2.3). The trophozoites were capable of rhythmic bending and coiling. A spherical nucleus was 14 µm in diam. (range = 13-15 µm). The posterior end was conspicuously compressed, forming a spade-like cell terminus, whereas the anterior end was more cylindrical, especially near the mucron. The surface of the trophozoites was covered with 20-22 longitudinal folds that ran nearly the entire length of the cell; epicytic folds were absent at the extreme posterior end. Transverse striations were absent on the entire cell, except near the base of the mucron; these transverse striations continued only 10-15 µm away from the mucron region and then disappeared. A conspicuous collar that was 2 µm in diam. marked the base of the mucron (Figure 2.3).

2.3.4 *Veloxidium leptosynaptae* n. gen., n. sp.

Trophozoites were vermiform, 74 µm long (range = 62-97 µm; N = 9) and 11 µm wide at the middle of the cell (range= 9-13 µm) (Figure 2.4). The most distinctive feature of these trophozoites was their relatively rapid bending and thrashing motility. A spherical nucleus was located in the middle of the trophozoite and measured 10 µm in diam. (range = 9-11 µm). The cell surface consisted of raised transverse striations or rings at a density of 3-4 per micron; the transverse striations were most pronounced near the posterior end of the cell, and relatively faint towards the anterior end. Longitudinal epicytic folds were absent. The mucron was inconspicuous and the anterior end was generally dome-shaped. The posterior end of the cell tapered to a point starting from the middle of the
trophozoite. The posterior end of the trophozoites often formed a loop by curving back toward the middle of the cell. Syzygy was head-to-head. Conspicuous spherical granules or vacuoles, ~1 to 2 µm in diam., were observed throughout the cytoplasm of the trophozoites (Figure 2.4).

2.3.5 Molecular phylogenetic positions of the new taxa.

Analyses of the 87-taxon data set resulted in a weakly supported backbone that gave rise to a dinoflagellate clade as outgroup and several different subclades of apicomplexans, including cryptosporidians, rhytidocystids, and a clade consisting of piroplasmids and coccidians (Figure 2.5). Most of the gregarine sequences fell into three main clades: (1) a terrestrial gregarine clade consisting of monocystids, neogregarines, and eugregarines from the intestines of insects; (2) a clade of eugregarines from the intestines of crustaceans; and (3) a clade of marine lecudinids and urosporids (excluding *Lecudina polymorpha* and related environmental sequences) (Figure 2.5). The eleven sequences from archigregarines did not cluster together as a separate clade and, instead, branched in several different positions along the apicomplexan backbone. The three new *Selenidium* sequences reported here (i.e. *S. cf. mesnili*, *S. boccardiellae* n. sp., and *S. idanthyrsae* n. sp.) formed a robust clade with *Selenidium serpulae*. The distance analysis across 1,653 base pairs showed that the SSU rDNA of *S. serpulae* differed from those collected from *S. cf. mesnili*, *S. idanthyrsae*, and *S. boccardiellae* by 8.6%, 9.6% and 9.1%, respectively. The SSU rDNA of *S. cf. mesnili* differed from those of *S. idanthyrsae* and *S. boccardiellae* by 8.3% and 7.5%, respectively, and the SSU rDNA of *S. idanthyrsae* differed from that of *S. boccardiellae* by 8.1% (Table 2.2). This clade formed the nearest sister lineage to robust clade consisting of *Selenidium orientale* and *Selenidium pisinnus*, albeit with weak statistical support. The following sequences branched in unresolved positions from the apicomplexan backbone: (1) *Selenidium terebellae*, (2) a clade consisting of *Filipodium phascolosomae* and *Platyproteum vivax*, and (3) a clade consisting of
two environmental sequences of unknown cellular identity (AY179975 and AY179976) (Figure 2.5).

The SSU rDNA sequences from *V. leptosynaptae* n. gen. n. sp. isolate 1 and *V. leptosynaptae* n. gen., n. sp. isolate 2 were identical; this sequence formed a robust clade with an environmental sequences (AB275006). This 'Veloxidium clade' branched as the nearest sister lineage to the major clade of marine lecudinids and urosporids, excluding *L. polymorpha* and related environmental sequences, with robust statistical support (Figure 2.5).
Figure 2.1. Light and scanning electron micrographs (SEM) of *Selenidium boccardiellae* n. sp. A–B. SEMs showing elongated and slightly compressed trophozoites with 10–12 shallow epicytic folds (arrowhead). Distinct lateral ridges are visible near the anterior region of the cell (asterisk). The mucron is pointed.
(arrow), free of epicytic folds and narrows toward the anterior end. The posterior end contains epicytic folds that twist near the posterior (double arrowhead). C–F. Differential interference contrast (DIC) micrographs illustrating the bending and thrashing movements of the trophozoites. G. SEM showing the mucron region (arrow) and longitudinal epicytic folds (arrowhead) at high magnification. Scale bars: A–B = 10 µm; C–F = 25 µm; G = 2 µm.
Figure 2.2 Light and scanning electron micrographs (SEM) of *Selenidium cf. mesnili*. A–B. SEM showing the general shape and surface morphology of the trophozoites. The surface of the trophozoites is adorned with 22–24 longitudinal epicytic folds (arrowhead) that disappear at the anterior (arrow) and posterior ends. The epicytic folds (arrowhead) bifurcate (asterisk) near the anterior and posterior ends. Transverse striations (double arrowhead) on the epicytic folds are densely packed in some regions of the cell. The mucron region (arrow) and the posterior end are pointed. C–F. Differential interference contrast (DIC) micrographs illustrating the characteristic bending and thrashing motility of the trophozoites, including cell extension and contraction. Epicytic folds are visible in the DIC micrographs (arrowhead). The nucleus (N) is situated at the anterior or posterior parts of the cell. G, H. High magnification SEMs of the trophozoite surface showing epicytic folds (arrowhead) and transverse striations (double arrowhead). Scale bars: A–B = 10 µm; C–F = 25 µm; G = 3 µm; H = 1 µm.
Figure 2.3 Light and scanning electron micrographs of *Selenidium idanthyrsae* n. sp.  

**A.** Differential interference contrast (DIC) micrograph showing the pointed anterior (arrow), bending movement and longitudinal epicytic folds (arrowhead). The nucleus (N) was in the middle to posterior region of the trophozoites.  

**B.** High magnification SEM of the pointed mucron (arrow); transverse striations (double arrowhead) are only visible at the base of the mucron.  

**C.** SEM showing the vermiform shape of the trophozoites and 20–22 epicytic folds (arrowhead), which are absent from the extreme anterior (arrow) and compressed posterior (triple arrowhead) region of the cell. Scale bars: A = 50 \( \mu\)m; B = 3 \( \mu\)m; C = 10 \( \mu\)m.
Figure 2.4 Light and scanning electron micrographs (SEMs) of *Veloxidium leptosynaptae* n. gen., n. sp. **A.** SEM showing general morphology and spindle-shape of the trophozoites. The mucron (arrow) is dome-like and blunt. Epicytic folds are not present. A dense array of transverse striations (double arrowhead) is present over most of the cell surface. **B.** Differential interference micrographs (DIC) image of “isolate 1” collected for single-cell PCR and subsequently molecular phylogenetic analysis. The nucleus (N) was located in the middle of the cell. **C–E.** DIC image of “isolate 2” collected for PCR and subsequently molecular phylogenetic analysis. The micrographs showing head-to-head syzygy. The junction (asterisk) between the two gamonts is the same width as the mucron of an individual trophozoite; the terminal ends of each gamont are tapered, curved, and pointed in a way that is identical to the posterior end of an
individual trophozoite. Prominent spherical granules, roughly 1 µm in diam., were observed within the cytoplasm of the cell. Scale bars: A = 10 µm; B = 50 µm; C–E = 50 µm.

Figure 2.5 Molecular phylogeny of Selenidium and Veloxidium based on SSU rDNA. Maximum likelihood (ML) tree based on 87 small subunit (SSU) rDNA sequences and 1,008 unambiguously aligned sites using the GTR + I + Γ substitution model (-ln L = 18528.74970, gamma shape = 0.6170, proportion of invariable sites = 0.1770). Bootstrap support values are given at the top of branches, and Bayesian posterior probabilities are given at the bottom. Thick
branches represent bootstrap support values and Bayesian posterior probabilities 95/0.95 or greater. Bootstrap values less than 55, and Bayesian posterior probabilities less than 0.90 were not added to this tree. The four sequences generated in this study are highlighted in black boxes
Table 2.1 Morphological comparisons of *Veloxidium* n. gen., *Selenidium pendula* (type species) and other *Selenidium* species. Archigregarines described in this study are highlighted in bold.

<table>
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<th></th>
<th><em>S. pendula</em> (type species)</th>
<th><em>S. terebellae</em></th>
<th><em>S. orientale</em></th>
<th><em>S. pisinnus</em></th>
<th><em>S. serpulae</em></th>
<th><em>S. cf. mesnili</em></th>
<th><em>S. boccardiellae</em> n. sp</th>
<th><em>S. spionis</em></th>
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<td>Themiste pyroides</td>
<td>Phascolosoma agassizii</td>
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<td>Myxicola infundibulum</td>
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<td>8-15 x 10-25</td>
<td>5 x 11</td>
<td>7-10 x 5-11</td>
<td>7-9 x 10-11</td>
<td>10-12 x 4-6</td>
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</tr>
<tr>
<td>Position of nucleus</td>
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<td>Posterior Half</td>
<td>Middle</td>
<td>Anterior half</td>
<td>Anterior half</td>
<td>Anterior to Posterior</td>
<td>Anterior half</td>
<td>Unknown</td>
</tr>
<tr>
<td>Motility behavior</td>
<td>Bending, twisting, pendulum-like</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Pending, twisting</td>
<td>Bending, twisting, contract/stretch</td>
<td>Bending, twisting, contract/stretch</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
</tr>
<tr>
<td>Number of long. epicytic folds</td>
<td>20 to 30</td>
<td>4 to 6</td>
<td>18 to 20</td>
<td>40 to 44</td>
<td>14 to 23</td>
<td>22 to 24</td>
<td>10 to 12</td>
<td>20 to 30</td>
</tr>
<tr>
<td>Transverse surface folds shape of mucron</td>
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<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pointed</td>
<td>Pointed to slightly round</td>
<td>Pointed</td>
<td>Pointed</td>
<td>Pointed to round</td>
<td>Pointed to round</td>
<td>Pointed to round</td>
<td>Sucker-like, depressed</td>
<td></td>
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Table 2.1 Continued

<table>
<thead>
<tr>
<th>S. cirratuli</th>
<th>S. polydorae</th>
<th>S. rayi</th>
<th>S. idanthyrsae n. sp</th>
<th>S. sabellariae</th>
<th>S. fallax</th>
<th>S. hollandei</th>
<th>V. leptosynaptae n. gen.</th>
<th>S. synapta</th>
</tr>
</thead>
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<tr>
<td><strong>Cirratulus cirratus</strong></td>
<td><strong>Polydora ciliata</strong></td>
<td><strong>Cirratulus filiformis</strong></td>
<td><strong>Idanthyrsus saxicavus</strong></td>
<td><strong>Sabellaria alveolata</strong></td>
<td><strong>Cirriformia tentaculata</strong></td>
<td><strong>Sabellaria alveolata</strong></td>
<td><strong>Leptosynaptae clarkii</strong></td>
<td><strong>Synapta sp.</strong></td>
</tr>
<tr>
<td>Intestines</td>
<td>Intestines</td>
<td>Intestines</td>
<td>Intestines</td>
<td>Intestines</td>
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<td>Intestines</td>
</tr>
<tr>
<td>W. Atlantic</td>
<td>W. Pacific</td>
<td>W. Pacific</td>
<td>W. Pacific</td>
<td>E. Atlantic</td>
<td>E. Atlantic</td>
<td>E. Atlantic</td>
<td>W. Pacific</td>
<td>E. Atlantic</td>
</tr>
<tr>
<td>Spindle-shaped, flattened</td>
<td>Spindle-shaped</td>
<td>Spindle-shaped</td>
<td>Spindle-shaped, partially flattened</td>
<td>Spindle-shaped</td>
<td>Spindle-shaped</td>
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<td>80-100 x 15-20</td>
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<td>220 x 16</td>
<td>300-500 x 10-30</td>
<td>Up to 500 x 20-30</td>
<td><strong>62-97 x 9-13</strong></td>
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<td>Unknown</td>
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<td>ovoid</td>
<td>Unknown</td>
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<td><strong>13-16 x 9-10</strong></td>
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<td><strong>9-10 x 9-11</strong></td>
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<tr>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting</td>
<td>Bending, twisting (rapid)</td>
<td>Bending, twisting</td>
</tr>
<tr>
<td>About 12</td>
<td>10 to 12</td>
<td>10 to 12</td>
<td><strong>20 to 22</strong></td>
<td>Unknown</td>
<td>About 90</td>
<td>About 16</td>
<td>None</td>
<td>4 to 8</td>
</tr>
<tr>
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<td>Unknown</td>
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<td>Unknown</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cone-shaped</td>
<td>Nipple-like</td>
<td>Knob-like</td>
<td><strong>Pointed</strong></td>
<td>Pointed, spine present</td>
<td>Knob-like</td>
<td>Heart-shaped</td>
<td>Round</td>
<td>Pointed</td>
</tr>
</tbody>
</table>

2.4 Discussion

2.4.1 Archigregarines and Selenidium

Archigregarines are a poorly understood, but diverse assemblage of marine gregarines that are important for understanding early stages in the evolutionary history of gregarines and perhaps apicomplexans as a whole (Leander 2007, 2008). The main goal of this study was to discover and characterize additional species of archigregarines, to more comprehensively infer the phylogenetic relationships of these lineages within the Apicomplexa. Trophozoites of *Selenidium cf. mesnili* were isolated from *M. infundibulum* collected from the north Pacific Ocean in this study. The host species, trophozoite motility and trophozoite morphology of *S. cf. mesnili* were indistinguishable from the original description of the type species (Brasil 1909; Ray 1930; Reed 1933; Schrével 1971a,b) (Table 2.1). However, because our isolate was collected from the Pacific Ocean and the type species of *S. mesnili* was collected from the Atlantic Ocean, we have chosen to designate our isolate as “*S. cf. mesnili*”; future work that determines a SSU rDNA sequence from the type host species, *Myxicola infundibulum*, collected in the east Atlantic, the type locality, will clarify whether or not this species has a broad geographical distribution. Nevertheless, this study
has expanded our knowledge of this species or species complex with SEM data and a SSU rDNA sequences.

*Selenidium idanthyrsae* n. sp. was isolated from the intestines of *Idanthyrsae saxicavus*, which has not been reported as a host species of archigregarines until now. The trophozoites of *S. idanthyrsae* n. sp. were on average 438 µm long and 15 µm wide, which is longer than previously described species of *Selenidium*, except for *S. sabellariae*, *S. fallax*, and *S. hollandei* (Levine 1971). However, the trophozoites in all three of these species differ from the isolate we describe here in several ways (Table 2.1): *S. fallax* has many more longitudinal folds (~ 90); *S. sabellariae* has transverse striations across the entire cell; and the anterior end of *S. hollandei* was reported to be “heart-shaped” (Table 2.1). Therefore, we established a new species for this isolate using host-specific, morphological, and molecular phylogenetic markers.

The trophozoites of *Selenidium boccardiellae* n. sp. were isolated from the intestines of *Boccardiellae ligerica* and were most similar in the number of longitudinal folds (10–12) and overall size with *S. spionis*, *S. cirratuli*, *S. polydorae*, and *S. rayi* (Levine 1971) (Table 2.1). However, all five species were isolated from a different host species and have trophozoites that differ in the shape of the mucron: *Selenidium boccardiellae* n. sp. has a pointed mucron, *S. spionis* has a depressed sucker-like mucron, *S. cirratuli* has a cone-shaped mucron, and both *S. polydorae* and *S. rayi* have mucrons described as “nipple-like” or “knob-like” (Levine 1971). Moreover, the flattened cell shape of the trophozoites in *S. boccardiellae* n. sp. distinguishes this species from these other four species, which are not flattened. Therefore, we established a new species for this isolate using host-specific, morphological, and molecular markers.

Our molecular phylogenetic analyses resulted in three different lineages of *Selenidium* species: a *S. terebellae* lineage, a clade consisting of *S. orientale*...
and *S. pisinnus* from the intestines of sipunculids, and a clade consisting of *S. boccardiellae*, *S. cf. mesnili*, *S. idanthrysae*, and *S. serpulae*. Whether or not these three lineages form a more inclusive “*Selenidium* clade” or occupy different positions along the apicomplexan backbone was inconclusive in our analyses.

The trophozoites of the *Selenidium* species share several morphological features: a vermiform cell, a pointed mucron and posterior tip, nematode-like bending motility, and relatively few (i.e. between 4 and 44) longitudinal epicytic folds (Rueckert and Leander 2009; Simdyanov and Kuvardina 2007) (Table 2.1). Within the context of available molecular phylogenetic data, the fewest numbers of longitudinal epicytic folds are found in *S. terebellae* (4–6) and *S. boccardiellae* (10–12); the largest number is found in *S. pisinnus* (40–44). The trophozoites of *S. cf. mesnili*, *S. idanthrysae*, *S. orientale*, and *S. serpulae* have an intermediate number of longitudinal epicytic folds (i.e. 18–30), suggesting that a gradual increase in the number of longitudinal folds occurred in the evolutionary history of archigregarines, presumably as a way to increasing surface area for acquiring nutrients within the intestines of their hosts. Moreover, like *P. vivax*, *S. boccardiellae* and *S. orientale* have trophozoites that are considerably flattened, which also increases surface area.

The different molecular phylogenetic positions of these three species suggest that flattened trophozoites have evolved several times independently. The presence of transverse striations on the surface of trophozoites is a distinctive feature of many archigregarines; in the context of our phylogenetic analysis, transverse striations are present in *S. cf. mesnili*, *S. serpulae*, *S. terebellae*, *P. vivax*, the anterior region of *S. idanthrysae*, and *V. leptosynaptae* n. gen., n. sp. (Leander 2006, 2007; Leander et al. 2003b; Rueckert and Leander 2009). Transverse striations have not been observed on the trophozoites of *S. boccardiellae*, *S. orientale*, and *S. pisinnus* (Rueckert and Leander 2009). The phylogenetic distribution of archigregarines without transverse striations suggests that this feature has been lost at least three times independently: once in *S. boccardiellae*, once in the most recent ancestor of *S. orientale* and *S. pisinnus*,
and once in the most recent ancestor of marine lecudinids and urosporids (excluding *L. polymorpha* and close relatives).

### 2.4.2 Veloxidium leptosynaptae n. gen., n. sp.

Twenty-five species of apicomplexans have been described from echinoderms thus far: 23 eugregarines, one *Selenidium* species, and one coccidian (Barel and Kramers 1977; Jangoux 1987). The eugregarine species constitute the Urosporidae and fall within three genera: *Cystobia*, *Lithocystis*, and *Urospora* (Coulon and Jangoux 1987; Jangoux 1987). Similar to so many other lineages of gregarines, molecular information available for gregarines from echinoderms is sparse. Only a single DNA sequence collected from a putative *Lithocystis* sp. isolated from an echinoid is available; this sequence was not definitively linked to any morphological features of the trophozoites or other life history stages (Leander et al. 2006). The description and phylogenetic analysis of *V. leptosynaptae* n. gen., n. sp., in this study, represents the first morphological description of a gregarine isolated from an echinoderm in tandem with molecular phylogenetic data.

The motility and morphological characteristics of *V. leptosynaptae* are reminiscent of other archigregarines, particularly behavioral traits exhibited by members of *Selenidium*. Syzygy in this species was head-to-head. Light micrographs and videos show trophozoites of *V. leptosynaptae* bending, twisting, and thrashing in an almost identical manner to that of many *Selenidium* spp. (e.g. *S. boccardiiellae*, *S. idanthyrsae*, and *S. cf. mesnili*, in this study). However, the motility of these trophozoites was also distinctive in the relatively rapid manner in which it occurred. The trophozoite surface of *V. leptosynaptae* was not adorned with longitudinal epicytic folds and instead was covered with a dense array of transverse striations, most similar to those observed in the archigregarines *P. vivax* and *Digyalum oweni* (Dyson et al. 1993, 1994; Gunderson and Small 1986; Leander 2006; Rueckert and Leander 2009). Mingazzini (1893) originally
described the only other *Selenidium* archigregarine from a holothurian, namely *Selenidium synaptae* from the intestines of *Synapta* sp., which was subsequently accepted by Levine (1971). The original description of *S. synaptae* documented four longitudinal epicytic folds on the surface of the trophozoites, a feature that is not present in *V. leptosynaptae* n. gen., n. sp. Therefore, the host species and the morphology of our isolates of *V. leptosynaptae* do not conform to the description of *S. synaptae* (Table 2.1). With the exception of *Platyproteum*, none of the other eight previously described archigregarine genera has a trophozoite stage that has transverse surface striations and lacks longitudinal epicytic folds (Rueckert and Leander 2009). The trophozoites of *Platyproteum*, however, differ markedly from those in *V. leptosynaptae* because they are greatly flattened, have a distinctive wave-like pattern of motility, and are found in sipunculids. Moreover, the SSU rDNA sequence generated from *V. leptosynaptae* is very distantly related to the sequence from *Platyproteum* (Figure 2.5). Therefore, it was necessary to establish a new genus for this unusual isolate.

**2.4.3 Character evolution and the “archigregarine” morphotype.**

There are two very important realizations in any interpretation of character evolution within the context of available molecular phylogenetic data from gregarines: (i) The archigregarine concept has never been adequately tested with molecular phylogenetic data and is, at most, a convenient category of trophozoite morphotypes with little or no taxonomic value; (ii) the deepest relationships within the Apicomplexa are almost completely unresolved with available molecular phylogenetic data. Analyses of SSU rDNA sequences do not provide (statistical) support for or against any of the relationships between the major clades of gregarines (especially genera referred to as “archigregarines”) and other groups of apicomplexans (rhytidicystids, coccidians + prioplasmids, and cryptosporidians). The current molecular phylogenetic data contain several different species of gregarines with trophozoites that conform to the “archigregarine” morphotype (e.g. spindle-shaped or vermiform cells with
transverse striations, bending motility, and relatively few or no longitudinal epicytic folds). These species fall within a minimum of five different clades/lineages, four of which do not have any sister group and therefore have unresolved phylogenetic positions along the apicomplexan backbone. The clustering of these lineages at the bottom of Figure 2.5 reflects convenience of illustration (i.e. it provides a way to bracket species with the archigregarine morphotype) and does not reflect any reliable evidence for phylogenetic relationships. Nevertheless, the strongly supported phylogenetic position of the Veloxidium clade was the most exciting discovery of this study because the new species reported here conforms to the archigregarine morphotype yet branches as the nearest sister group to a large and diverse clade of marine eugregarines (i.e. urosporids and most lecudinids). Two reasonable interpretations exist for this situation: (i) Veloxidium is a descendant of eugregarines, and the archigregarine morphotype in this species reflects convergent evolution with (distantly related) Selenidium species; or (ii) the archigregarine morphotype in Veloxidium reflects morphostasis of traits that are shared with Selenidium species and their most recent common (archigregarine) ancestor.

As articulated in Leander (2008), we favor the second interpretation, specifically that available data are consistent with the hypothesis that species with the archigregarine morphotype represent a large paraphyletic (stem) group from which many other clades of gregarines evolved (Cox 1994; Grassé 1953; Leander 2008; Leander and Keeling 2003; Leander et al. 2003a; Rueckert and Leander 2009; Théodoridès 1984; Vivier and Desportes 1990). An expectation of this hypothesis is that different species with the archigregarine morphotype will form the nearest sister lineage to several different major groups of eugregarines that currently branch from the apicomplexan backbone without resolution. The phylogenetic position of Veloxidium conforms to this expectation. This hypothesis would be unsupported/falsified if all or most gregarine species with the archigregarine morphotype clustered exclusively within one clade (i.e. monophyletic) and/or were phylogenetically scattered within several different
major groups of eugregarines (i.e. polyphyletic). This distribution of archigregarine species is not supported by current molecular phylogenetic data.

Three relatively large clades of eugregarines are usually recovered in molecular phylogenetic analyses of SSU rDNA sequences: (i) terrestrial eugregarines plus neogregarines, (ii) eugregarines form crustacean hosts, and (iii) marine lecudinid and urosporid eugregarines, excluding *L. polymorpha* and close relatives (Rueckert et al. 2011) (Figure 2.5). Most members of these clades have trophozoites with a cortex consisting of a dense array of longitudinal epicytic folds and with gliding motility that is distinct from the bending and twisting movements of archigregarines. The most parsimonious explanation from previous work was that this combination of features evolved in the most recent common ancestor of eugregarines (Leander et al. 2003a, 2006). However, the robust molecular phylogenetic position of *V. leptosynaptae* as the nearest sister group to a diverse clade of marine lecudinids and urosporids provides support not only to the hypothesis that archigregarines form a paraphyletic stem group from which other gregarines evolved, but also that trophozoites with gliding motility and dense arrays of longitudinal epicytic folds (e.g. most eugregarines) evolved several times independently. Continued exploration, discovery, and molecular characterization of new archigregarine species will test these inferences and provide some of the greatest insights into the earliest stage of apicomplexan evolution.

### 2.5 Taxonomic summary and descriptions

Apicomplexa Levine, 1970  
Gregarinea Bütschli, 1882, Embedded Grassé, 1953  
Archigregarinorida Grassé, 1953  
Selenidiidae Brasil, 1907
**Selenidium** Giard, 1884 *Selenidium boccardiellae* n. sp. Wakeman and Leander

**Diagnosis.** Trophozoites are vermiform, mean length = 158 µm (range= 87-250), mean width = 10 µm (range= 10-12). Cells are slightly compressed forming two lateral ridges, especially in the anterior region. The mucron tapers and is pointed. The surface is inscribed with 10-12 shallow epicytic folds. The epicytic folds swirl near the tapered posterior end. The nucleus is ellipsoidal (11 µm x 5 µm) and located in the middle upper half of the cell. Trophozoites are capable of bending, coiling and trashing movements.

**Gene sequence.** A sequence of the SSU rDNA is deposited as GenBank accession No. JN857969.

**Type locality.** Odgen Point, Victoria, British Columbia (48° 25' 43" N 123° 21' 56" W), Canada. Subtidal 7m.

**Type habitat.** Marine

**Type host.** *Boccardiella ligerica* (Ferronnière, 1898) (Metazoa, Annelida, Polychaeta, Spionida, Spionidae)

**Location in host.** Intestinal lumen

**Holotype.** Figure 2.1A is an image taken from the holotype fixed on a gold sputter-coated SEM stub. The stub has been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection; voucher: MI-PR114) at the University of British Columbia, Vancouver, Canada.

**Etymology.** The specific epithet is named for the genus of the polychaete host in which this species was found.

*Selenidium idanthyrsae* n. sp. Wakeman and Leander

**Diagnosis.** Trophozoites are elongated with a compressed and spade-like posterior end. Trophozoites have an average length of 438 µm (range= 450-543 µm) and an average width of 15 µm (range= 14-16). The spherical nucleus (14 µm x 14 µm) was position in the middle to posterior part of the cell. The cell surface was lined with 20-22 epicytic folds. Transverse striations are present on
parts of the anterior near the anterior part of the cell near the mucron. The mucron is pointed, protruding 2-3 µm. This species is capable of bending and coiling movements.

**Gene sequence.** A sequence of the SSU rDNA is deposited as GenBank accession No. JN857967.

**Type locality.** Wizard Islet (48° 50' 17'' N, 125° 08' 02'' W). Subtidal 20m. Bamfield, British Columbia, Canada.

**Type habitat.** Marine

**Type host.** *Idanthyrsae saxicavus* (Baird, 1863) (Metazoa, Annelida, Polychaeta, Sabellariidae)

**Location in host.** Intestinal lumen

**Holotype.** Figure 2.3 C. Image taken from the holotype fixed on a gold sputter-coated SEM stub. The stub has been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection; voucher: MI-PR115) at the University of British Columbia, Vancouver, Canada.

**Paratype.** Figure 2.3 A

**Etymology.** The specific epithet is named for the genus of the polychaete host in which this species was found.

*Veloxidium* n. gen. Wakeman and Leander

**Diagnosis.** Trophozoites are vermiform with a spherical nucleus located in the middle of the cell. The trophozoite surface lacks longitudinal epicytic folds and has transverse striations. Syzygy is head-to-head. Trophozoites are capable of dynamic and distinctively rapid bending movements.

**Type species.** *Veloxidium leptosynaptae*

**Etymology.** The genus name is taken from ‘*Velox*’ (Latin) = “rapid” and refers to the fast bending and thrashing motility of the trophozoites.

*Veloxidium leptosynaptae* n. sp. Wakeman and Leander
**Diagnosis.** Trophozoites are vermiform, mean length = 74 µm (range = 62-97 µm) and mean width = 11 µm (range = 9-13 µm). A spherical nucleus (10 µm x 10 µm) is located in the middle of the cell. The surface of the trophozoites lacks longitudinal epicytic folds and is covered with a dense array of transverse striations. The anterior mucron region is dome-like. The posterior end tapers to a point and in some individuals bends characteristically at an angle. Syzygy is head-to-head. The trophozoites are capable of dynamic bending and twitching movements.

**Gene sequence.** A sequence of the SSU rDNA is deposited as GenBank accession No. JN857966.

**Type locality.** Grappler Inlet. Muddy intertidal. Bamfield, British Columbia (48°49' 49" N 125° 8'15" W), Canada.

**Type habitat.** Marine

**Type host.** *Leptosynapta clarki* (Heding, 1928) (Metazoa, Echinodermata, Holothuroidea, Synaptidae, Leptosynapta)

**Location in host.** Intestinal lumen.

**Holotype.** Figure 2.4 A. Image taken from the holotype fixed on a gold sputter-coated SEM stub. The stub has been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection; voucher: MI-PR116) at the University of British Columbia, Vancouver, Canada.

**Paratypes.** Figure 2.4 C-E

**Etymology.** The specific epithet is named for the genus of the polychaete host in which this species was found.
3 Molecular phylogeny of marine gregarine parasites (Apicomplexa) from tube-forming polychaetes (Sabellariidae, Cirratulidae and Serpulidae), including descriptions of two new species of Selenidium

3.1 Synopsis

Gregarine parasites within the genus Selenidium have retained several morphological and ecological traits inferred to be ancestral for apicomplexans as a whole, such as monoxenous lifecycles involving extracellular trophozoites (feeding stages) that feed by myzocytosis within the intestinal lumen of marine invertebrate hosts (Leander 2007, 2008; Schrével 1968, 1970, 1971a, b, Simdyanov and Kuvardina 2007). However, molecular phylogenetic data from this genus are rare, which severely limits our understanding of these evolutionarily significant parasites. Small subunit (SSU) rDNA sequences, for instance, are currently available from only seven of the approximately 60 described species of Selenidium, and it is expected that the vast majority of Selenidium species have yet to be discovered and characterized. As such, the phylogenetic relationships between Selenidium species and other lineages of marine gregarines remain unclear (Leander et al. 2003; Leander 2007, 2008; Rueckert and Leander 2009; Wakeman and Leander 2012).

Selenidium species have been reported predominantly from polychaete worms (e.g., spionids, sabellids, and serpulids), but have also been described from sipunculids, sea cucumbers, hemichordates, and tunicates (Levine 1971; Schrével 1971a; Rueckert and Leander 2009; Wakeman and Leander 2012). The intestinal trophozoites are either spindle-shaped or vermiform and exhibit a type of bending motility that is reminiscent of nematodes. The cell shape, pattern of motility, and specific ultrastructural features associated with the trophozoite stage in Selenidium (e.g., an apical complex associated with myzocytosis and a robust
corset of microtubules beneath the inner membrane complex) are most similar to the traits of apicomplexan sporozoites in general and, taken together, are indicative of the traditional “archigregarine” concept (Grassé 1953; Leander 2008, Mellor and Stebbings 1980; Schrével 1970, 1971a, b; Simdyanov and Kuvardina 2007; Stebbings et al. 1974; Vivier and Schrével 1964; Wakeman and Leander 2012).

This concept promotes the inference that *Selenidium* species have retained several morphological, ecological, and life history traits from distant ancestors that provide compelling insights into the earliest stages of apicomplexan evolution (Barta and Thompson 2006; Cox 1994; Dyson et al. 1994; Leander 2006; Leander 2008; Leander and Keeling 2003b). Therefore, continued exploration of *Selenidium* diversity is expected to shed considerable light onto the deepest relationships within the phylogeny of apicomplexans and onto patterns of co-evolution between gregarine apicomplexans and marine metazoan hosts (Leander 2008; Rueckert and Leander 2009; Théodoridès 1984).

Unlike many species of terrestrial gregarines (from insect hosts), marine gregarines are particularly challenging to work with because they are generally encountered only as trophozoites and in low numbers within a small percentage of individual hosts that are difficult to obtain from oceanic environments (Leander 2007, 2008; Rueckert and Leander 2010).

In this study, we discovered five different morphotypes of *Selenidium* isolated from the intestines of three different species of tube-forming polychaetes collected from the Pacific Ocean. These *Selenidium* isolates were compared to one another and to previously described species using a combination of molecular, ecological, and morphological data. SSU rDNA sequences were generated from single-cell (trophozoite) isolates that were first imaged with light microscopy. These data enabled us to evaluate cryptic diversity among gregarines, establish two new species of *Selenidium*, and link SSU rDNA sequences to a previously described species of *Selenidium*. These data
underscored the practical advantages of DNA sequences in advancing the field of gregarine systematics and the importance of reciprocal reinforcement between molecular, morphological and ecological data in building arguments for the establishment of new species (Leander 2008; Rueckert et al. 2011a; Wakeman and Leander 2013).

3.2 Materials and methods

3.2.1 Collection of organisms.

Neosabellaria cementarium Moore, 1906 and Dodecaceria concharum Örsted, 1843 were collected in February 2012 off rocks in Victoria, British Columbia, Canada while SCUBA diving at a depth of 10-20 meters near Ogden Point 48˚24’46.10”N 123˚23’24.67”W and Clover Point 48˚24’14.18”N 123˚21’00.91”W, respectively. Spirobranchus giganteus Pallas, 1766 was purchased in March 2012 from J&L Aquatics, Burnaby, British Columbia, Canada. Host guts from seven, nine and four individuals of N. cementarium, D. concharum and S. giganteus, respectively, were removed with forceps and teased apart in seawater on a well slide. All gut material from N. cementarium and D. concharum was infected with gregarine parasites; 75% of the material (i.e., three individuals) from S. giganteus was infected.

3.2.2 Light and scanning electron microscopy.

Hand-drawn glass pipettes were used to isolate and clean individual trophozoites using an inverted microscope (Zeiss Axiovert 200, Carl-Zeiss, Göttingen, Germany) attached to a PixeLink Megapixel color digital camera (PL-A662-KIT, Ottawa, Canada). Some isolates were photographed on well slides, washed in autoclaved, filtered seawater and collected for DNA extraction and single-cell PCR amplification (SC-PCR). Isolates were also photographed using a Leica DC 500 color camera attached to a Zeiss Axioplan 2 microscope (Carl-Zeiss,
Göttingen, Germany); as noted below, some of these cells were also recovered for SC-PCR.

To generate scanning electron micrograph (SEM) images, individual trophozoites were pooled in 2% glutaraldehyde on ice. A 10-µl polycarbonate membrane filter was placed within a Swinnex filter holder (Millipore Corp. Billerica, MA, USA). Trophozoites were filtered onto the membrane using a syringe with distilled water, and the holder was placed in a small beaker (4 cm diam. and 5 cm tall) that was filled with distilled water. Ten drops of 1% OsO₄ were added to the opening of the filter holder, and the samples were post-fixed on ice for 30 minutes. The syringe was used to slowly run distilled water over all samples. A graded series of ethanol washes (30%, 50%, 75%, 85%, 95% and 100%) was then used to dehydrate the fixed cells using the syringe system. Following dehydration, the polycarbonate membrane filters containing the trophozoites were transferred from the Swinnex filter holders into an aluminum basket submerged in 100% ethanol in preparation for critical point drying with CO₂. The dried polycarbonate membrane filters containing the trophozoites were mounted on aluminum stubs, sputter coated with 5 nm gold and viewed under a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

3.2.3 DNA isolation, gene amplification, cloning and sequencing of the Selenidium species.

Each of the single-cell isolates (13 total) were placed in a 1.5 ml eppendorf tube containing cell lysis buffer. Genomic DNA was extracted with the standard protocol provided by the MasterPure complete DNA & RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). However, the final elution step was lowered to 4 µl, with the goal of concentrating extracted DNA prior to SC-PCR amplification. Outside primers, PF1 5’ – GCGCTACCTGGTTGATCCTGCC
– 3’ and SSUR4 5’ – GATCCTTCTGCAGGTTCACCTAC – 3’ (Leander et al. 2003), were used in a 25 µl PCR reaction with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI, USA). The following program was used on the thermocycler for the initial amplification: Initial denaturation at 94°C for 2:00 m; 35 cycles of denature at 94°C for 0:30 s, anneal at 52°C for 0:30 s, extension at 72 °C for 1:50 m., final extension 72°C 9:00 m. Subsequently, internal primers F2 5’ – GGTAGYGACAAGAATAACAC – 3’ and R2 5’ – GAYTACGACGGTATCTGATCGTC – 3’ were paired with outside primers in a nested PCR reaction using the following program on a thermocycler: initial denaturation for 94°C for 2:00 m; 25 cycles of denature at 94°C for 0:30 s, anneal at 55°C for 0:30 s., extension at 72 °C for 1:30 m.; final extension at 72°C for 9:00 m. All SC-PCR products were separated on agarose gels and isolated using the UltraClean15 DNA Purification Kit (MO BIO, Laboratories, Inc., Carlsbad, CA, USA), and cloned into a pCR 2.1 vector using a StrataClone PCR cloning kit (Aligent Technologies, Santa Clara, CA, USA). Clones were screen for size and sequenced using vector primers and ABI Big-dye reaction mix. Novel sequences (i.e., one from each of the single-cell isolates) were identified using the National Center for Biotechnology Information’s (NCBI) BLAST tool, confirmed with molecular phylogenetic analyses, and deposited into GenBank (KC110863-KC110875).

3.2.4 Molecular phylogenetic analyses.

Two separate datasets were constructed de novo and analyzed in this study: (1) A comprehensive 82-taxon alignment (1,085 unambiguously aligned sites) containing a representative SSU rDNA sequence from each of the five _Selenidium_ morphotypes described here, three dinoflagellate sequences (outgroup), and 74 sequences representing gregarine and other apicomplexan subclades; and (2) a 17-taxon alignment (1,610 unambiguously aligned sites) containing the 13 _Selenidium_ sequences generated in this study by SC-PCR (from 5 different morphotypes) and four sequences published previously from
closely related *Selenidium* species. Both alignments were initially constructed using MUSCLE (Edgar 2004) and were subsequently edited using MacClade 4 (Maddison and Maddison 2004); gaps and ambiguously aligned regions were excluded from the analyses.

Jmodeltest 0.1.1 selected a GTR + I + I' model of evolution under Akaike Information Criterion (AIC) and AIC with correction (AICc) for both alignments (82-taxon alignment: proportion of invariable sites = 0.1820, gamma shape = 0.6230; 17-taxon alignment: proportion of invariable sites = 0.3290, gamma shape = 0.3770) (Posada and Crandall 1998). Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) was used to infer a maximum likelihood (ML) tree and for ML bootstrap analyses (500 pseudoreplicates, one heuristic search per pseudoreplicate) (Zwickl 2006). Bayesian posterior probabilities were calculated for both alignments using the following parameter on the program MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003): [GTR (Lset nst = 6); gamma distribution (of rate among sites) and Monte Carlo Markov Chains [starting trees = 4; default temperature = 0.2; generations = 7,000,000; sample frequency = 100; prior burn-in = 500,000 trees]; stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate)]. Burn-in was confirmed manually, and majority-rule consensus trees were constructed; posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees. PAUP 4.0 (Swofford 1999) was used to calculate percent differences between the SSU rDNA sequences in the 17-taxon alignment.

3.3 Results

3.3.1 *Selenidium cf. echinatum*.

The trophozoites isolated from the gut material of *Dodecaceria concharum* were spindle-shaped, 173 µm (95-205 µm; n = 14) long and 10 µm (8-11 µm; n = 14)
wide (Table 3.1 and Figure 3.1). The cells were capable of bending and twisting. The spherical nucleus had an average width of 10 \( \mu m \) \( (n = 14) \) and was positioned in the anterior-half of the trophozoites. Syzygy of gamonts was tail-to-tail (Figure 3.1). The mucron terminated as a nipple-like projection at the anterior tip of the cell, where a single apical opening was observed. Five to six longitudinal striations were observed on one side of the cell (Figure 3.1).

Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR124.

### 3.3.2 Selenidium neosabellariae n. sp.

Trophozoites isolated from the intestines of Neosabellaria cementarium were elongated and vermiform in shape, 292 \( \mu m \) (125-350 \( \mu m \); \( n = 27 \)) long, and 11 \( \mu m \) (9-12 \( \mu m \); \( n = 27 \)) wide (Table 3.1 and Figure 3.2). The spherical nucleus (10 \( \mu m \); \( n = 20 \)) was positioned in the center of the trophozoite cell (Figure 3.2). The posterior end tapered to a fine point, and the base of a cone-shaped mucron was defined by a cluster of transverse striations. Five to six longitudinal striations were observed on one side of the cell (Figure 3.2). Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR122.

### 3.3.3 Selenidium sensimae n. sp.

This species represented one of three trophozoite morphotypes isolated from the intestines of Spirobranchus giganteus. These trophozoites were spindle shaped, 155 \( \mu m \) (130-170 \( \mu m \) \( n = 11 \)) long, and 12 \( \mu m \) (10-13 \( \mu m \); \( n = 11 \)) wide (Table 3.1 and Figure 3.3). An ellipsoidal nucleus (10 \( \mu m \) x 4-6 \( \mu m \); \( n = 11 \)) was centrally located in the cell. The posterior end of the cell tapered distinctly to a blunt point with a terminal indentation (Figure 3.3); the anterior region of the cell either
tapered to a blunt point or was rounded. There were eight to nine longitudinal striations observed on one side of the cell. A row of micropores were positioned in the grooves between the epicytic folds and extended nearly the entire length of the cell (Figure 3.3). Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR123.

3.3.4 *Selenidium* sp. 1.

This species represented one of three trophozoite morphotypes isolated from the intestines of *Spirobranchus giganteus*. These trophozoites were relatively cylindrical in shape, elongated, undulated, 170 µm (105-220 µm; n = 9) long, and 13 µm (9-14 µm; n = 9) wide (Table 3.1 and Figure 3.3). The posterior and anterior ends of the cell were distinctly bulbous in shape. The spherical nucleus (10 µm; n = 10) was positioned in the anterior half of the cell (Figure 3.3).

3.3.5 *Selenidium* sp. 2.

This species represented one of three trophozoite morphotypes isolated from the intestines of *Spirobranchus giganteus*. These trophozoites were spindle shaped, 160 µm (150-185 µm; n = 13) long, and 13 µm (10-15 µm; n = 13) wide (Table 3.1 and Figure 3.3). The posterior end of the cell was distinctly spade-like in appearance; the anterior region of the cell was pointed in association with a cone-shaped mucron. The ellipsoidal nucleus (10 µm x 4-6 µm; n = 13) was centrally located in the trophozoite cell. Nine to ten longitudinal striations were observed on one side of the cell (Figure 3.3).
3.3.6 Molecular phylogenetic analyses.

Analyses of the 82-taxon alignment resulted in four relatively large clades of marine gregarines: crustacean gregarines, capitellid gregarines, lecudinids, and Selenidium species isolated from Pacific tube-forming polychaetes (Figure 3.4). All of the sequences reported in this study clustered within the Selenidium clade from Pacific tube-forming polychaetes with very strong statistical support (Figure 3.4). The Selenidium species isolated from serpulid polychaetes (S. serpulae, S. sensimae n. sp. Selenidium sp. 1 and Selenidium sp. 2) formed a robust subclade within the more inclusive Selenidium clade from Pacific tube-forming polychaetes. The two Selenidium species isolated from sipunculids (S. orientale and S. pisinnus) formed a separate clade that branched from an unresolved apicomplexan backbone; S. terebellae, isolated from a spaghetti worm (Thelepus sp.), did not cluster with either of the two Selenidium clades (Figure 3.4).

Our analyses also recovered separate clades consisting of coccidians, cryptosporidians, rhytidocystids, and two different compositions of terrestrial gregarines (terrestrial gregarines I and II). Generally speaking, statistical support values for branches reflecting more recent relationships were strong, but the overall apicomplexan backbone was poorly resolved (Figure 3.4)

Molecular phylogenetic analyses of the 17-taxon alignment focused on the internal relationships within the Selenidium clade from Pacific tube-forming polychaetes and contained all of the SSU rDNA sequences derived from SC-PCR and four SSU rDNA sequences from previous work.

These analyses resulted in distinct and well-supported subclades of Selenidium species that correlated with host affiliation and trophozoite morphology (Figure 3.5). Genetic distances between the SSU rDNA sequence of Selenidium sp. 2 and all other Selenidium species in the analysis ranged from 8.3-14.0%; intraspecific variation between the SC isolates of Selenidium sp. 2 ranged from 0.3-0.8% (Table 3.2). Genetic distances between the SSU rDNA sequence of
Selenidium sp. 1 and all other Selenidium species in the analysis ranged from 4.8-11.5%; intraspecific variation between the SC isolates of Selenidium sp. 1 ranged from 0.18-0.48% (Table 3.2). Genetic distances between the SSU rDNA sequence of S. sensimae n. sp. and all other Selenidium species in the analysis ranged from 5.8-12.8%; intraspecific variation between the SC isolates of S. sensimae n. sp. was 0.6% (Table 3.2).

Genetic distances between the SSU rDNA sequence of S. cf. echinatum and all other Selenidium species in the analysis ranged from 8.9-11.0%; intraspecific variation between the SC isolates of S. cf. echinatum n. sp. was 1.7% (Table 3.2). The lowest interspecific genetic distance found within this clade ranged from 2.2-2.4%, between S. neosabellariae n. sp. and S. indanthryrsa. Intraspecific variation between the SC isolates of S. neosabellariae n. sp. ranged from 0.24-0.42% (Table 3.2).
Figure 3.1 Micrographs of *Selenidium cf. echinatum*. C-E. Differential interference contrast (DIC) light micrographs (LMs) showing the general spindle-shaped morphology of the trophozoites that were used for single-cell (SC) PCR. The nipple-like mucron is oriented upwards. The spherical nucleus (N) is positioned in the anterior region of the trophozoites. Longitudinal epicytic folds (arrowhead) run nearly the entire length of the trophozoite. E. LM showing two gamonts in tail-to-tail syzygy. F. High-magnification SEM showing the mucron region of the trophozoite with a single apical opening at the tip (arrow). G. SEM showing the general morphology of the trophozoite. Five to six relatively weakly developed epicytic folds (arrowhead) run along the longitudinal axis on each side of the cell. Scale bars: 1--5 = 25 µm; 6 = 1 µm; 7 = 10 µm.
Figure 3.2 Micrographs of *Selenidium neosabellariae*. **A-D.** Differential interference contrast (DIC) light micrographs (LMs) showing the vermiform and highly undulated morphology of the trophozoites with a nucleus (N) located in the center of the cell. Images **B-D.** show the specific cells that were recovered for single-cell (SC) PCR. **E.** SEM showing the general vermiform-shape of the cell and epicytic folds (double arrowhead) that run along the longitudinal axis of the cell. The nipple-like mucron (arrowhead) is defined by a basal cluster of transverse striations (arrow). **F.** High-magnification SEM showing the mucron region (arrowhead) of the cell, the transverse striations (arrow), and the longitudinally arranged epicytic folds (double arrowhead). **G.** High magnification SEM of the longitudinally arranged epicytic folds. Scale bars: 8--11 = 35 μm; 12 = 10 μm; 13 = 3 μm; 14 = 1.5 μm.
Figure 3.3 Light and scanning electron micrographs of *Selenidium sensimae* n. sp., *Selenidium* sp. 1 and *Selenidium* sp. 2. A-I. Differential interference contrast (DIC) light micrographs showing trophozoites of *Selenidium* that were used for single-cell (SC) PCR. A-C. Light micrographs of *S. sensimae* n. sp. The rounded mucron (M) is oriented upwards, the posterior end is blunt with a terminal indentation (I), and epicytic folds (arrowhead) run along the longitudinal axis of the cell. C. LM of a single-cell of *S. sensimae* n. sp. that was subsequently isolated and prepared for SEM. D-F. LMs showing the general vermiform and cylindrical shape of *Selenidium* sp. 1. The nucleus (N) is positioned near the rounded mucron (M). The posterior end is distinctively bulbous (B). G-I. Light micrographs showing the trophozoites of *Selenidium* sp. 2. The pointed mucron (M) is oriented upwards and eighteen to twenty epicytic folds (arrowhead) run along the longitudinal axis of the cell. An ellipsoidal nucleus (N)
is positioned in the middle of the cell. The posterior end of the trophozoite is distinctly flattened and spade-shaped (S). J. Scanning electron micrograph showing the general cell morphology of *S. sensimae* n. sp. with eight to nine epicytic folds (arrowhead) that run along the longitudinal axis on one side of the cell. K. High-magnification SEM showing rows of pores running along the longitudinal axis of *S. sensimae* n. sp. Scale bars: 15--18, 19 = 1 µm, 20--25 = 10 µm.

Figure 3.4 Phylogenetic analyses of *Selenidium*. Maximum likelihood (ML) tree derived from phylogenetic analysis of the 82-taxon dataset (1,085 unambiguously aligned sites) of small subunit (SSU) rDNA sequences. This tree
was inferred using the GTR+Γ substitution model (\( - \ln L = 21,138.89056 \) gamma shape = 0.6230, proportion of invariant sites = 0.1820). Bootstrap support values are listed above, and Bayesian posterior probabilities are listed below. Black dots on branches denote bootstrap support values and Bayesian posterior probabilities of 95/0.95 or greater, respectively. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree. Representative sequences from the five species of *Selenidium* described in this study are highlighted in black boxes. Some branches were shortened by the length (e.g., X1) of the substitutions/site scale bar.

![Phylogenetic tree](image)

**Figure 3.5 Phylogenetic analyses of single-cell isolates of *Selenidium*.** Unrooted maximum likelihood (ML) tree derived from phylogenetic analysis of the 17-taxon dataset (1,610 unambiguously aligned sites) containing small subunit (SSU) rDNA sequences from 13 single-cell isolates representing the five species of *Selenidium* described here (bold) and four closely related species of *Selenidium* published previously. This tree was inferred using the GTR+Γ substitution model (\( - \ln L = 5,903.65704 \), gamma shape = 0.3770, proportion of invariant sites = 0.3290). Bootstrap support values are listed above Bayesian posterior probabilities; support values of 100 and 1.00 for bootstrap and Bayesian analyses, respectively, are represented by black dashes. Morphological traits that distinguish the species from one another are indicated by letters. **A**: Spindle-shaped trophozoites, 160 \( \mu \)m long and 13 \( \mu \)m wide, with
spade-like posterior end; host: *Spirobranchus giganteus*. **B**: Spindle-shaped trophozoites, 155 µm long and 12 µm wide, with blunt posterior end containing a terminal indentation; host: *Spirobranchus giganteus*. **C**: Vermiform trophozoites, 170 µm long and 13 µm wide, with bulbous posterior and anterior ends; host: *Spirobranchus giganteus*. **D**: Vermiform trophozoites, 292 µm long and 10 µm wide, with a pointed posterior end and a cluster of transverse striations that define the base of a nipple-like mucron; host: *Neosabellaria cementarium*. **E**: Spindle-shaped trophozoites, 173 µm long and 10 µm wide, with pointed posterior end, a nipple-like mucron, and five to six subtle epicytic folds on each side of the cell; host: *Dodecaceria concharum*. Some branches were shortened by multiple lengths (e.g., X2) of the substitutions/site scale bar.
Table 3.1. Morphology of *Selenidium*. Comparison of trophozoites between species within the *Selenidium* clade and the type species, *S. pendula*.

<table>
<thead>
<tr>
<th>Host</th>
<th>Host tissue</th>
<th>Locality</th>
<th>Trophozoite shape</th>
<th>Tophozoite size (L x W, µm)</th>
<th>Nucleus shape</th>
<th>Nucleus size (L x W, µm)</th>
<th>Position of nucleus</th>
<th>Shape of posterior end</th>
<th>Number of long. epicytic folds</th>
<th>Transverse surface folds</th>
<th>Shape of mucron</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host</td>
<td>Host tissue</td>
<td>Locality</td>
<td>Trophozoite shape</td>
<td>Tophozoite size (L x W, µm)</td>
<td>Nucleus shape</td>
<td>Nucleus size (L x W, µm)</td>
<td>Position of nucleus</td>
<td>Shape of posterior end</td>
<td>Number of long. epicytic folds</td>
<td>Transverse surface folds</td>
<td>Shape of mucron</td>
<td>Literature</td>
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<tr>
<td>Host</td>
<td>Host tissue</td>
<td>Locality</td>
<td>Trophozoite shape</td>
<td>Tophozoite size (L x W, µm)</td>
<td>Nucleus shape</td>
<td>Nucleus size (L x W, µm)</td>
<td>Position of nucleus</td>
<td>Shape of posterior end</td>
<td>Number of long. epicytic folds</td>
<td>Transverse surface folds</td>
<td>Shape of mucron</td>
<td>Literature</td>
</tr>
<tr>
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<td>Host tissue</td>
<td>Locality</td>
<td>Trophozoite shape</td>
<td>Tophozoite size (L x W, µm)</td>
<td>Nucleus shape</td>
<td>Nucleus size (L x W, µm)</td>
<td>Position of nucleus</td>
<td>Shape of posterior end</td>
<td>Number of long. epicytic folds</td>
<td>Transverse surface folds</td>
<td>Shape of mucron</td>
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<td>Host tissue</td>
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<td>Shape of posterior end</td>
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<td>Literature</td>
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Table 3.2 Pairwise genetic distances of *Selenidium*. Summary of percent sequence divergences of small subunit rDNA sequences from different species of *Selenidium*. The five *Selenidium* discussed in this study are shown in bold font. Percent divergences are based on an alignment across 1,685 nucleotides. Intraspecific variation among sequences from four single-cell isolates of each new species are indicated along the diagonal.

<table>
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<tr>
<th></th>
<th><em>Selenidium</em> sp. 2</th>
<th><em>Selenidium</em> sp. 1</th>
<th><em>S. sensimae n. sp.</em></th>
<th><em>S. serpulae</em></th>
<th><em>S. cf. echinatum</em></th>
<th><em>S. cf. mesnili</em></th>
<th><em>S. boccardiellae</em></th>
<th><em>S. neosabellariae n. sp.</em></th>
<th><em>S. idanthyrsae</em></th>
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<td>9.1 - 9.8%</td>
<td>8.3 - 9.0%</td>
<td>8.5 - 9.1%</td>
<td>13.4 - 14.0%</td>
<td>11.7 - 12.2%</td>
<td>12.2 - 12.6%</td>
<td>12.3 - 12.8%</td>
<td>12.4 - 12.7%</td>
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<td>9.2 - 9.5%</td>
<td>9.9 - 10.2%</td>
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<td>10.4 - 10.5%</td>
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<td></td>
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<td>9.1%</td>
<td>9.6%</td>
<td>8.9 - 9.0%</td>
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<td>1.7%</td>
<td>10.9 - 11.0%</td>
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Table 3.2 Pairwise genetic distances of *Selenidium*. Summary of percent sequence divergences of small subunit rDNA sequences from different species of *Selenidium*. The five *Selenidium* discussed in this study are shown in bold font. Percent divergences are based on an alignment across 1,685 nucleotides. Intraspecific variation among sequences from four single-cell isolates of each new species are indicated along the diagonal.
3.4 Discussion

The majority of species descriptions of *Selenidium* species, and gregarines generally, are based on morphological observations using light microscopy, electron microscopy and line drawings (Schrével 1968, 1971a, b; Vivier and Schrével 1964; Levin 1971, 1976, 1977a, b; 1981). Linking these older species descriptions to modern day species-discovery surveys is challenging because definitive traits for species identification are often lacking, especially when new isolates have been collected in environments that are very distant from the type locality. For instance, how does one reconcile a new Pacific isolate that is very similar in morphology to a gregarine species described from an Atlantic type locality? This issue is made increasingly formidable because: (1) infection rates may be low both within an individual host, and among a host population; (2) gregarines have never been cultivated; (3) different life history stages of gregarines are either ambiguous or difficult to observe within an individual host; and (4) host material may be rare and only opportunistically available (e.g., acquired from a marine sediment dredge).

The literature is rich with examples of how molecular phylogenetic data can discriminate different species of microbial eukaryotes (protists) from one another, especially species that are rarely encountered, have very dissimilar life history stages, and are prone to cryptic speciation and convergent evolution (Adl et al. 2007; LaJeunesse et al. 2012). Along these lines, the use of molecular markers, especially SSU rDNA sequences, for evaluating the diversity of marine gregarines has been very informative for delimiting closely related species from one another and for discovering major clades of gregarine species (Leander et al. 2003b; Rueckert and Leander 2010; Rueckert et al. 2010; Rueckert et al. 2011a, b; Rueckert et al. 2012, Wakeman and Leander 2013). Genetic distances have also shed light onto patterns of biogeography and host affinity in different gregarine species (Landers and Leander 2005; Leander et al. 2003b; Rueckert et al. 2011b).
The variation in the SSU rDNA sequences generated here combined with host affinity, type locality, and trophozoite morphology suggest that all five gregarine morphotypes discovered in this study are different species. Two of the host species, namely Neosabellaria cementarium and Spirobranchus giganteus, were examined for gregarines for the first time in this study. A previous study of Dodecaceria concharum in 1899 resulted in the description of one Selenidium species, namely S. echinatum (Caullery and Mesnil 1899). While the type locality of this species is in the Eastern Atlantic Ocean, the trophozoites we encountered within the intestines of Dodecaceria concharum collected from the Eastern Pacific Ocean shared general morphological features with the original line drawings and description of S. echinatum: 10-12 longitudinal epicytic folds, spindle-shape, and tail-to-tail syzygy (Caullery and Mesnil, 1899). Therefore, we chose to designate our isolate “S. cf. echinatum” until molecular phylogenetic data from gregarines isolated from D. concharum collected in the type locality have been generated; these data will further our understanding of biogeographical patterns associated with Selenidium species.

The trophozoites of S. neosabellariae n. sp. were isolated from the intestines of the tube worm Neosabellaria cementarium, which is closely related to the type host of Selenidium idanthyrsae, namely Idanthyrsus saxicavus (Wakeman and Leander 2012). The SSU rDNA sequences from these two Selenidium species were also very similar, with a genetic distance of only 2.2% to 2.4%. However, intraspecific variability within the three different sequences of S. neosabellariae ranged from 0.24% to 0.42%, and the sequences formed a robust clade to the exclusion of S. idanthyrsae in the molecular phylogenetic analyses (Figure 3.5). Moreover, along with an affiliation with different host species, the trophozoites of these two gregarine species can be distinguished from one another at the morphological level; the trophozoites of S. neosabellariae n. sp. are half the average length and have half the number of longitudinal folds (visible) as the trophozoites of S. idanthyrsae (Table 3.1).
The three different morphotypes of trophozoites discovered within the intestines of *Spirobranchus giganteus* also had distinctive SSU rDNA sequences, which taken together formed the basis of establishing *S. sensimae* n. sp. and the recognition of two additional putative species (*Selenidium* sp. 1 and 2). Differences in the form of the posterior end of the trophozoites between *S. sensimae* n. sp., *Selenidium* sp. 1, and *Selenidium* sp. 2 were diagnostic for each: *Selenidium* sp. 1 has a bulbous posterior end, *Selenidium* sp. 2 has a spade-like posterior end, and *S. sensimae* n. sp. has a posterior end that tapers to a blunt point with a terminal indentation. A more focused molecular phylogenetic analyses of the SSU rDNA sequences from these three species (in the 17-taxon alignment) allowed us to include almost 600 additional homologous sites (compared to the more comprehensive 82-taxon alignment consisting of more distantly related gregarines and apicomplexans). The 17-taxon alignment resulted in robust clades that were congruent with the observed morphological difference between the trophozoites. Interspecific variability between these three clades ranged from 5.7% to 9.8%, while intraspecific variability ranged from 0.18% to 0.80% (Table 3.2 and Figure 3.4). The intraspecific variation between the SC isolates was localized to the hyper-variable regions in the SSU rDNA sequences (i.e., not randomly scattered throughout the sequences). Similarly, most of the sequences from the SC isolates of each morphotype shared identical indels and nucleotide substitutions. These results suggest that this variability reflects the population rather than artifacts of PCR or sequencing error. Nonetheless, the hyper-variable regions and ambiguous sites (e.g., indels) were excluded from the phylogenetic analyses.

Molecular phylogenetic analyses of the 82-taxon alignment, including representative sequences from each of the five morphotypes discovered in the study, demonstrated a robust clade of nine *Selenidium* species isolated from Pacific tube-forming polychaetes. This clade does not include *Selenidium* species collected from sipunculids (*S. orientale* and *S. pisinnus*) or other lineages.
of polychaetes such as spaghetti worms (S. terebellae). As such, these data demonstrate that host affinity can be a predictor of gregarine phylogenetic relationships and offers additional insights into emerging co-evolutionary patterns between gregarine parasites and their marine invertebrate hosts. The (unresolved) phylogenetic positions of S. terebellae, Veloxidium leptosynaptae, and the clade consisting of S. orientale and S. pisinnus leave open the possibility that the Selenidium morphotype (i.e., the “archigregarine” concept) reflects a paraphyletic stem group from which all other gregarines, and perhaps apicomplexans, generally evolved (Leander 2008; Wakeman and Leander 2012). Phylogenetic analyses of additional molecular markers (e.g., heat shock protein 90 and perhaps cytoskeletal protein genes) from Selenidium species offer the most promising way forward for evaluating the strength of the phylogenetic hypotheses inferred from SSU rDNA sequences.

It is worthwhile noting here that a paper published this year (Clopton 2012) argued strongly against the predominant use of molecular phylogenetic data for describing gregarine species, and perhaps organisms in general, in favor of a set of ideological rules based on detailed analyses of morphometric data; the promotion of these rules is intended to govern the way new species of gregarines should be described in the future. As reflected in our previous studies (Landers and Leander 2005; Leander 2007; Leander et al. 2003; Rueckert and Leander 2010; Rueckert et al. 2010; Rueckert et al. 2011a, b; Rueckert et al. 2012, Wakeman and Leander 2012, 2013), we advocate a very different point of view and path forward using SC-PCR and comparative analyses of molecular markers (e.g., SSU rDNA) to more precisely determine the boundaries between gregarine species. The advantages and insights gained from molecular data are varied and have been repeatedly demonstrated in a large body of molecular systematic studies on a vast array of lineages (e.g., determining biogeographical patterns, convergent evolution of morphological traits, cryptic species, and the connections between disparate life history stages of the same species) (Bucklin et al. 2011; Hebert and Gregory 2005).
Molecular phylogenetic approaches are particularly powerful and pragmatic for the systematics of gregarine parasites because their life histories involve several distinct developmental stages (e.g., cysts, sporozoites, and trophozoites at different phases of maturation) that complicate species identification based on morphology alone, no matter how detailed the morphometric data might be (Leander 2008; Wakeman and Leander 2013; Rueckert et al. 2011b.). Moreover, not all life history stages are available to observe at any given time (especially in species of marine gregarines) and there tends to be high levels of intraspecific variation coupled with low levels of interspecific variation associated with trophozoite morphology, which is the most conspicuous life history stage in most gregarine species. The SSU rDNA sequences reported here from *S. cf. echinatum* provide an important example of how molecular data will help us reconcile new discoveries with previous species descriptions based on line drawings and/or light-micrographs. The SSU rDNA sequences from our Pacific Ocean isolates can eventually be compared with SSU rDNA sequences generated from isolates of *S. echinatum* collected from hosts living in the type locality in the Atlantic Ocean. We were unable to make a definitive species identification based on comparative morphology alone because of the ambiguities associated with the trophozoite traits and the original description itself. The intraspecific and interspecific variation associated with molecular markers, such as SSU rDNA sequences, will provide great insight into whether or not *S. echinatum* has a biogeographical distribution that extends into both the Pacific and Atlantic Oceans or represents two different (cryptic) species. The SSU rDNA sequence data also places the diversity of gregarine species into a molecular phylogenetic context, which so far has demonstrated several unexpected clades and relationships that are steadily refining our overall understanding of apicomplexan evolution.
3.5 Taxonomic summary and descriptions

Apicomplexa Levine, 1970
Gregarinea Bütschli, 1882, Embedded Grassé, 1953
Archigregarinorida Grassé, 1953
Selenidiidae Brasil, 1907
Selenidium Giard, 1884

*Selenidium neosabellariae* n. sp. Wakeman and Leander

**Diagnosis.** Trophozoites vermiform with an average length and width, at the widest part, of 292 µm and 11 µm, respectively. Cells light-brown. The posterior end tapers to a fine point; the anterior end tapers to a cone-shaped mucron defined at the base by a series of transverse striations. A spherical nucleus (10 µm x 12-14 µm) is positioned in the center of the cell. Trophozoites move by undulating, bending and twisting. Five to six deep longitudinal striations occur on each side of the trophozoite surface.

**Gene sequence.** SSU rDNA sequence (GenBank KC110871).

**Type locality.** Ogden Point (48°24'46.10"N 123°23'24.67"W), Victoria, British Columbia, Canada. Host in tubes on rocks; subtidal; 20 m below mean sea level.

**Type habitat.** Marine.

**Type host.** *Neosabellaria cementarium* Moore, 1906 (Annelida, Polychaeta, Sabellida, Sabellariidae).

**Location in host.** Intestinal lumen.

**Holotype:** Figure 3.1G. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR122.

**Etymology.** The species name, *neosabellariae*, refers to the genus of the type host.
Selenidium sensimae n. sp. Wakeman and Leander

**Diagnosis.** Trophozoites spindle-shaped with an average length and width, at the widest part, is 155 µm and 12 µm, respectively. Cells dark-brown. An ellipsoidal nucleus (10 µm x 4-6 µm) is positioned in the central part of the cell. The posterior end of the cell tapers to a blunt point with a terminal indentation; the anterior region of the cell forms a rounded or pointed mucron. Trophozoites bend and twist slowly. Eight to nine longitudinal striations occur on each side of the trophozoite surface.

**Gene sequence.** SSU rDNA sequence (GenBank KC110863).

**Type locality.** Coral specimen purchased from J&L Aquatics, Burnaby, British Columbia, Canada, collected in the Eastern Pacific Ocean (Fiji) in coral reef habitat.

**Type habitat.** Marine.

**Type host.** Spirobranchus giganteus Pallas, 1766 (Annelida, Polychaeta, Sabellida, Serpulidae).

**Location in host.** Intestinal lumen.

**Holotype:** Figure 3.3J. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR123.

**Etymology.** The specific epithet, sensimae, means “slowly” in Latin and refers to the slow bending and twisting movements observed in the trophozoites.
4 Identity of environmental DNA sequences using descriptions of four novel marine gregarine parasites, *Polyplicarium* n. gen. (Apicomplexa), from capitellid polychaetes

4.1 Synopsis

Marine gregarine apicomplexans are a diverse but poorly understood assemblage of endoparasites that infect the intestines and other extracellular spaces in a wide range of marine invertebrates (Grassé 1953; Levine 1971; Levine 1976; Perkins et al. 2002). Only a tiny fraction of the known diversity of marine gregarines is represented in molecular phylogenetic datasets; the most widely explored marker so far has been small-subunit (SSU) rDNA sequences (Leander 2007; Leander and Keeling 2004; Leander et al. 2003; Leander et al. 2006; Rueckert et al. 2010; Rueckert and Leander 2008; Rueckert and Leander 2009; Rueckert and Leander 2010; Rueckert et al. 2011a, b; Wakeman and Leander 2012). Nonetheless, phylogenetic analyses of DNA sequences used in tandem with high-resolution microscopy of trophozoite stages has helped shape our understanding of gregarine diversity and evolutionary history (Leander 2008). This approach has also been vital for the delimitation and identification of different gregarine species and for establishing the cellular identities of ambiguous environmental DNA sequences generated from several different PCR surveys of marine biodiversity (Berney et al. 2004; Cavalier-Smith 2004; Dawson and Pace 2002; Edgcomb et al. 2002; Leander and Ramey 2006; López-García et al. 2007; Moreira and López-García 2003; Stoeck and Epstein 2003; Stoeck et al. 2007; Takishita et al. 2005; Takishita et al. 2007).

Environmental PCR surveys targeting SSU rDNA sequences are informative approximations for the overall composition of species in an ecosystem, especially when considering the vast assortment of uncultivated lineages of microbial
eukaryotes present in these systems (e.g., intertidal areas, salt marshes, and deep sea hydrothermal vents). However, the cellular identities of numerous environmental sequences generated from marine environments remain ambiguous. This is mainly due to an inability to establish the sister lineages of highly divergent sequences using molecular phylogenetic datasets with only a limited sample of taxa that have also been characterized at the morphological level (Leander and Ramey 2006; Rueckert et al. 2011a, b). This situation has led some authors to conclude that the variation observed in some SSU rDNA sequences represents novel lineages of eukaryotic diversity that are largely or completely unknown (Dawson and Pace 2002; López-García et al. 2007; Stoeck and Epstein 2003; Stoeck et al. 2007). Other authors interpret ambiguous environmental sequences as representing known species or more inclusive taxonomic groups that have yet to be characterized at the molecular level (Cavalier-Smith 2004; Rueckert et al. 2011a, b). These contrasting interpretations are difficult to evaluate when the molecular phylogenetic relationships between lineages of interest are unresolved (Leander 2008).

The exploration of gregarine diversity using molecular phylogenetic data has established the cellular identities of several different environmental sequence clades within the Apicomplexa (Leander and Ramey 2006; Rueckert et al. 2011a, b). The lifecycle of marine gregarine apicomplexans includes a cyst stage that, in marine environments, is dispersed in the sediment and eventually ingested by a new individual host (Leander 2008; Vivier and Desportes 1990). The amplification of DNA sequences in environmental PCR surveys suggest that the cysts of marine gregarines are prevalent in marine sediments and show that the extreme divergence of some gregarine SSU rDNA sequences make them difficult to analyze (Cavalier-Smith 2004; Leander 2007; Leander and Ramey 2006; Takishita et al. 2005; Takishita et al. 2007). A study of gregarines isolated from the intestines of crustaceans, for instance, demonstrated that the highly divergent SSU rDNA sequences from these particular species were only identifiable in PCR surveys after establishing direct links between the DNA sequences and
other cellular traits (e.g., trophozoite morphology). Therefore, species discovery surveys that aim to characterize novel organisms using culture-independent methods to acquire data at both morphological and molecular levels provide the necessary context for identifying clades of ambiguous environmental sequences.

Like most groups of microbial eukaryotes (protists), gregarine parasites are particularly prone to having a low degree of morphological diversity between different species and a high degree of morphological plasticity within species (Rueckert et al. 2011b); moreover, like other parasites, gregarines have different life cycle stages that vary considerably at the morphological level (e.g., gametocysts, oocysts, and the developmental stages between sporozoites and trophozoites). Therefore, using extensive and tedious morphometric measurements to delimit one gregarine species from another is not only inadvisably time consuming for delimiting one species from another but also largely impenetrable, impractical and misleading.

In this study, we discover and characterize the morphology and molecular phylogenetic markers of four novel species of Pacific marine gregarines isolated from the intestines of two capitellid polychaetes, Notomastus tenuis and Heteromastus filiformis. These combined data enabled us to establish a new genus of marine gregarines that provides the cellular identity of a clade of SSU rDNA environmental sequences isolated from various marine environments around the globe.

4.2 Materials and methods

4.2.1 Collection of organisms.

The capitellid polychaete Notomastus tenuis Moore, 1909 was collected at low tide from Boundary Bay, Tsawwassen (Vancouver), British Columbia, Canada in August 2011. A second capitellid polychaete, Heteromastus filiformis Claparède,
1864 was collected from the rocky intertidal area at Jericho Beach, Vancouver, British Columbia, Canada in September 2011. No specific permits were required for the collection of worms in these field sites. Tide levels were estimated and acquired through Fisheries and Oceans Canada. Host material was transported and kept in chilled seawater prior to dissection. All dissections were completed within 24 hours of collection. No fixatives were used either during dissections or while taking photographs of the trophozoites. Three different morphotypes of gregarine trophozoites (*Polyplicarium lacrimae* n. gen., n. sp., *P. curvarae* n. sp. and *P. translucidae* n. sp.) were collected from the intestines of *N. tenuis*; one distinct morphotype of a gregarine trophozoite (*P. citrusae* n. sp.) was collected from the intestines of *H. filiformis*.

### 4.2.2 Light microscopy and single-cell DNA extraction, amplification and sequencing

Hand-drawn glass pipettes were used to collect individual trophozoites representing four distinct morphotypes using an inverted microscope (Zeiss Axiovert 200, Carl-Zeiss, Göttingen, Germany). Four single-cell (SC) isolates were collected from each of the four distinct morphotypes (a total of 16 SC isolates) and prepared for light microscopy and DNA extraction. The SC isolates were washed three times (until clean) in chilled, autoclaved seawater and photographed either on glass slides with a Leica DC 500 color camera connected to a Zeiss Axioplan 2 microscope (Carl-Zeiss, Göttingen, Germany) or on well-slides with a PixeLink Megapixel color digital camera (PL-A662-KIT, Ottawa, Canada) connected to an inverted Zeiss Axiovert 200 microscope (Carl-Zeiss, Göttingen, Germany). Each of the SC isolates was then placed in a 1.5 ml Eppendorf tube containing cell lysis buffer. Genomic DNA was extracted with the standard protocol provided by the MasterPure complete DNA & RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). However, the final elution step was lowered to 4 µl with the goal of concentrating extracted DNA prior to PCR amplification.
Sixteen novel SSU rDNA sequences were generated by nested PCR with primers specific for the gregarine parasite species (Table 1). Initially, outside primers PF1 and SSUR4 (Leander et al. 2003) were used in a 25 µl PCR reaction with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI, USA). The following program was used on the thermocycler for the initial amplification: initial denaturation at 94°C for 2:00 m; 35 cycles of denature at 94°C for 0:30 s, anneal at 52°C for 0:30 s, extension at 72 °C for 1:50 m., final extension 72°C 9:00 m. Subsequently, a pair of internal primers, namely F1 and R2 (Table 1), were used in a nested PCR with 1 µl of template DNA generated from the first PCR reaction in order to amplify a 1,000 bp region of the SSU rRNA gene using the following program on a thermocycler: Initial denaturation for 94°C for 2:00 m; 25 cycles of denature at 94°C for 0:30 s., anneal at 51°C for 0:30 s., extension at 72 °C for 1:20 m.; final extension at 72°C for 9:00 m.

From the initial sequences, specific primers were then designed and paired with outside (universal eukaryotic) primers (e.g., PF1-PlacrimaeR and PlacrimaeF-SSUR4) in semi-nested PCR reactions in order to attain the final SSU rDNA sequences (1650 – 1800 bp) (Table 1). All PCR products were separated on agarose gels and isolated using the UltraClean15 DNA Purification Kit (MO BIO, Laboratories, Inc., Carlsbad, CA, USA). All sequencing reactions were performed using ABI Big Dye reaction mix with appropriate primers (Table 4.1). Novel sequences were initially identified using the National Center for Biotechnology Information’s (NCBI) BLAST tool and confirmed with molecular phylogenetic analyses. All unique sequences generated in this study were deposited in GenBank (Accession numbers JX535336-JX535351).

### 4.2.3 Scanning electron microscopy

Between 20 and 65 individual trophozoites representing each morphotype were pooled in 2% glutaraldehyde in seawater on ice. A 10 µl polycarbonate
membrane filter was placed within a Swinnex filter holder (Millipore Corp. Billerica, MA, USA). Trophozoites were then collected with a hand-drawn glass pipette and placed in the filter holder. The filter holder was then placed in a small beaker (4 cm diam. and 5 cm tall) that was filled with 2% glutaraldehyde in seawater. Ten drops of 1% OsO₄ were added to the opening of the filter holder, and the samples were post-fixed on ice for 30 minutes. A syringe was used to slowly run distilled water over all samples. A graded series of ethanol washes (30%, 50%, 75%, 85%, 95% and 100%) was then used to dehydrate the fixed cells using the syringe system. Following dehydration, the polycarbonate membrane filters containing the trophozoites were transferred from the Swinnex filter holders into an aluminum basket submerged in 100% ethanol in preparation for critical point drying with CO₂. The dried polycarbonate membrane filters containing the trophozoites were mounted on aluminum stubs, sputter coated with 5 nm gold and viewed under a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

4.2.4 Molecular phylogenetic analyses

Two separate phylogenetic analyses were conducted in this study. A comprehensive 79-taxon dataset contained a representative SSU rDNA sequence from each of the four novel morphotypes described here, five closely related environmental DNA sequences, three dinoflagellate sequences (outgroup), and 67 sequences representing major clades of gregarines and other apicomplexans. The 79-taxa alignment was visually fine-tuned using MacClade 4 (Maddison and Maddison 2000); gaps and ambiguously aligned regions were excluded resulting in 1,007 unambiguously aligned sites. JModeltest (Guindon and Gascuel 2003; Posada and Crandall 1998) selected a GTR + I + Γ model of evolution under AIC and AICc (proportion of invariant sites=0.1280, gamma shape= 0.5250). Garli-GUI (Zwickl 2006) was used to generate a maximum
likelihood (ML) tree, and ML bootstrap analysis (100 pseudoreplicates, one heuristic search per pseudoreplicate).

Bayesian posterior probabilities were calculated for the larger dataset using the program MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We set our program for four Monte Carlo Markov Chains starting from a random tree (MCMC; default temperature= 0.2), a gamma distribution and stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate). A sum of 5,000,000 generations was calculated. Trees were sampled every 100 generations, with a prior burn-in of 500,000 generations. Burn-in was confirmed manually, and a majority-rule consensus tree was constructed. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

A more restricted phylogenetic analysis focused on the interrelationships between the 16 SSU rDNA sequences generated from four different single cell isolates from each of the four different morphotypes described here; the alignment also contained the five closely related environmental DNA sequences identified in GenBank. This 21-taxon alignment was visually fine-tuned with MacClade 4, excluded gaps and ambiguous sites, and contained 1,407 sites. An unrooted ML tree and ML Bootstrap percent values were calculated using Garli-GUI under a GTR + I + Γ model of evolution selected by JModeltest (proportion of invariable sites=0.4970, gamma shape= 0.5830). Like the larger 79-taxon data set, posterior probabilities were calculated using MrBayes 3.1.2. using the same criteria described previously. A sum a 700,000 generations was calculated with a prior burn-in of 70,000 generations. PAUP 4.0 (Swofford 1999) was used to calculate percent differences between the 16 novel SSU rDNA sequences generated in this study.
4.3 Results

4.3.1 Morphological traits of the four new species

4.3.1.1 Polyplicarium lacrimae n. gen., n. sp.

The trophozoites were teardrop-shaped (Figure 4.1). The anterior end of the cell was bulbous, having an average width of 54 µm (range 38-66 µm, n = 42) at its widest part. The average length of the cell was 197 µm (range 183-207 µm, n = 42). Cells appeared dark-brown from large amounts of amylopectin. The nucleus was circular to ovoid (17-22 µm x 15-21 µm, n=17) and located in the central part of the bulbous anterior of the cell (Figure 4.1). The anterior end tapered slightly toward a blunt and otherwise inconspicuous mucron. The posterior end tapered to a point. Longitudinal epicytic folds covered the surface of the cell with a density of 4-5/µm. Gliding motility was present. A distinct region of shallow epicytic folds was observed on the surface of cells examined under SEM (Figure 4.1). This region was observed in two out of the six samples recovered for viewing.

4.3.1.2 Polyplicarium curvarae n. sp.

The trophozoites were slightly curved, 156 µm long (range 98-167 µm, n = 47) and 42 µm wide (range 34-53 µm, n = 47) (Figure 4.2). The nucleus was ovoid (16-21 µm x 10-12 µm, n = 21) and located in the posterior region of the cell. A conspicuous mucron was visible in cells under light microscopy; otherwise, the mucron appeared flat and inconspicuous. Cells appeared brown from the accumulation of amylopectin. SEMs demonstrated longitudinal epicytic folds covering the cell surface with a density of 4-5/µm (Figure 4.2). The posterior end of the trophozoites tapered slightly to a blunt and slightly compressed end. The trophozoites were capable of gliding motility. The surface of the cell contained a distinct region of 12-17 shallow epicytic folds that were 2-3 microns wide and
taller than the other epicytic folds (Figure 4.2). This pattern of epicytic folds was observed in 11 out of 20 cells observed under SEM.

4.3.1.3 *Polyplicarium translucidae* n. sp.

Trophozoites were 163 µm long (range 112-183 µm, *n* = 59) and 27 µm wide (range 25-32 µm, *n* = 59) (Figure 4.3). The nucleus was circular to ovoid (12-15 µm x 7-13 µm) and located in the middle to posterior end of the cell. The trophozoites were capable of gliding motility. The cells appeared translucent under light microscopy, having a low accumulation of amylopectin granules. The posterior end was slightly tapered and sometimes tapered to a nipple-like point (Figure 4.3). Longitudinal epicytic folds covering surface had a density of 4-5/µm. A distinct region of 10-13 shallow epicytic folds was observed on the surface of the cell (Figure 4.2). This pattern of epicytic folds was observed in 17 out of 30 cells observed under SEM.

4.3.1.4 *Polyplicarium citrusae* n. sp.

The trophozoites were extremely flat, ovoid to lemon-shaped, 47 µm long (range 39-53, *n* = 49) and 32 µm wide (range 28-42, *n* = 49) (Figure 4.4). The circular to ovoid nucleus (4-7 µm x 4-8 µm, *n* = 15) was located in the central part of the cell. Cells appeared translucent to light brown, depending on the amount of amylopectin granules present within the cell. Both the anterior and posterior ends were slightly tapered. The trophozoites were capable of gliding motility. The anterior end was differentiated from the posterior end mainly by observing the direction of “forward” gliding motility. Syzygy was side-to-side (Figure 4.4). The cell surface was covered with longitudinal epicytic folds with a density of 4/µm (Figure 4.4). No distinct region of shallow epicytic folds was present on the 32 cells observed with SEM.
4.3.2 Molecular phylogenetic analyses of SSU rDNA sequences.

Our phylogenetic analysis of the 79-taxon data set recovered a clade of apicomplexans with moderate support. Our analyses also recovered groups of coccidians, piroplasmids, rhytidocystids, cryptosporidians and terrestrial gregarines, ranging in support from moderate to robust (Figure 4.5). Four major subgroups of marine gregarines were recovered in this analysis: crustacean gregarines, lecudinids clade I, lecudinids clade II plus urosporids, and the novel clade established here from capitellid hosts (Figure 4.5). Species of *Selenidium* (i.e., archigregarines) branched from the unresolved apicomplexan backbone as three separate lineages. The four novel sequences representing *P. lacrimal*, *P. curvata*, *P. translucida*, and *P. citrusae* formed a well-supported clade with five environmental sequences of previously unknown origin within the Apicomplexa (AY179976, EF100216, EF100199, AB275013 and AY179975) (Figure 4.5).

Molecular phylogenetic analyses with the 21-taxon data set containing five environmental sequences and the 16 new SSU rDNA sequences (i.e., four from single-cell isolates of each of the four species in this study) are shown in Figure 4.6. The analysis recovered four distinct, well-supported clades that represent the single-cell isolates from each species described in this study. A fifth clade consisted of four environmental sequences (AB275013, EF100199, EF100216 and AY179976), and environmental sequence AY179975 branched as the sister lineage to *P. citrusae* with strong statistical support (Figure 4.6).

Intraspecific variation of the four SSU rDNA sequences generated from each of the four species ranged from 0.98-2.12% (*P. lacrimal*), 0.22-1.46% (*P. curvata*), 0.68-1.73% (*P. translucida*), and 0.82-1.35% (*P. citrusae*) (Table 4.2). Interspecific variation between isolates of *P. lacrimal*, *P. curvata*, *P. translucida* and *P. citrusae* ranged from 7.42-14.98% (Table 4.2).
Figure 4.1 Micrographs of Polyplicarium lacrimae n. gen., n. sp. A-D.
Differential interference contrast (DIC) light micrographs (LMs) were taken of individual cells on a well slide just before they were removed for DNA extraction and single-cell (SC) PCR. Individual trophozoites with a blunt mucron (arrow) and bulbous anterior containing a centrally located nucleus (N). The posterior region tapers to a point. E. LM showing the general morphology of P. lacrimae showing the inconspicuous mucron (arrow), centrally located nucleus (N) and a pointed posterior end. F. High-magnification SEM of the cell surface of P. lacrimae showing the epicytic folds (arrowhead). G. SEM showing the general morphology, the inconspicuous mucron (arrow), the pointed posterior end of the cell, and the distinct region of wider folds on a trophozoite (triple-arrowhead). H. High-magnification SEM of the distinct region of wider folds (triple-arrowhead) on trophozoites. Scale bars: A–D = 20.0 µm; E = 25 µm; F = 1 µm; G = 15 µm; H = 1.5 µm.
Figure 4.2 Micrographs of Polyplicarium curvarae n. sp. A–D. Differential interference contrast (DIC) light micrographs (LMs) were taken of individual cells on a well slide just before they were removed for DNA extraction and single-cell (SC) PCR. General morphology of cup-like A–B or projected D. mucrons (arrow). The nucleus (N) is located in the posterior or middle-posterior part of the cell. The posterior end tapers slightly to a blunt end. E. LM showing the general morphology of a trophozoite with the mucron (arrow), nucleus (N), and a distinct fold (triple arrowhead) in the center of the trophozoite. F. High-magnification SEM showing the mucron (arrow) and dense epicytic folds (arrowhead) on surface of the cell. G. SEM of a trophozoite attached to host gut (HG) material. The interface between the host gut and trophozoite is marked by an arrow. The posterior region of the cell is blunt and slightly compressed. H. SEM showing the general morphology of the cell, the mucron (arrow), and a blunt posterior end. A distinct region of wider folds (triple-arrowhead) was observed on the cell surface. Scale bars: A–D = 30 µm; E = 30 µm; F = 3.5 µm; G,H = 10 µm.
Figure 4.3 Micrographs of *Polyplicarium translucidae* n. sp. A–D. Differential interference contrast (DIC) and light micrographs (LMs) were taken of individual cells on a well slide just before they were removed for DNA extraction and single-cell (SC) PCR. Trophozoites have a posterior nucleus (N), a rounded mucron (arrow), and a posterior end that tapers slightly to a nipple-like point (P) B–C. E. LM showing the general cell morphology, the mucron (arrow), the posterior nucleus (N), and a region of wider folds (triple arrowhead). F. High-magnification SEM showing the dense epicytic folds (arrowhead) and a region of wider folds (triple arrowhead) on the cell surface. H. SEM showing the mucron (arrow), the nipple-like posterior end of the cell, and the distinct region of wider folds (triple-arrowhead). Scale bars: A–E. = 25 µm; F. = 2.0 µm; G. = 10 µm.
Figure 4.4 Micrographs of *Polyplicarium citrusae* n. sp. A–D. Differential interference contrast light micrographs (LMs) were taken of individual cells just before they were removed for DNA extraction and single-cell (SC) PCR. Individual trophozoites were extremely flattened and had a centrally located nucleus (N). The mucron (arrow) was identified based on the forward direction of movement. 

E. SEM showing two gamonts (G1 and G2) in side-to-side syzygy and epicytic folds running along the longitudinal axis of the cell. 

F. SEM of a single trophozoite showing the epicytic folds (arrowhead). The anterior and posterior ends of these cells was difficult to distinguish under SEM. Scale bars: A–E = 10 µm; F = 5 µm.
Figure 4.5 Molecular phylogenetic analyses of Polyplicarium n. gen and associated environmental sequences. Maximum likelihood (ML) tree based on 1,007 unambiguously aligned sites from 79 SSU rDNA sequences using the GTR+I+Γ substitution model (-ln L = 16800.87694, gamma shape = 0.5250, proportion of invariable sites = 0.1280). Bootstrap supports are given at the top of branches, and Bayesian posterior probabilities are given at the bottom. Black dots on branches represent bootstrap support values and Bayesian posterior probability 95/0.99 or greater. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree. Representative sequences from the four novel species described in this study are highlighted in black boxes.
Figure 4.6 Molecular phylogenetic analyses of single-cell isolates of *Polyplicarium* n. gen. and associated environmental sequences. Unrooted maximum likelihood (ML) tree of four single-cell isolates from each of the four novel species of *Polyplicarium* n. gen. described in this study, as well as five closely related environmental sequences. This tree is based on 1,407 unambiguously aligned sites from 21 SSU rDNA sequences using the GTR+I+Γ substitution model (\(-\text{ln L} = 16800.87694, \gamma\text{ shape} = 0.5830, \text{proportion of invariable sites} = 0.4970\)). Bootstrap supports are given at the top of branches, and Bayesian posterior probabilities are given at the bottom. Black dots on branches represent bootstrap support values and Bayesian posterior probability 95/0.99 or greater. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree.
Table 4.1 Primers designed for amplification of *Polyplicarium* SSU rDNA. The annealing regions refer to one of the sequences derived from *Polyplicarium lacrimae* n. gen., n. sp.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence 5'-3'</th>
<th>Annealing region</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Forward</td>
<td>5'-GATTAAGCCATGCATGTCTAAG-3'</td>
<td>47 to 70</td>
</tr>
<tr>
<td>P.lacrimaeF</td>
<td>Forward</td>
<td>5'-CGTTTCTACGATTATCAATTGG-3'</td>
<td>486 to 508</td>
</tr>
<tr>
<td>P.curvaraeF</td>
<td>Forward</td>
<td>5'-CGTTTCTATGAGTACCCATTGG-3'</td>
<td>486 to 508</td>
</tr>
<tr>
<td>P.translucidaeF</td>
<td>Forward</td>
<td>5'-CTTTCTACGAGTACCAATTGG-3'</td>
<td>486 to 508</td>
</tr>
<tr>
<td>P.citrusaeF</td>
<td>Forward</td>
<td>5'-CTTTCTACGAGTACCAATTGG-3'</td>
<td>486 to 508</td>
</tr>
<tr>
<td>R1</td>
<td>Reverse</td>
<td>5'-CGGTGTGTACAAACGGCAGGGAC-3'</td>
<td>1762 to 1740</td>
</tr>
<tr>
<td>P.lacrimaeR</td>
<td>Reverse</td>
<td>5'-CTGACAGGGCCGAGGTCTATCG-3'</td>
<td>671 to 648</td>
</tr>
<tr>
<td>P.curvaraeR</td>
<td>Reverse</td>
<td>5'-CGGATAAGACGGGAAGTCCTATCG-3'</td>
<td>671 to 648</td>
</tr>
<tr>
<td>P.translucidaeR</td>
<td>Reverse</td>
<td>5'-GGGATAGGACGGGAAGTCCTATAG-3'</td>
<td>671 to 648</td>
</tr>
</tbody>
</table>
Table 4.2 Summary of intraspecific and interspecific divergences of small subunit rDNA. Sequences were generated from four isolates of each of the four species of *Polyplicarium* n. gen. described in this study. Percent divergences are based on comparisons of 1,678 nucleotides.

<table>
<thead>
<tr>
<th></th>
<th><em>Polyplicarium lacrimae</em> n. gen., n. sp.</th>
<th><em>P. curvarae</em> n. sp.</th>
<th><em>P. translucidae</em> n. sp.</th>
<th><em>P. citrusae</em> n. sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Polyplicarium lacrimae</em> n. gen., n. sp.</td>
<td>0.98-2.12%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. curvarae</em> n. sp.</td>
<td>10.67-12.53%</td>
<td>0.22-1.46</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. translucidae</em> n. sp.</td>
<td>9.39-11.10%</td>
<td>7.42-9.02%</td>
<td>0.68-1.73</td>
<td>-</td>
</tr>
<tr>
<td><em>P. citrusae</em> n. sp.</td>
<td>13.21-14.98%</td>
<td>8.58-10.07%</td>
<td>10.17-12.09%</td>
<td>0.82-1.35</td>
</tr>
</tbody>
</table>
Table 4.3 Comparative morphology of *Polyplicarium*. Trophozoite morphology for the four new species of *Polyplicarium* n. gen. described in this study.

<table>
<thead>
<tr>
<th></th>
<th><em>Polyplicarium lacrimae</em> n. gen., n. sp. (type species)</th>
<th><em>P. curvarae</em> n. sp.</th>
<th><em>P. translucidae</em> n. sp.</th>
<th><em>P. citrusae</em> n. sp.</th>
<th>Heteromastus filiformis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host</strong></td>
<td><em>Notomastus tenuis</em></td>
<td><em>Notomastus tenuis</em></td>
<td><em>Notomastus tenuis</em></td>
<td></td>
<td><em>Heteromastus filiformis</em></td>
</tr>
<tr>
<td>Host tissue</td>
<td>E. Pacific</td>
<td>Intestines</td>
<td>E. Pacific</td>
<td></td>
<td>Intestines</td>
</tr>
<tr>
<td>Localitiy</td>
<td>E. Pacific</td>
<td>Intestines</td>
<td>E. Pacific</td>
<td></td>
<td>E. Pacific</td>
</tr>
<tr>
<td><strong>Trophozoite shape</strong></td>
<td>Elongate, bulbous anterior, posterior end tapered to point</td>
<td>Elongate, cylindrical, posterior end slightly compressed</td>
<td>Elongate, compressed, posterior blunt</td>
<td>Round to ovoid, highly flattened</td>
<td></td>
</tr>
<tr>
<td><strong>Trophozoite size</strong></td>
<td>183-207 x 38-66</td>
<td>98-167 x 34-53</td>
<td>112-183 x 25-32</td>
<td>39-53 x 28-42</td>
<td></td>
</tr>
<tr>
<td>(L x W, µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nucleus shape</strong></td>
<td>Ovoid</td>
<td>Ovoid</td>
<td>Ovoid</td>
<td></td>
<td>Ovoid</td>
</tr>
<tr>
<td><strong>Nucleus size</strong></td>
<td>17-22 x 15-21</td>
<td>16 x 21 x 10-12</td>
<td>12-15 x 7-13</td>
<td>4-7 x 4-8</td>
<td></td>
</tr>
<tr>
<td>(L x W, µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Position of nucleus</strong></td>
<td>Middle anterior</td>
<td>Middle posterior</td>
<td>Middle posterior</td>
<td>Middle</td>
<td></td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>Gliding motility</td>
<td>Gliding motility</td>
<td>Gliding motility</td>
<td>Gliding motility</td>
<td></td>
</tr>
<tr>
<td><strong>Density of epicytic folds</strong></td>
<td>4-5/µm</td>
<td>4-5/µm</td>
<td>4-5/µm</td>
<td>4/µm</td>
<td></td>
</tr>
<tr>
<td><strong>Shape of mucron</strong></td>
<td>Simple, blunt</td>
<td>Simple, blunt</td>
<td>Simple, blunt</td>
<td>Simple, blunt</td>
<td></td>
</tr>
<tr>
<td><strong>Region with alternative fold pattern</strong></td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td></td>
</tr>
</tbody>
</table>

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4.4 Discussion

Species of marine gregarines have been established using a wide variety of criteria, including host affinity, geographical distribution, detailed morphological dimensions of different life history stages, ultrastructural patterns on the surface of trophozoites, and more recently, SSU rDNA sequence variability (Leander et al. 2003; Rueckert and Leander 2008; Rueckert et al. 2010; Rueckert et al. 2011a, b). Traditionally, new species were justified mainly on just one of these criteria (e.g., detailed morphological dimensions of different life history stages). More recent studies suggest that one criterion alone is inadequate to convincingly delimit different species of marine gregarines from one another (Rueckert et al. 2011a, b). For instance, the SSU rDNA sequences from very different morphotypes of Lecudina polymorpha were over 99% identical (Leander et al. 2003; Rueckert et al. 2010). By contrast, the SSU rDNA sequences from morphologically similar gregarines isolated from two different species of nemerteans differed by 14.1%, demonstrating two different species of Difficilina that were correlated with two different host species (Rueckert et al. 2010). A similar study described two different species of Lankesteria isolated from two different species of tunicates (Rueckert and Leander 2008). Because the SSU rDNA sequences from these two species differed only by 2.1% – 3.1%, the separation of the two species was also based on morphological variation (e.g., L. chelyosomae was over 10 times larger than L. cystodytae), different host affinities, and the fact that multiple isolates from each species clustered into two separate clades (Rueckert and Leander 2008). Another study addressed the SSU rDNA sequence variation in several different morphotypes of Lecudina cf. tuzetae isolated from different hosts collected in two different geographical regions (Rueckert et al. 2011b). The range of variation in these sequences was 0.0 – 3.9%; however, the sequences did not cluster into clades according to morphotype, location or host, suggesting that the degree of variation found in this study was intraspecific. Taken together, these studies illustrate the importance of considering multiple criteria to justify the establishment of new species and
genera (Leander et al. 2003; Rueckert et al. 2010; Rueckert et al. 2011a, b).
Arguably, the most pragmatic approach for describing new species of gregarines is to consider host affinity, morphological features of the most conspicuous life history stage (usually trophozoites), and a widely sampled molecular marker with sufficient interspecific variation (e.g., SSU rDNA sequences) using a single-cell PCR approach (Rueckert et al. 2011b).

In practice, marine gregarines are encountered only as trophozoites and often in very low numbers (fewer than 10 specimens in an individual host) within a small percentage of individual hosts, which are also difficult to encounter and collect (e.g., the hosts might have been collected on a research cruise that was a one time opportunity). More importantly, the challenges emphasized within the principles listed by Clopton (2012) are needless and overcome by efficient and pragmatic DNA-based approaches to systematics, and the literature is rich with excellent studies that demonstrate this in a wide variety of organisms and a broad range of contexts (e.g., biogeography, cryptic lifecycle stages, and cryptic species). Although reciprocal reinforcement of molecular data and other traits (e.g., trophozoite features and host affiliations) provide the most compelling arguments for species discrimination, a viewpoint that insists on an absolute set of morphometric details in gregarine systematics simply stifles the enterprise and provides a stark counter-example for why DNA-based approaches to biogeography, the delimitation of species, and phylogenetic reconstruction have become so predominant in advancing our understanding of biodiversity, especially within the context of protists.

4.4.1 Justification for establishing the new genus and species

Nearly 1,700 species of gregarines have been formally described, often with only very limited morphological information; only a tiny fraction of these have been examined with molecular phylogenetic data (Leander 2008; Perkins et al. 2002; Levine 1971; Levine 1976; Levine 1977a, b; 1979). Molecular markers like SSU
rDNA sequences that have been obtained from manually isolated trophozoites have been helpful in our understanding of gregarine species boundaries and phylogenetic relationships (Leander 2007; Leander et al. 2006; Rueckert and Leander 2008; Rueckert and Leander 2009; Rueckert and Leander 2010; Rueckert et al. 2010; Rueckert et al. 2011a, b; Wakeman and Leander 2012). Analyses of SSU rDNA sequences derived directly from known species have also been able to establish the cellular identities of ambiguous environmental DNA sequences that have accumulated from PCR surveys of biodiversity (Cavalier-Smith 2004; Rueckert et al. 2011a, b). Nonetheless, the absence of molecular data from the vast majority of described gregarine species severely constrains the comparative power needed to place newly discovered species within the context of known gregarine species.

In this study, we established four novel species within Polyplicarium n. gen. using comparative morphology and SSU rDNA sequences from four different single-cell isolates of each of the four different species. The trophozoite morphology of Polyplicarium species was most similar to the following genera within the Lecudinidae: Hyperidion, Ancora, Ulvina, Zygosoma and Lecudina. The trophozoites in all of these genera have “simple mucrons” but differ from one another in several ways (Lee et al. 2000; Perkins et al. 2002). For instance, the trophozoites of Hyperidion are 175 µm – 350 µm long, contractile, have longitudinal folds with a density of about 2-3/µm, and have a textured projection from the mucron (Lee et al. 2000; Mackinnon and Ray 1931). The trophozoites of Polyplicarium are shorter and almost twice as wide and have longitudinal folds with a density of 4-5/µm. The trophozoites of Ancora have two-three lateral processes running from the mucron towards the posterior end (Hoshide 1998; Levine 1977). Like Polyplicarium, two species of Ancora (A. sagitatta and A. lutzii) were isolated from a capitellid polychaete (Capitella capitata); however, Polyplicarium species do not have the lateral processes that define Ancora (Hasselmann 1918). The morphology of Polyplicarium is also different from Ulvina and Zygosoma (Lee et al. 2000; Mackinnon and Ray 1931). Unlike
*Polyplicarium*, the trophozoites of *Ulvina* have an incomplete septum dividing the cell into a “pseudoprotomerite” and “pseudodeutomerite”, and the trophozoites/gamonts of *Zygosoma* are covered with nipple-like projections (Lee et al. 2000). *Lecudina* is the largest and most widely studied genus within the Lecudinidae (Clausen 1993; Hasselmann 1918; Leander et al. 2003; Lee et al. 2000; Levin 1976). Several species of *Lecudina*, including the type species *L. pellucida*, have trophozoites with a density of epicytic folds in the range of 2-3/µm (Leander et al. 2003; Rueckert et al. 2011a, b; Vivier 1968), which is about half the density of folds on the surface of trophozoites in *Polyplicarium*. Moreover, all species of *Lecudina* that have been examined with SSU rDNA sequences do not cluster with or within the *Polyplicarium* clade in molecular phylogenetic analyses.

Aside from the molecular phylogenetic data, the main features that delimit the trophozoites of *Polyplicarium* from other gregarine genera within the Lecudinidae are longitudinal epicytic folds with density of 4-5/µm, a distinct region of wider epicytic folds (usually), and host affiliation. The four different species of *Polyplicarium* n. gen. described here can be distinguished from one another based on details of trophozoite morphology and SSU rDNA sequence variation (Tables 2 and 3). Unlike the other species, the trophozoites of *P. lacrimae* have a bulging region in the anterior-middle region of the cell, a centrally located nucleus, and a posterior end that tapers to a distinct point. A previously described species by Lankester (1866), namely *Lecudina eunicae*, had a similar cell shape (bulbous anterior and a pointed posterior) and size (254 µm) to that of *P. lacrimae*. However, the species described by Lankester (1866) was isolated from a different host, *Eunice harassii*, and the original drawing of the trophozoite of *L. eunicae* shows a distinct bulbous anterior region that is about half of the cell’s total length. In contrast, the anterior region of the trophozoites of *P. lacrimae* appear to be about 2/3 the total length of the cell. The trophozoite stage and host affinity of *L. eunicae* is most similar to that of *Trichotokara eunicae*, recently described by Rueckert et al. (2012), and most likely represents a close relative of gregarines isolated from Eunicid polychaetes (Rueckert et al. 2012).
The trophozoites of *P. curvarae* had a posteriorly positioned nucleus and were distinctly curved and cylindrical, compared to *P. lacrimae*, and *P. translucidae* (Table 4.3). The trophozoites of *P. translucidae* lacked a large number of amyllopectin granules within the cytoplasm, giving this species a characteristic transparent appearance under light microscopy. In contrast to the other species of *Polyplicarium*, *P. citrusae* is highly flattened and relatively small with an average length and width of only 53 µm and 29 µm, respectively. The trophozoites of *P. citrusae* were also isolated from the intestines of different capitellid host, namely *Heteromastus filiformis*. The general outline shape of *P. citrusae* was distinctly ovoid, reminiscent of a lemon (Table 4.3).

In contrast to *P. citrusae*, the surface of the trophozoites in *P. lacrimae*, *P. curvarae* and *P. translucidae* had a distinct region of wider and shallower epicytic folds. Although the functional significance of this particular region of epicytic folds in these three species is uncertain, it is plausible that the folds facilitate the acquisition of nutrients by expanding and contracting, thereby moving contents in the host gut around the cell. The consistent presence of this region of shallow folds in repeated observations of three different species, each prepared multiple times, minimized the chance that this distinct feature reflects a preparation artifact.

Our molecular phylogenetic analyses of SSU rDNA grouped the four single-cell isolates from each of the four species into four separate and corresponding clades (Fig. 6). Intraspecific variation of the SSU rDNA sequence within each clade was low, ranging from 0.22-2.12% (Table 2). Interspecific variation between the four clades was relatively high, ranging from 7.42-14.98% (Table 2). The morphological features of the trophozoites combined with the phylogenetic pattern of SSU rDNA sequence variation provided strong evidence for the delimitation of all four species of *Polyplicarium* from one another.
4.4.2 Environmental SSU rDNA sequences and the *Polyplicarium* clade

The four species of *Polyplicarium* that we described here grouped strongly with five SSU rDNA environmental sequences of similar branch length that were retrieved from GenBank. Environmental sequences AY179975 and AY179976 were generated from a PCR survey of sediment in a salt marsh near Cape Cod, Massachusetts, USA; environmental sequences EF100199 and EF100216 generated from a PCR survey of sediment from a marine tidal flat off the coast of Greenland (Stoeck and Epstein 2003); environmental sequence AB275013 was generated from sediment from a deep sea methane cold seep near Sagami Bay, Japan (Takishita et al. 2007). Until now, the cellular identity of these environmental sequences was either considered uncertain within gregarine apicomplexans (Cavalier-Smith 2004; Leander 2007; Leander et al. 2006; Rueckert and Leander 2008; Rueckert and Leander 2009; Rueckert and Leander 2010; Rueckert et al. 2010; Rueckert et al. 2011a, b) or entirely misinterpreted (e.g., novel jacobid-like sequences) (López-García et al. 2007; Stoeck and Epstein 2003). Nonetheless, the vastly different geographical locations from which the environmental DNA sequences were generated indicate that the *Polyplicarium*-clade has a global distribution and that we are at an early stage of understanding the total composition of this clade.

4.4.3 Concluding remarks

This study represents the first molecular phylogenetic data gathered from gregarines isolated from capitellid polychaetes. The combination of SC-PCR approaches, molecular phylogenetic analyses of SSU rDNA sequences, and comparative morphological data demonstrated the cellular identity of a previously unidentified environmental SSU rDNA sequence clade and enabled us to establish four new species within one novel genus: *Polyplicarium lacrimae* n. gen., n. sp. (type species), *P. curvae* n. sp., *P. translucidae* n. sp. and *P.*
*citrusae* n. sp. These data highlight significant limitations of environmental PCR surveys of biodiversity, mainly that accurate interpretations of the resulting DNA sequences require a comprehensive sample of reference species that have also been characterized at the cellular level. Hopefully, an appreciation for this organismal context will inspire future exploration into the overall diversity of marine gregarines using an approach that combines single-cell PCR, molecular phylogenetic analyses, and comparative morphology.

### 4.5 Taxonomic summary and descriptions

Apicomplexa Levine, 1970
Gregarinea Bütschli, 1882, stat. nov. Grassé, 1953
Eugregarinorida Léger, 1900

**Polyplicarium n. gen. Wakeman and Leander**

**Diagnosis.** Ovoid to elongate trophozoites with a blunt mucron. The posterior end is either blunt or tapers to a point. Longitudinal epicytic folds with density of 4-5/µm; most trophozoites also have a distinct region of wider, shallower epicytic folds. Gliding motility. The genus name, *Polyplicarium*, is Latin, translates to “many folds”, and refers to the high density of epicytic folds on the surface of the trophozoite stages.

**Type species.** *Polyplicarium lacrimalae* Wakeman and Leander.

**Polyplicarium lacrimalae n. sp. Wakeman and Leander**

**Diagnosis.** Trophozoites teardrop shaped with a bulbous mucron. Average length and width, at the widest part, is 197 µm and 54 µm, respectively. Cells dark-brown. The posterior end tapered to a point; anterior end tapered slightly toward a blunt, inconspicuous mucron. Nucleus is circular to ovoid (17-22 µm x 15-21µm) and located in the anterior or central part of the cell. Gliding motility.
Longitudinal epicytic folds with a density of 4-5/µm. A distinct region of wider, shallower epicytic folds may be present on the surface of trophozoite stages.

**Gene Sequence.** SSU rRNA gene sequence (GenBank JX535336).

**Type locality.** Boundary Bay (49°00'54.88"N, 123°02'12.72"W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sea level.

**Type habitat.** Marine.

**Type host.** Notomastus tenuis Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

**Location in host.** Intestinal lumen.

**Holotype.** Figure 4.1G. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR117.

**Etymology.** The species name, *lacrimae*, stems from Latin meaning “tear” and refers to the “teardrop-shape” of the trophozoite stage.

*Polyplicarium curvae* n. sp., Wakeman and Leander

**Diagnosis.** Trophozoites slightly curved with an average length and width of 156 µm and 42 µm, respectively. Cells brown. The posterior end of trophozoite slightly tapered and compressed. Ovoid nucleus located in the posterior region of the cell. Mucron usually flat and inconspicuous but sometimes pointed. Gliding motility. Longitudinal epicytic folds with a density of 4-5/µm. A distinct region of 15-17 wider and shallower epicytic folds present on the trophozoite surface.

**Gene sequence.** SSU rRNA gene sequence (GenBank JX535340).

**Type locality.** Boundary Bay (49°00’54.88”N, 123°02’12.72”W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sea level.

**Type habitat.** Marine.
Type host. *Notomastus tenuis* Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

Location in host. Intestinal lumen.

Holotype. Figure 2H. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR118.

Etymology. The species name, *curvarae*, stems from Latin meaning “curved” and refers to the curved cell shape of the trophozoites stage.

*Polypticarium translucidae* n. sp., Wakeman and Leander

Diagnosis. Elongated trophozoites 163 µm long and 27 µm wide on average. The circular to ovoid nucleus was located in the posterior half of the cell. The posterior end tapered to a nipple-like tip. Gliding motility. Trophozoites with an inconspicuous mucron and distinctively translucent under light microscopy. Longitudinal epicytic folds with a density of 4-5/µm over the trophozoite surface. A distinct swelling of 6-10 wider epicytic folds was present on one side of the trophozoite surface.

Gene sequence. SSU rRNA gene sequence (GenBank JX535344).

Type locality. Boundary Bay (49°00'54.88''N, 123°02'12.72''W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sea level.

Type habitat. Marine.

Type host. *Notomastus tenuis* Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

Location in host. Intestinal lumen.

Holotype. Figure 3G. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at

**Etymology.** The species name, *translucidae*, stems from Latin meaning “transparent”, and refers to the “see-through” quality of the trophozoites stages of this species.

*Polyplicarium citrusae* n. sp., Wakeman and Leander

**Diagnosis.** Trophozoites of *P. citrusae* extremely flattened and lemon-shaped with an average length and width of 47 µm and 32 µm, respectively. Trophozoites translucent to light brown under light microscopy. Circular nucleus positioned in the center of trophozoites. Gliding motility. The anterior mucron region was inconspicuous and difficult to distinguish from the posterior end, without observing the direction of gliding motility. The cell surface was covered longitudinal epicytic folds with a density of 4/µm. Syzygy side-to-side.

**Gene sequence.** SSU rRNA gene sequence (GenBank JX535348).

**Type locality.** Jericho Beach (49°16’24.39”N, 123°11’07.18”W), Point Grey (Vancouver), British Columbia, Canada. Host in black sediment; mid-low intertidal; 0.80 m below mean sea level.

**Type habitat.** Marine.

**Type host.** *Heteromastus filiformis* Eisig, 1887 (Annelida, Polychaeta, Scolecida, Capitellidae).

**Location in host.** Intestinal lumen.

**Holotype.** Figure 4F. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR120.

**Etymology.** The species name, *citrusae*, stems from Latin meaning “citrus”, and refers to the general “lemon-shape” of the trophozoites and gamonts.
5 Molecular phylogeny and ultrastructure of *Surculinium glossobalanae* n. gen. et sp. (Apicomplexa) from a Pacific *Glossobalanus minutus* (Hemichordata) confounds the relationships between marine and terrestrial gregarines

5.1 Synopsis

Gregarine apicomplexans are single-celled parasites of the intestines and other body cavities of marine, freshwater and terrestrial invertebrates. Nearly 1,700 species of gregarines have been described thus far, and likely millions more await discovery and characterization (Grassé 1953; Levine 1971, 1976, 1977a, b, 1979; Perkins et al. 2002). The most obvious life history stage in gregarines is an extracellular feeding cell called a “trophozoite”, and species descriptions have focused on distinctive traits associated with this life history stage. Although the morphology of trophozoites is very diverse in gregarines as a whole (Leander 2008), high degrees of intraspecific variation combined with relatively low degrees of interspecific variation make the delimitation of different species based on trophozoite traits alone difficult (Wakeman and Leander 2012, 2013, Rueckert et al. 2010, 2011b). Moreover, the overall diversity of marine gregarines is very poorly understood because of limited available expertise and the challenges associated with the collection, isolation, and characterization of these single-celled parasites. The most recent research on marine gregarines has coupled molecular phylogenetic data with comparative morphology in order to shed considerable light onto the boundaries and interrelationships of different species (Leander 2007, 2008; Leander et al. 2003; Rueckert and Leander 2009; Rueckert et al. 2010; Rueckert et al. 2011; Rueckert et al. 2012; Wakeman and Leander 2012).
Archigregarines (Selenidiidae) have several traits inferred to have been retained from the most recent common ancestor of gregarines and perhaps apicomplexans as a whole, such as trophozoites with (1) an apical complex supporting a myzocytotic mode of feeding within the intestines of marine invertebrate hosts and (2) relatively few (<50) longitudinal epicytic folds supported by robust layers of microtubules (Dyson et al. 1994; Hoshide and Todd 1996; Leander 2006, 2007, 2008; Leander and Keeling 2003; Levine 1971; Macgregor and Thomasson 1965; MacKinnon and Ray 1933; Mellor and Stebbings 1980; Ray 1930; Rueckert and Leander 2009; Schrével 1968, 1970, 1971a, 1971b; Schrével 1971b; Simdyanov and Kuvardina 2007; Stebbings et al. 1974; Vivier and Schrével 1964; Wakeman and Leander 2012). The microtubules facilitate the distinctive bending, twisting and nematode-like thrashing motility of archigregarines. Approximately 60 of all described gregarine species are considered archigregarines; the majority of these fall within a single genus, Selenidium sensu lato (Levine 1971; Ray 1930; Rueckert and Leander 2009; Schrével 1971a, b; Wakeman and Leander 2012). As explained previously (Rueckert and Leander 2009), our interpretation of Selenidium reflects a contemporary view that does not split the genus into two different “families”, namely the Selenidiodidae and Selenidiidae, based on the presence or absence of merogony (Levine 1971).

The combination of inferred ancestral traits in archigregarines suggest that these species form a (paraphyletic) stem group from which all other gregarines evolved (Grassé 1953; Leander 2008; Théodoridès 1984; Wakeman and Leander 2012). Molecular phylogenetic analyses of small subunit (SSU) rDNA sequences have reinforced this view by showing that species of archigregarines tend to have relatively short branches and represent five different lineages along the unresolved backbone of the apicomplexan tree; one of which, Veloxidium, forms the sister lineage to a diverse clade of marine lecudinids (Wakeman and Leander 2012). These results provide a compelling example for how the use of molecular phylogenetic approaches and increased sampling of new species have improved
our understanding of gregarine diversity and evolutionary history (Leander 2007, 2008; Rueckert and Leander 2009; Wakeman and Leander 2012, 2013).

In this study, we report the discovery of a novel species of an archigregarine-like apicomplexan isolated from the hemichordate Glossobalanus minutus collected from the Western Pacific Ocean. We characterized this parasite using light microscopy (LM), transmission electron microscopy (TEM) and molecular phylogenetic analyses of SSU rDNA sequences. This report represents the first ultrastructural and molecular phylogenetic data from any gregarine infecting this distinctive group of hosts. The molecular phylogenetic analyses also incorporated environmental DNA sequences, which allowed us to evaluate some unexpected relationships between marine and terrestrial gregarines and the overall distribution of archigregarine-like lineages within the tree of apicomplexans.

5.2 Materials and methods

5.2.1 Collection of organisms.

The host, G. minutus Kowalevsky, 1866 (Hemichordata) was collected in October and November 2012 in the intertidal zone (26°28’58.48’’ N 127°50’14.25’’E) near Onna, Okinawa, Japan. Host material was transported to the lab in buckets filled with seawater, and stored for no more than 24 hours prior to dissection. Contents from the hepatic region of the host were placed on well slides and observed with an inverted microscope (Olympus CKX41, Olympus Corporation, Tokyo, Japan). Individual trophozoites were isolated with hand-drawn glass pipettes, washed in chilled, filtered and autoclaved seawater, and subsequently prepared for LM, TEM and DNA extraction.
5.2.2 Light microscopy and transmission electron microscopy.

Differential interference contrast (DIC) images of living trophozoites were taken with an Olympus BX51TF (Olympus Corporation, Tokyo, Japan), connected to a Nikon DS-L3 color digital camera (Nikon Corporation, Tokyo, Japan). Additional material was preserved in 2.5% glutaraldehyde in seawater for 1 hour, washed in distilled water, and dehydrated with a graded series of ethanol washes (50%, 60% and 70%), and mounted in glycerol on glass slides.

Because trophozoites were infrequently encountered, single-cells of the trophozoite stage were collected for TEM in Eppendorf tubes over a period of three to four hrs. The trophozoites were fixed in 2.5% glutaraldehyde in seawater on ice for 30 minutes. After being washed three times with cacodylate buffer (0.2 M pH 7.2), cells were fixed in 1% OsO₄ in cacodylate buffer for 90 minutes. Following this fixation period, cells were washed three times with cacodylate buffer and suspended in ~1.5% low melting point agarose (temperature ~37°C). The cell/agarose solutions were placed on ice for two to three minutes to solidify. The agarose containing the cells was then removed from the tube and the individual cells were found using an Olympus SZ61 stereomicroscope (Olympus Corporation, Tokyo, Japan). Agar blocks containing the cells were cut out using a razor blade, placed in an Eppendorf tube and dehydrated through a graded series of ethanol washes (70%, 80% 85%, 90%, 95%, 100%, 100%, 100%), lasting five minutes each. The material was then placed in a 1:1 mixture of ethanol and acetone, and 100% acetone for five minutes. Cells were embedded in 1:1 acetone and Epon 812 resin for 30 minutes, and then transferred to 100% resin overnight. After changing the 100% resin one time, material was polymerized for 32 hours at ~ 68°C. Ultra-thin sections through five different trophozoites were cut using a diamond knife on a Leica EM UC6 ultra-microtome and double-stained with 2% (w/v) uranyl acetate and lead citrate. Sections were observed using a Hitachi H7600 electron microscope.
5.2.3 DNA extraction, amplification, cloning and sequencing.

We extracted DNA from two single cell isolates of the new gregarine. For each isolate, an individual trophozoite was placed in a 1.5 ml Eppendorf tube containing cell lysis buffer. Genomic DNA was extracted with the standard protocol provided by the MasterPure complete DNA & RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). However, the final elution step was lowered to 4 µl, in order to concentrate extracted DNA prior to single-cell (SC) PCR amplification. Outside primers, PF1 5’–
GCGCTACCTGGTTGATCTGCC – 3’ and SSUR4 5’–
GATCCTTCTGCAGGTTCACCTAC – 3’ (Leander et al. 2003), were used in a 25 µl PCR reaction with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI, USA). The following program was used on the thermocycler for the initial amplification: Initial denaturation at 94°C for 2:00 m.; 35 cycles of denature at 94°C for 0:30 s., anneal at 52°C for 0:30 s., extension at 72 °C for 1:50 m., final extension 72°C 9:00 m. Subsequently, internal primers F2 5’–
GGTAGYGACAAGAAATAACAAC – 3’ and R2 5’–
GAYTACGACGGTATCTGATCGTC – 3’ were paired with outside primers in a nested PCR reaction using the following program on a thermocycler: Initial denaturation for 94°C for 2:00 m.; 25 cycles of denature at 94°C for 0:30 s., anneal at 55°C for 0:30 s., extension at 72 °C for 1:30 m.; final extension at 72°C for 9:00 m (Wakeman and Leander 2013). All SC-PCR products were separated on agarose gels and isolated using the UltraClean15 DNA Purification Kit (MO BIO, Laboratories, Inc., Carlsbad, CA, USA), and cloned into a pCR 2.1 vector using a StrataClone PCR cloning kit (Aligent Technologies, Santa Clara, CA, USA). Sixteen clones from each cloning reaction were screen for size, digested with HaeIII restriction enzyme (Invitrogen, Frederick, MD, USA) and sequenced using vector primers and ABI Big-dye reaction mix. A novel sequence was identified using the National Center for Biotechnology Information’s (NCBI) BLAST tool and confirmed with molecular phylogenetic analyses. The SSU rDNA sequence from this gregarine was confirmed using multiple single trophozoite
isolates separated by at least one month. The different isolates had identical SSU rDNA sequences (GenBank KC890798).

5.2.4 Molecular phylogenetic analyses.

Two datasets were constructed to analyze the phylogenetic position of the new isolate among gregarines and other apicomplexans: (1) an 80-taxon dataset representing the full diversity of known marine and terrestrial gregarine sequences (1,054 sites), and (2) a 61-taxon dataset that excluded the longest branches in the 80-taxon set, contained fewer marine gregarine sequences, and included a more comprehensive set of terrestrial gregarine sequences (1,200 sites). Each dataset in this study was aligned with MUSCLE (Edgar 2004) and subsequently fine-tuned using MacClade 4 (Maddison and Maddison 2004); gaps and ambiguously aligned regions were excluded from the analyses.

Jmodeltest selected a GTR + I + Γ model of evolution under AIC and AICc for both alignments (80-taxon alignment: proportion of invariable sites = 0.1980, gamma shape = 0.624080; 61-taxon alignment: proportion of invariable sites = 0.2220, gamma shape = 0.4880) (Posada and Crandall 1998). Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) was used to infer a maximum likelihood (ML) tree and for ML bootstrap analyses (500 pseudoreplicates, one heuristic search per pseudoreplicate) (Zwickl 2006). Bayesian posterior probabilities were calculated for both alignments using the program MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We set our program for four Monte Carlo Markov Chains starting from a random tree (MCMC; default temperature= 0.2), a gamma distribution and stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate). 7,000,000 and 5,000,000 generations were calculated for the 80-taxon and 61-taxon datasets, respectively. Trees were sampled every 100 generations, with a prior burn-in of 600,000 and 500,000 generations for the 80-taxon and 61-taxon datasets, respectively. Burn-in was
confirmed manually, and majority-rule consensus trees were constructed; posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

5.3 Results

5.3.1 General ultrastructure of the trophozoites.

The trophozoites were vermiform, crescent-shaped, and on average 90 µm long and 11 µm wide (range = 60--110 µm x 10--14 µm, n = 21) (Fig. 5.1A-D). The nucleus was 10 µm wide, 14 µm long (n = 21), and positioned in the center of the cell; the nucleus also contained a large nucleolus (Fig. 5.1; Fig. 5.3F). None of the cells observed showed any evidence of motility (e.g., bending, twisting or gliding). A total of five individual trophozoites were sectioned for TEM: two were sectioned transversely and three were sectioned along the longitudinal axis of cell. Microtubules were not observed in any of these sections. Transverse TEM sections demonstrated 26 epicytic folds running along the longitudinal axis of the cell (Fig. 5.1E). TEM images also revealed distinct patches of mitochondria with tubular cristae underlying the epicytic folds (Fig. 5.2A-B; Fig. 5.3A-C). Amylopectin granules and lipid droplets were very common throughout the cytoplasm (Fig. 5.2A; Fig5.3A). Darkly-stained lysosomes with a striated internal organization were also distributed throughout the cytoplasm and particularly numerous near the nuclear envelope (Fig. 5.2C,D; Fig5.3F).

Longitudinal TEM sections demonstrated a sucker-like mucron consisting of a sunken dome encircled by a raised lip (Fig. 5.3A-C). The base of the mucron was also defined by 3-4 transverse striations (Fig. 5.3B,C). Tangential TEM sections near the base of the mucron passed through the bases of the grooves between the transverse folds (Fig. 5.3D). Pinocytotic whorled vesicles formed from invaginations of the trilayered membrane complex, were common within the mucron region (Fig. 5.3B,C). Pinocytotic whorled vesicles and associated
micropores were also present in rows along the sides of the cell, and in close association with the superficial patches of mitochondria (Fig. 5.3C). We did not observe rhoptories, micronemes, a conoid, or microtubules in any of our sections through the mucron region.

5.3.2 Molecular phylogenetic analyses of SSU rDNA sequences.

Analyses of the 80-taxon dataset resulted in seven different clades of marine gregarines: (1) the Cephaloidophoroidea (i.e., gregarines from crustaceans), (2) *Polyplicarium*, (3) paralecudinids, (4) lecudinids, urosporids, and *Veloxidium*, (5) *Selenidium* from tube-forming polychaetes, (6) *Selenidium* from sipunculids, and (7) *Platyproteum* and *Filipodium* (Fig. 5.4). The relationships between these clades were unresolved. The terrestrial gregarines in the analyses formed a weakly supported clade that contained, in part, two robust subclades: (1) *Prismatospora, Monocystis*, and *Syncystis* and (2) *Gregarina, Protomagalhaensia, Leidyana* and *Amoebogregarina*. The sequence from the new marine gregarine branched with the terrestrial subclade containing *Gregarina* with very strong support; however, the internal relationships within this subclade were unresolved (Fig. 5.4). This result was consistent with the phylogenetic analyses of the 61-taxon dataset, which included a more comprehensive set of sequences from terrestrial gregarines (data not shown).
Figure 5.1 Micrographs of the trophozoite stage of *Surculinium glossobalanae* n. gen. et sp. A-D. Differential interference contrast light micrographs showing the sucker-like mucron (M), ovoid nucleus (N) and longitudinal epicytic folds (arrowhead). Sticky host material (arrow) covers the surface of the cell. E. Transverse TEM showing 26 epicytic folds on the surface of the trophozoite and a low magnification view of the cell contents. Scale bars: Fig. A--D = 15 µm; Fig. E = 500 nm.
Figure 5.2 Transmission electron microscope (TEM) images of the trophozoites stage of *Surculinium glossobalanae* n. gen., n. sp. A-D. Dense patches of mitochondria (MT) beneath the plasma membrane (arrowhead) and inner membrane complex. The TEMs also show epicytic folds (EF), amylopectin granules (G), lipids (L), pinocytotic whorled vesicles (WH), lysosomes (LY), the nucleus (N) and the nucleolus (NO). 8 (inset). High-magnification TEM of a lysosome with a striated appearance. Scale bars: Fig. A = 500 nm; Fig. B = 2 µm; Fig. C = 500 nm (inset = 100 nm); Fig. D = 500 nm.
Figure 5.3 Transmission electron microscope (TEM) images of the trophozoites stage of Surculinium glossobalanae n. gen., n. sp. A. Longitudinal TEM showing the mucron (M) and dense patches of mitochondria (arrows) beneath the surface of the epicytic folds. B-C. Longitudinal TEM showing a higher magnification view of the mucron consisting of a dome encircled by a mucron lip (ML). Transverse folds (TF) can be seen just below the
mucron lip. Pinocytotic whorled vesicles (WH) and mitochondria (MT) are present within the mucron. D. Glancing sections through the bases of folds (BF) were observed just below the mucron lip. E. High-magnification TEMs showing a micropore (P) and the associated inclusion (I). F. TEM showing the nucleus (N), nucleolus (NO) and clusters of electron dense lysosomes (LY) surrounding the nuclear envelope. Scale bars: Fig. A = 20 µm; Fig. B = 2 µm; Fig. C = 1 µm; Fig. D; Fig. 14 = 500 nm; Fig. E = 5 µm.
Figure 5.4 Phylogenetic analyses of SSU rDNA of *Surculinium glossobalanae* gen., n. sp. Maximum likelihood (ML) tree derived from phylogenetic analysis of the 80-taxon dataset (1,054 unambiguously aligned sites) of small subunit (SSU) rDNA sequences. This tree was inferred using the GTR+I+Γ substitution model (-ln L= 19,526.461 gamma shape = 0.6240, proportion of invariable sites = 0.1980). Bootstrap support values are listed above Bayesian posterior probabilities. Black dots on branches denote bootstrap support values and Bayesian posterior probabilities of 95/0.99 or greater, respectively. Bootstrap and Bayesian values less than 55 and 0.99, respectively, were not added to this tree. A representative sequence from *Surculinium glossobalanae* n. gen. et sp. is highlighted in a black box. Long branches were shortened by multiple lengths of the substitutions/site scale bar.
Table 5.1 Comparisons of traits in known species of marine gregarines isolated from hemichordates. *Surculinium glossobalanae* n. gen. et sp. is highlighted in bold.

<table>
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<th>Host</th>
<th><em>Glossobalanus minutus</em></th>
<th><em>Balanoglossus clavigerus</em></th>
<th><em>Glossobalanus minutus</em></th>
<th><em>Glossobalanus minutus</em></th>
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<td>Glossobalanus minutus</td>
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<td>E. Atlantic Ocean</td>
<td>E. Atlantic Ocean</td>
<td>W. Pacific Ocean</td>
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<td>Vermiform</td>
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<td>Théodoridès &amp; Desportes (1968)</td>
<td>Fernandez (1982)</td>
<td>This study</td>
</tr>
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</table>
5.4 Discussion

Two previous studies isolated and described gregarines from hemichordates: Léger and Duboscq (1917) described *Selenidium metchnikowi* from an E. Atlantic *G. minutus* and Théodoridès and Desportes (1968) described *Selenidium grassei* from an E. Atlantic *Balanoglossus clavigerus* delle Chiaje, 1829. These two species of gregarines differ in the size of the trophozoites, the number of longitudinal epicytic folds and the type host (Table 5.1). An additional study used histological staining to investigate “*Selenidium* sp.” from the hepatic region of an E. Atlantic *G. minutus* (Fernandez 1982). However, this author chose not to establish a new species or determine whether this isolate represented one of the two previously described species because of overlapping similarities with the two morphological descriptions (e.g., host affiliations, the size of the trophozoites and the number of longitudinal epicytic folds) (Table 5.1). Host affinity and the lack of motility are similarities between the trophozoites isolated in our study and those reported by Léger and Duboscq (1917) (*S. metchnikowi*) and photographed by Fernandez (1982) (Table 1). However, the size of the trophozoites and the sucker-like mucron in our isolate are more consistent with *S. grassei* (Table 5.1). The primary differences between our isolate and the two previously described species are the number of longitudinal epicytic folds on the surface of the trophozoites (26 rather than 5-8) and the geographical location from which the hosts were collected (W. Pacific Ocean rather than the E. Atlantic Ocean) (Table 5.1). Moreover, our study is the first to perform TEM on individual trophozoites, so it is possible that the previous studies were unable to clearly distinguish the total number of longitudinal epicytic folds on the trophozoite surface.

Our study of the ultrastructure of *Surculinium glossobalanae* n. gen. et sp. also encountered conspicuous, electron dense structures that were distributed throughout the cytoplasm of the cell (Fig. 5.2C,D). One interpretation of these data could be that these structures are mitochondria with densely-packed cristae like those reported in *Pterospora* (Landers 2002). However, these dense
structures were not surrounded by two membranes (Fig. 5.2C), and canonical mitochondria with tubular cristae were abundant under the epicytic folds (Fig. 5.2A). Therefore, we have interpreted these structures as lysosomes at different stages of development (Fig. 5.2C,D).

The active bending, coiling and twisting movement in the trophozoite stages of *Selenidium* species is facilitated by an extensive array of cortical microtubules organized beneath the inner membrane complex (Leander 2006, 2007, 2008; Mellor and Stebbings 1980; Schrével 1971b; Simdyanov and Kuvardina 2007; Stebbings et al. 1974; Vivier and Schrével 1964). The lack of motility, cortical microtubules, and a conoid in our isolate suggests that it is not a member of *Selenidium* Giard 1884 (Dyson et al. 1994; Schrével, 1971a,b; Vivier and Schrével 1964). Additional evidence that our isolate falls outside of *Selenidium* comes from the molecular phylogenetic data; the sequence from the new isolate is not closely affiliated with the SSU rDNA sequences from the twelve *Selenidium* species currently available for molecular phylogenetic analyses (Rueckert and Leander 2009; Wakeman and Leander 2012, 2013). A SSU rDNA sequence from the type species of *Selenidium*, *S. pendula*, is currently unavailable. Acquiring the data from its original locality and type host will inevitably inform the use of *Selenidium* in future molecular phylogenetic trees and classification schemes.

Nonetheless, molecular phylogenetic analyses of the SSU rDNA sequence from the new marine isolate unexpectedly clustered within a clade of terrestrial gregarines containing *Gregarina, Leidyana, Protomagalhaensia*, and *Amoebogregarina*. We verified this result by acquiring identical SSU rDNA sequences from two different single-cell isolates of the gregarine from two hosts, each collected on different trips separated by over 4 weeks. Clopton (2009) acquired several of the SSU rDNA sequences from the terrestrial gregarines that were closely allied with our marine isolate and performed phylogenetic analyses on a selective group of species in the so-called "Septorina". The underlying assumption of this study was that terrestrial septorinids represent a monophyletic
group, and therefore, all sequences from marine gregarines (and other lineages of apicomplexans) were excluded from the analyses (Clopton 2009). The SSU rDNA sequence derived from the gregarine isolated from *G. minutus* provides the first direct link between a marine gregarine species and gregarine species isolated from terrestrial hosts. This result shows that marine gregarines can branch in unanticipated positions and should therefore be included in all molecular phylogenetic analyses of gregarine diversity. Moreover, it is possible that terrestrial gregarines are polyphyletic and represent more than one clade that independently descended from marine ancestors (Rueckert et al. 2011a). It has already been demonstrated, for instance, that gregarines from marine amphipods are more closely related to gregarines found in freshwater amphipods than to other marine gregarines (Rueckert et al. 2011a). Ultimately, isolation of other gregarines like *Surculinium glossobalanae* n. gen., n. sp. from hemichordates from distinct geographic regions will provide the best evidence to refute or support our conclusions of the phylogenetic analyses in this study.

In conclusion, we characterized the first ultrastructural and molecular phylogenetic data from a gregarine infecting a hemichordate host. The general morphology of the trophozoite stage demonstrated that the new isolate from the W. Pacific Ocean was similar but distinct from the two previously described species of *Selenidium* from E. Atlantic hemichordates, namely *S. metchnikowi* and *S. grassei*. The absence of a conoid, cortical microtubules and motility suggest that the new isolate was not a member of *Selenidium*. These ultrastructural data were consistent with the molecular phylogenetic data showing that the new isolate was not closely affiliated with the other species of *Selenidium* and instead was nested within a clade of terrestrial gregarines, including *Gregarina*. Although this phylogenetic result was unexpected, it potentially sheds light onto the origins of terrestrial gregarines from marine ancestors and is consistent with the hypothesis that archigregarine-like apicomplexans form a (paraphyletic) stem group from which all other gregarines evolved. Overall, these
data justified the establishment of a new binomial for the new isolate, *Surculinium glossobalanae* n. gen. et sp.

5.5 Taxonomic summary and diagnosis.

Apicomplexa Levine, 1970
Gregarinea Bütschli, 1882, stat. nov. Grassé, 1953
Archigregarinorida Grassé, 1953

*Surculinium* n. gen. Wakeman, Reimer, Jenke-Kodama et Leander 2013

**Diagnosis.** Trophozoites non-motile, vermiform, crescent-shaped and with an oval nucleus located in the middle of the cell. Mucron sucker-like consisting of a depressed dome surrounded by raised lip. The trophozoite surface inscribed by longitudinal epicytic folds and transverse striations near the base of the mucron. Cortical microtubules and conoid absent.

**Type Species.** *Surculinium glossobalanae*

**Etymology.** The genus name is taken from ‘*surculus’* (Latin) = “sucker” and refers to the sucker-like mucron of the trophozoites.

*Surculinium glossobalanae* n. sp. Wakeman, Reimer, Jenke-Kodama et Leander 2013

**Description.** Trophozoites are vermiform, mean length = 90 µm (range = 60-110 µm) and mean width = 11 µm (range = 10-14 µm). An oval nucleus (10 µm x 14 µm) is located in the middle of the cell. The trophozoite surface inscribed by 26 longitudinal epicytic folds and 4-5 transverse striations near the base of the sucker-like mucron.

**Gene sequence.** SSU rDNA sequence (GenBank KC890798)

**Type locality.** Onna Village (26°28’58.48” N 127°50’14.25”E). West Pacific Ocean intertidal zone sand near Onna, Okinawa, Japan.
**Type host.** *Glossobalanus minutus*

**Location in host.** Hepatic region

**Type Material.** Parasites fixed in resin blocks and on glass slides have been deposited in the Beaty Biodiversity Research Centre at the University of British Columbia, Vancouver, Canada (Marine Invertebrate Collection; voucher: MI-PR126).

**Etymology.** The specific epithet is named for the genus of the hemichordate host in which this species was found.
6 Comparative ultrastructure and molecular phylogeny of *Selenidium melitzanae* n. sp. and *S. terebellae* Ray, 1930 demonstrate niche partitioning in marine gregarine parasites (Apicomplexa)

6.1 Synopsis

Gregarine apicomplexans are single-celled parasites with morphologically diverse feeding stages, called “trophozoites”, that infect the extracellular spaces of invertebrates. Traits associated with trophozoite morphology and life history dynamics (e.g., asexual division in the trophozoite stage) form the basis for organizing gregarine species into three main groups: archigregarines, eugregarines, and neogregarines (Grassé 1953; Levine 1971, 1976, 1977a, b, 1979; Schrével 1971a; Théodoridès 1984; Cox 1994; Perkins et al. 2002; Leander 2008; Adl et al. 2012). However, molecular phylogenetic data are beginning to refine inferences about the relationships between different species of gregarines by demonstrating several unexpected patterns of divergence, including examples of convergent evolution and significant levels of intraspecific variation in trophozoites (Leander 2007, 2008; Leander et al. 2003; Rueckert and Leander 2009; Rueckert et al. 2010; Rueckert et al. 2011a; Rueckert et al. 2012; Wakeman and Leander 2012, 2013a, b). Some species of gregarines, by contrast, have trophozoites with morphological traits that have remained relatively unchanged over long periods of time (Leander 2007, 2008; Leaner and Keeling 2003; Rueckert and Leander 2009; Schrével 1971a; Simdyanov and Kuvardina 2007; Théodoridès 1984). The molecular phylogenetic data combined with a deeper knowledge of ultrastructural traits in different species of gregarines are challenging some of the original concepts for the three traditional groupings of gregarines and bring to light a much richer evolutionary history for these
Archigregarines are found only in marine environments and are inferred to have maintained a set of morphological and life history characteristics from the most recent common ancestral of all gregarines and perhaps apicomplexans as a whole (Grassé 1953, Kuvardina et al. 2002; Leander 2006, 2007, 2008; Leander and Keeling 2003; Leander et al. 2003; Myl'nikov 2009; Rueckert and Leander 2009; Schrével 1968, 1971b; Simdyanov and Kuvardina 2007; Stebbings et al. 1974). Phylogenetic trees inferred from small subunit (SSU) rDNA sequences are consistent with the interpretation that archigregarines form a paraphyletic stem group from which other gregarine lineages evolved (Leander 2007, 2008; Rueckert and Leander 2009; Wakeman and Leander 2012). Therefore, improved knowledge of archigregarine diversity at both molecular and ultrastructural levels is expected to shed considerable light onto the earliest stages of apicomplexan evolution.

To this end, we set out to characterize the ultrastructure of *Selenidium terebellae* Ray, 1930 collected from the intestinal lumen of *Thelepus japonicas*. In the process, we discovered a second species that primarily inhabits the coelom of the same host and had trophozoites with a fundamentally different set of morphological traits compared to *S. terebellae*. We comprehensively characterized both species using differential interference contrast microscopy (DIC), fluorescence microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and molecular phylogenetic analyses of SSU rDNA sequences derived from single-cell PCR. We also used alpha-tubulin antibodies to visualize (1) the distribution of microtubules within trophozoites and (2) the presence or absence of a conoid within the mucron of trophozoites. These
data demonstrated novel ultrastructural traits in marine gregarines and the power of molecular phylogenetic data for inferring patterns of morphological divergence that would otherwise be inaccessible from comparative morphology alone.

6.2 Materials and methods

6.2.1 Collection or organisms.

_Thelepus japonicus_ Marenzeller, 1884 was collected in January 2013 under rocks in the low intertidal to sub tidal zone while SCUBA diving at a depth < 5 m near Clover Point 48°24′11.36″ N 123°21′01.94″ W, Victoria, British Columbia, Canada. Host material was stored in containers of seawater on ice for 24 hours prior dissection. The complete gut was removed from the host using fine forceps. Some of the whole gut material was prepared for SEM and TEM. Other gut material was teased apart with fine forceps on well slides to release individual trophozoites that were individually isolated for light microscopy, SEM, TEM and DNA extraction.

6.2.2 Light microscopy.

Hand-drawn glass pipettes were used to isolate and clean individual trophozoites using an inverted microscope (Zeiss Axiovert 200, Carl-Zeiss, Göttingen, Germany) attached to a PixeLink Megapixel color digital camera (PL-A662-KIT, Ottawa, Canada). Some isolates were photographed on well slides, washed in autoclaved, filtered seawater and collected for DNA extraction and single-cell PCR amplification (SC-PCR). Isolated trophozoites were also photographed with differential interference contrast using a Leica DC 500 color camera attached to a Zeiss Axioplan 2 microscope (Carl-Zeiss, Göttingen, Germany).
6.2.3 Immunolocalization.

Trophozoites of *S. terebellae* and *S. melitzanae* n. sp. were isolated from the host and washed at least twice (or until clean) in chilled, filtered seawater. Isolates were initially fixed for 5 minutes in 4% paraformaldehyde in PBS containing 50 mM K-EGTA (Heintzelman 2004). Isolates were washed three times with PBS/K-EGTA and permeabilized for 30 minutes in PBS/K-EGTA containing 0.1% BSA and 0.1% Triton X-100. Cells were then incubated overnight with a polyclonal alpha-tubulin primary antibody (Sigma-Aldrich, Ontario, Canada). Cells where then washed four times in PBS/K-EGTA containing 0.1% BSA and 0.1% Triton X-100, and incubated with a secondary antibody (Molecular Probes, Eugene, Oregon) for five hours. Controls were made for each species by following this procedure; however, the primary antibody was not added to the control samples. Specimens where then mounted on glass slides and sealed with clear fingernail polish. Slides were viewed on an Olympus FV10i Confocal microscopy (Olympus, Tokyo, Japan). At 546 nm, the autoflorescence of the specimens and non-specific binding of the secondary antibody was negligible.

6.2.4 Scanning electron microscopy.

Individual trophozoites were pooled in 2% glutaraldehyde on ice. A 10 µl polycarbonate membrane filter was placed within a Swinnex filter holder (Millipore Corp. Billerica, MA, USA). Trophozoites were filtered onto the membrane using a syringe with distilled water, and the holder was placed in a small beaker (4 cm diam. and 5 cm tall) that was filled with distilled water. Ten drops of 1% OsO₄ were added to the opening of the filter holder, and the samples were post-fixed on ice for 30 minutes. The syringe was used to slowly run distilled water over all samples. A graded series of ethanol washes (30%, 50%, 75%, 85%, 95% and 100%) was then used to dehydrate the fixed cells using the syringe system. Following dehydration, the polycarbonate membrane
filters containing the trophozoites were transferred from the Swinnex filter holders into an aluminum basket submerged in 100% ethanol in preparation for critical point drying with CO$_2$. The dried polycarbonate membrane filters containing the trophozoites were mounted on aluminum stubs, sputter coated with 5 nm gold and viewed under a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, California).

6.2.5 Transmission electron microscopy.

Single trophozoites of *S. terebellae* and *S. melitzanae* n. sp., as well as small sections (5 mm) of host gut tissue were collected for TEM in Eppendorf Tubes. Initially, these preparations were fixed in 2.5% glutaraldehyde in seawater on ice for 30 minutes. After being washed three times with cacodylate buffer (0.2 M pH 7.2), the material was post-fixed in 1% OsO$_4$ in cacodylate buffer for 90 minutes. Following this fixation period, cells were washed three times with cacodylate buffer and suspended in ~1.5% low melting point agarose (temperature ~37°C). The cell/agarose solutions were placed on ice for two to three minutes to solidify. The agarose containing the cells and sections of gut tissue was then removed from the tube and regions of the agar with trophozoites were excised using a razor blade under a stereo microscope (LeicaL2, Wetzlar, Germany). These chunks of agar were placed in an Eppendorf tube and dehydrated through a graded series of ethanol washes (70%, 80% 85%, 90%, 95%, 100%, 100%, 100%), lasting five minutes each. The material was then placed in a 1:1 mixture of ethanol and acetone, and 100% acetone for five minutes. Cells were embedded in 1:1 acetone and Epon 812 resin for 30 minutes, and then transferred to 100% Epon resin overnight. After changing the 100% Epon resin one time, material was polymerized for 32 hours at ~ 68°C. Ultra-thin sections were cut using a diamond knife on a Leica EM UC6 ultra-microtome and double-stained with 2% (w/v) uranyl acetate and 2% lead citrate. Sections were observed using a Hitachi H7600 electron microscope.
6.2.6 DNA extraction, amplification and sequencing.

Individual trophozoites were placed in a 1.5 ml Eppendorf tube containing cell lysis buffer. Genomic DNA was extracted with the standard protocol provided by the MasterPure complete DNA & RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). However, the final elution step was lowered to 4 µl, with the goal of concentrating extracted DNA prior to SC-PCR amplification. Outside primers, PF1 5’ – GCGCTACCTGGTTGATCCTGCC – 3’ and SSUR4 5’ – GATCCTTCTGCAGGTTCACCTAC – 3’ (Leander et al. 2003), were used in a 25 µl PCR reaction with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI, USA). The following program was used on the thermocycler for the initial amplification: Initial denaturation at 94°C for 2:00 m; 35 cycles of denature at 94°C for 0:30 s, anneal at 52°C for 0:30 s, extension at 72 °C for 1:50 m., final extension 72°C 9:00 m. Subsequently, internal primers F2 5’ – GGTAGYGACAAGAAATAACAAC – 3’ and R2 5’ – GAYTACGACGGTATCTGATCGTC – 3’ (Wakeman and Leander 2013) were paired with outside primers in a nested PCR reaction using the following program on a thermocycler: Initial denaturation for 94°C for 2:00 m; 25 cycles of denature at 94°C for 0:30 s., anneal at 55°C for 0:30 s., extension at 72 °C for 1:30 m.; final extension at 72°C for 9:00 m. All SC-PCR products were separated on agarose gels and isolated using the UltraClean15 DNA Purification Kit (MO BIO, Laboratories, Inc., Carlsbad, CA, USA), and cloned into a pCR 2.1 vector using a StrataClone PCR cloning kit (Aligent Technologies, Santa Clara, CA, USA). Sixteen clones from each cloning reaction were screen for size, digested with HAEIII restriction enzyme (Invitrogen, Frederick, MD, USA) and sequenced using vector primers and ABI Big-dye reaction mix. Novel sequences were identified using the National Center for Biotechnology Information’s (NCBI) BLAST tool, confirmed with molecular phylogenetic analyses, and deposited into GenBank (KC890799-KC890806).
6.2.7 Molecular phylogenetic analyses.

A comprehensive 84-taxon alignment (1,072 unambiguously aligned sites) containing SSU rDNA sequences from the eight single-cell isolates of *S. terebellae* and *S. melitzanae* n. sp., as well as three dinoflagellate sequences (outgroup) and 73 sequences representing the major apicomplexan subclades. The alignment was initially constructed using MUSCLE (Edgar 2004) and was subsequently edited and fine-tuned using MacClade 4.08 (Maddison and Maddison 2005); gaps and ambiguously aligned regions were excluded from the analyses.

Jmodeltest 0.1.1 selected a GTR + I + Γ model of evolution under Akaike Information Criterion (AIC) and AIC with correction (AICC) (proportion of invariable sites = 0.1940, gamma shape = 0.5710 (Posada and Crandall 1998). Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) was used to infer a maximum likelihood (ML) tree and for ML bootstrap analyses (500 pseudoreplicates, one heuristic search per pseudoreplicate) (Zwickl 2006).

Bayesian posterior probabilities were calculated using the following parameter on the program MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003): [GTR (Lset nst = 6); gamma distribution (of rate among sites] and Monte Carlo Markov Chains [starting trees= 4; default temperature= 0.2; generations= 7,500,000; sample frequency= 100; prior burn-in= 750,000 trees]; stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate)]. Burn-in was confirmed manually, and majority-rule consensus trees were constructed; posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees. PAUP 4.0 (Swofford 1999) was used to calculate percent differences between the SSU rDNA sequences of both isolates. The alignment use in this study is available from the authors by request.
6.3 Results

6.3.1 Morphology of *Selenidium terebellae*.

The vermiform trophozoites inhabited the intestinal lumen of the host and were on average 273 µm (range 175 µm - 430 µm, n = 86) long and 12 µm (range = 10 µm - 15 µm, n = 86) wide (Fig. 6.1). Cells were capable of bending and twisting and were light brown from the presence of amylopectin granules. A spherical nucleus was located in the center of the cell and had an average diameter of 10 µm (range = 9 µm - 12 µm, n = 52) (Fig. 6.1). The nucleus was also indented in seven locations to fit the contours of seven longitudinal epicytic folds that ran along the surface the trophozoites (Fig. 6.2). The cytoplasm contained pinocytotic whorled vesicles, amylopectin granules, Golgi bodies, mitochondria with tubular cristae, putative lysosomes, and four-membrane bound organelles that were reminiscent of apicoplasts in other apicomplexans (Fig. 6.3). The surface of the trophozoites was supported by dense arrays of microtubules, usually in bundles of two to three, that ran under the seven epicytic folds (Fig. 6.3A,B). Trophozoites also had transverse striations positioned in the middle regions of the cell (Fig. 6.2, 6.3, 6.4). Micropores leading to the pinocytotic whorled vesicles were present in the grooves between the epicyctic folds, transverse folds and the mucron region (Fig. 6.2A,C,D; Fig. 6.4A). The mucron region of the trophozoites contained a closed conoid and rhoptry-like vesicles (Fig. 6.4). Alpha-tubulin was localized below the surface of the trophozoite in association with the surface microtubules and in the mucron region in association with the closed conoid (Fig. 6.6).

6.3.2 Morphology of *Selenidium melitzanae* n. sp.

The trophozoites were on average 125 µm (range = 30 µm - 155 µm, n = 57) long and 30 µm (range = 10 µm - 41 µm, n = 57) wide (Fig. 6.7A-D). The nucleus was located in the center of the trophozoites and was spherical with an average
diameter of 15 µm (range = 7 µm - 19 µm, n = 33) (Fig. 6.7A-D). The trophozoites were bottle-shaped or egg plant-shaped with a rounded posterior end and an anterior end narrowing to a neck-like mucron (Fig. 6.7, 6.8). Cells were light to dark-brown in color from the presence of amylopectin granules. The trophozoites inhabited the coelom of the host and attached to the outside wall of the intestines (Fig. 6.8). The trophozoites were not capable of gliding or bending. The surface of the trophozoites was covered with approximately 30 epicytic folds that were helically arranged and supported by a layer of electron-dense filaments; cortical microtubules were not observed (Fig. 6.8, 6.9). The mucron region contained an apical cluster of tubule-like material (Fig. 6.10). Alpha-tubulin was localized in the mucron, corresponding to a conoid, and beneath the entire surface of the trophozoites, corresponding to the layer of fibrils present under the cell membrane (Fig. 6.11).

6.3.3 Molecular phylogenetic analyses of SSU rDNA.

We generated SSU rDNA sequences from four single-cell isolates for each of the two species, resulting in eight new sequences. Phylogenetic analyses of these SSU rDNA sequences within the context of an alignment encompassing the diversity of apicomplexans demonstrated two highly supported sister clades representing S. terebellae and S. melitzanae n. sp., respectively (Fig. 6.12). The four sequences generated in this study from S. terebellae were nearly identical to the previously published sequence from S. terebellae (Leander et al. 2003). Intraspecific variation between the four isolates of S. melitzanae n. sp. and the five isolates of S. terebellae ranged from 0.11% - 0.60% and 0.11% - 0.37%, respectively. The variation between the two sister clades ranged from 11.7% - 12.8%.
Figure 6.1 Light micrographs of *Selenidium terebellae*. A-D. Differential interference contrast light micrographs showing the general morphology and bending motility in the trophozoite stage of *Selenidium terebellae*. A-D. The nucleus (N) is centrally located in the trophozoite. Epicytic folds (arrowhead) run along the longitudinal axis of the cell. Micrographs are oriented with the mucron towards the top of the figure. Scale bars = 15 µm.
Figure 6.2 Scanning and transmission electron micrographs of *Selenidium terebellae*. Transmission electron micrographs (TEMs) and scanning electron micrographs (SEM) showing the general ultrastructure and surface morphology of the trophozoite stage of *Selenidium terebellae*. A. Longitudinal TEM showing the nucleus (N), nucleolus (NO) and transverse striations (TS) on the surface of the cell. The cytoplasm contains mitochondria (MT), pinocytotic whorled vesicles
(WH), and putative lysosomes (LY). B. Transverse TEM showing the centrally located nucleus (N) and nucleolus (NO). There are seven epicytic folds that correspond with nuclear indentations (NI). The cytoplasm contains mitochondria (MT), and pinocytotic whorled vesicles (WH) were positioned beneath the grooves between the epicytic folds. C. Low magnification SEM showing the general morphology of a trophozoite, the mucron (M), and epicytic folds (EF) that run along the longitudinal axis of the cell. D. High-magnification SEMs showing transverse striations (TS) on the surface of the cell. Scale bars: A = 2 µm; B = 1 µm; C = 10 µm, D = 3 µm.
Figure 6.3 High magnification transmission electron micrographs (TEMs) of the trophozoite stage of *Selenidium terebellae*. **A-C.** Transverse TEMs showing microtubules (MI) in layers of two to three under the trilayered membrane complex (TM). The inclusions (I) of micropores were present beneath the grooves between the epicytic folds, and led to the formation of pinocytotic whorled vesicles (WH). The cytoplasm contained lipid droplets (L), putative lysosomes (LY), mitochondria (MT) and Golgi bodies (GO). Nuclear indentations
beneath the grooves between epicytic folds were also visible. **D, E.** Longitudinal TEMs showing transverse striations (TS), superficial microtubules (MI), mitochondria with tubular cristae (MT), lipid droplets (L), and pinocytotic whorled vesicles (WH). The outside of the cell was reinforced by a robust trilayed membrane complex (TM). **F,G.** TEMs showing four membrane-bound organelles (MBO) that are reminiscent of the apicoplasts in other apicomplexans. Scale bars: A—D = 500 nm; D = 1 µm; E = 500 nm; F—G = 500 nm.

**Figure 6.4** Transmission electron micrographs (TEMs) through the mucron region of the trophozoites of *Selenidium terebellae*. **A.** High magnification TEM showing host gut cells (HG), host microvilli (MV), and the contents of the mucron. The mucron had transverse striations (TS), rhoptries (RH), mitochondria (MT), and vacuoles (VA). **B.** A high magnification TEM of the mucron showing the conoid (CO), rhoptries (RH) and vacuoles (VA). Scale bars: A = 5 µm; B = 500 nm.
Figure 6.5 Confocal images of the trophozoite of *Selenidium terebellae*. A-F. Phase contrast (*phase*), alpha-tubulin (*tubulin*) and merged (*merge*) images showing the localization of alpha-tubulin throughout the trophozoite and the mucron.
Figure 6.6 Alpha tubulin (α-tubulin) staining of the mucron of *Selenidium terebellae*. A-C. High-magnification confocal images of the mucron (M) showing distinct longitudinal striations (S). D, E. Scanning electron micrographs of the “apical tip” (A) and apical pores (AP) of the mucron, located within the anterior end of the grooves between the striations (S). Scale bars: A-C = 1 µm; D = 500 nm; E = 1 µm.
Figure 6.7 Light micrographs of *Selenidium melitzanae* n. sp. Differential interference contrast (DIC) light micrographs showing the general morphology of the trophozoite stage of *Selenidium melitzanae* n. sp. The mucron is oriented towards the top of the figures. **A.** One cell imaged at two different focal planes. **A-D.** The nucleus (N) is centrally located, and a sheath of host material (arrow) is visible on some of the trophozoites. Scale bar: 15 µm.
Figure 6.8 Scanning electron micrographs of *Selenidium melitzanae* n. sp. General morphology of the trophozoites and their attachment to the outside surface of the host intestine (*Thelepus japonicus*). **A-C.** Low-magnification SEMs showing the distribution and attachment of trophozoites on the outside surface of the gut. **D.** SEM showing the general morphology of the trophozoite with the mucron oriented upwards. A sheath of host gut material (arrow) was still present around the mucron. Epicytic folds (arrowheads) were present on the mucron, and spiraled in a helical arrangement over the entire surface of the cell. Scale bars: A = 100 µm; B = 80 µm; C = 25 µm D = 10 µm.
Figure 6.9 Transmission electron microscope (TEM) images showing the general ultrastructure of trophozoite stages of *Selenidium melitzanae* n. sp.  
A. Transverse TEM showing the nucleus (N), nucleolus (NO), and host microvilli (MV)  
B. Tangential TEM showing the trilayered membrane complex (TM) of the helically arranged epicytic folds, mitochondria (MT) and fibrils (F).  
C. Longitudinal TEM showing epicytic folds (EF) and a general view of the cytoplasm of the trophozoite.  
D. Transverse TEM through the epicytic folds (EF) showing fibrils (F) and mitochondria (MT) with tubular cristae positioned under each fold.  
Scale bars: A. = 3 µm; B = 1.5 µm; C, D = 2 µm; E = 2 µm.
Figure 6.10 Transmission electron micrographs of the mucron of *Selenidium melitzanae* n. sp. Transmission electron micrographs (TEM) of longitudinal sections through the mucron region of *Selenidium melitzanae* n. sp. A—D. TEMs showing epicytic folds (EF), the trilayered inner membrane complex (TM), and an apical cluster (AP) of tubular material (T). Scale bars: A = 2 μm; B,C = 500 nm; D = 1 μm.
Figure 6.11 Confocal and phase contrast images showing the localization of alpha-tubulin (tubulin) in the trophozoites of Selenidium melitzanae n. sp. A-C. Confocal and phase contrast images showing the localization of alpha-tubulin within the entire cell. D-E. Higher magnification confocal images showing the localization of alpha-tubulin (tubulin) within the mucron. F. Confocal image showing the localization of alpha-tubulin (tubulin) within the helically arranged epicytic folds (arrowheads). Scale bars: A-C, F = 10 µm; D-E = 5 µm.
Figure 6.12 Phylogenetic analyses of SSU rDNA from *Selenidium terebellae* and *Selenidium melitzanae* n. sp. Maximum likelihood (ML) tree derived from phylogenetic analyses of the 84-taxon dataset (1,072 unambiguously aligned sites) of small subunit (SSU) rDNA sequences. This tree was inferred using the GTR+I+Γ substitution model (-lnL = 18,398.84797, gamma shape = 0.5710, proportion of invariable sites = 0.1940). Bootstrap support values are listed above Bayesian posterior probabilities. Black dots on branches denote bootstrap support values and Bayesian posterior probabilities of 95/0.95 or greater, respectively. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree. Four single-cell isolations each from *Selenidium melitzanae* n. sp. and *Selenidium terebellae* are highlighted in grey boxes. Some branches were shortened by multiples of the length of the substitutions/site scale bar.

6.4 Discussion

6.4.1 Comparative ultrastructure.

The ultrastructure of *S. terebellae* is very similar to other *Selenidium* species examined with TEM; these species have vermiform trophozoites with relatively few epicytic folds supported by layers of microtubules beneath the inner membrane complex (Leander 2007; Simdyanov and Kuvardina 2007; Stebbings et al. 1974; Vivier and Schrével 1964; 1968, 1971b). The trophozoites of *Selenidium* species also have transverse striations, bending/twisting motility, and a conoid within the mucron (Leander 2007; Mellor and Stebbings 1980; Simdyanov and Kuvardina 2007; Schrével 1968, 1971a, b; Stebbings et al. 1974; Vivier and Schrével 1964). In this study, the localization of alpha-tubulin antibodies throughout the trophozoites of *S. terebellae* provided a straightforward method for visualizing the distribution of superficial microtubules and the presence of a conoid. The presence of a conoid in *S. terebellae* was inferred previously from SEM images, which demonstrated a cone-shaped mucron and "rhoptry docks" within the tip of the mucron (Leander et al. 2003). Our TEM and immunofluorescence data confirmed this inference. Although we observed a conoid and rhoptries within the mucron, it is unclear from our data if *S. terebellae* also feeds by myzocytosis (Simdyanov and Kuvardina 2007, Schrével 1968).
Nonetheless, the TEM data showed evidence for surface-mediated nutrition via rows of micropores and associated pinocytotic whorled vesicles within the grooves between the epicytic folds. This ultrastructural pattern has been observed in other species of *Selenidium*, such as *S. serpulae*, and appears to be a shared trait for the genus (Leander 2007).

Four-membrane bound organelles that differed from the pinocytotic whorled vesicles in size, in shape and in having a consistent number of enveloping membranes were also observed throughout the cytoplasm (Fig. 6.3F-G). Similar organelles were reported in previous ultrastructural studies of *Selenidium* (i.e., *Selenidium pendula*) and strongly resemble the apicoplasts in other lineages of apicomplexans (Schrével 1971; Hopkins et al. 1999; Köhler 2005; Leander and Ramey 2006; Tomova et al. 2006). These organelles were clustered throughout the cytoplasm within the trophozoites of *S. terebellae*. Because *Selenidium* species are inferred to have retained several ancestral traits in gregarines and perhaps apicomplexans as a whole, an improved understanding of the putative apicoplast in *S. terebellae* using genomic approaches is expected to shed considerable light onto the evolution of this enigmatic organelle.

### 6.4.2 Justification of *Selenidium melitzanae* n. sp.

The trophozoites of this new species were very different from those in other gregarine species described previously. *S. melitzanae* n. sp. has trophozoites with 30 to 40 epicytic folds that were helically arranged along the longitudinal axis of the cell. No movement (e.g., gliding motility) or changes in cell shape were observed in this species. TEM images showed that the epicytic folds are supported by arrays of fibrils rather than microtubules *per se*. However, the alpha-tubulin immunofluorescence demonstrates that fibrils were tubulin-based and localized just below the surface of the helical folds. The alpha-tubulin immunofluorescence data also showed that an apical cluster of tubule-like elements was present within the mucron. These data also demonstrate how
immunofluorescence approaches offer a more reliable way to detect the presence of a conoid and other tubulin-based structures in gregarine trophozoites, which would otherwise need to be tediously sectioned for TEM (see Heintzelman 2004; Kuriyama et al. 2005; Valigurová 2012). Future work using immunofluorescence approaches on other gregarine lineages that have been inferred to possess ancestral traits (e.g., Veloxidium) will enhance our ability to study character evolution within Selenidium and beyond.

The only gregarine species we could find in the literature with trophozoites that are vaguely similar to those in S. melitzanae n. sp. is Merogregarina amaroucii Porter 1908, which was isolated from the intestines of an ascidian host. The trophozoites of M. amaroucii have an overall shape that is similar to S. melitzanae n. sp., but there is no mention of helically arranged epicytic folds in the original description (Porter 1908). The five to seven epicytic folds that were reported in M. amaroucii were restricted to the anterior region of the cell (Porter 1908; Perkins et al. 2002). This, along with the fact that M. amaroucii was isolated from an ascidian, suggest that S. melitzanae n. sp. and M. amaroucii are two different species of marine gregarines; ultrastructural and molecular data from M. amaroucii would be necessary to evaluate this relationship further.

6.4.3 Niche partitioning in gregarine apicomplexans.

The molecular phylogenetic analyses demonstrated a close sister relationship between S. terebellae and S. melitzanae n. sp., which reflects a pattern of morphological divergence that would otherwise be undetectable from comparative morphology alone. S. terebellae has a set of molecular, morphological and motility traits inferred to be ancestral for gregarines, such as trophozoites with a conoid, a small number of longitudinal epicytic folds, dense layers of microtubules beneath the cell surface, and bending/twisting movement. Collectively, these characteristics convey the contemporary archigregarine concept (Schrével 1971a, Leander 2008, Wakeman and Leander 2012). By
contrast, the trophozoites of *S. melitzanae* n. sp. have traits that are more consistent with species of marine eugregarines like lecudinids and urosporids than with *Selenidium* (Leander 2008; Leander et al. 2003; Rueckert et al. 2010; Schrével 1971b; Wakeman and Leander 2012). The trophozoites of this species were non-motile and lacked superficial arrays of microtubules beneath the cell surface. Instead, these trophozoites had a dense array of helically arranged epicytic folds supported by a network of tubulin-based fibrils and a conoid-like scaffold within the mucron.

The distinct morphological differences between these two closely related gregarine species are correlated with two different locations within the polychaete host: *S. terebellae* infects the intestinal lumen, and *S. melitzanae* n. sp. was predominately observed attached to the outer intestinal wall. The molecular and morphological data combined suggest that this pattern of morphological divergence is associated with niche partitioning within the host. Other coelomic gregarines like *Lithocystis*, *Urospora* and *Pterospora* have a wide variety of surface morphologies, ranging from folds to bumps and ridges (Coulon and Jangoux 1987; Landers and Leander 2005; Leander 2008). One of the features that these gregarines share is a motility pattern of dynamic surface undulations (Leander and Landers 2006). Molecular phylogenetic data demonstrate that *Lithocystis*, *Urospora* and *Pterospora*, although found in distantly related hosts (holothuroideans and maldinid polychaetes), are part of a diverse monophyletic group that also contains marine lecudinids (Leander et al. 2006; Rueckert and Leander 2009; Wakeman and Leander 2012). *Selenidium melitzanae* n. sp. has a helical pattern of epicytic folds on the surface of the trophozoites, but the cell membrane does not pulsate like *Lithocystis*, *Urospora* and *Pterospora*. This suggests that there is a fundamental difference in how *S. melitzanae* n. sp. and urosporid coelomic gregarines acquire nutrients. Our observations from combined SEM, TEM and confocal micrographs of *S. melitzanae* n. sp. suggest that the cluster of tubulin-based elements at the anterior region of the trophozoite stage enable this parasite to penetrate the outer intestinal wall of its host in order
to feed, presumably, via myzocytosis; a strategy of acquiring nutrients known in other species of *Selenidium*.

Although one way to distinguish some gregarine species is by their host affiliation, there are still many examples of different gregarine species that occupy the same host (Coulon and Jangoux 1987; Levine 1971, 1976; Rueckert and Leander 2009; Wakeman and Leander 2013). For example, Coulon and Jangoux (1987) reported on five species of gregarines that co-infect one species of echinoderm. Some of these host-parasite relationships have been shown to be highly specific to even certain life stages of the host. For instance, four species of *Gregarina* specialize on either adult or larval stages of a single species of arthropod (Clopton 1992, 1993). In these cases, the use of molecular data is extremely important for understanding the boundaries between species and their interrelationships where morphology alone may be insufficient, and subsequently misleading. The data we report here from *S. terebellae* and *S. melitzanae* n. sp. demonstrates an unprecedented case of morphological divergence in two highly unanticipated sister species.

### 6.5 Taxonomic summary and diagnosis.

Phylum Apicomplexa Levine, 1970  
Gregarinea Bütschli, 1882, Embedded Grassé, 1953  
Order Eugregarinorida Léger, 1900  
Selenididae Brasil, 1907  
*Selenidium* Giard, 1884

*Selenidium melitzanae* Wakeman, Heintzelman and Leander n. sp.

**Diagnosis.** Trophozoites light to dark-brown, bottle-shaped or egg-plant-shaped, with a rounded posterior end and a narrow, neck-like mucron. Trophozoite surface with approximately 30 epicytic folds arranged in a helical pattern.
Trophozoites averaged 125 µm in length and 30 µm in width. Mucron contains an apical cluster of tubular elements. Trophozoites without gliding motility or bending movement.

**Gene sequence.** Small subunit rDNA (Genbank Accession KC890799).

**Type locality.** Low intertidal to subtidal zone (<5 m) at Clover Point, Victoria, British Columbia, Canada (48°24’11.36" N 123°21’01.94" W).

**Type host.** *Thelepus japonicus* Marenzeller 1884.

**Location in host.** Coelom, attached to outer wall of intestine and inner intestine.

**Hapantotype.** Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR127. Resin blocks and fixed slides containing infected gut material and/or individual trophozoites are held under the same code.

**Etymology.** The epithet "*melitzanae*" stems from the Greek “melitzana” meaning "eggplant", and refers to the shape of the trophozoites.
7 Conclusion

7.1 Contributions to understanding the diversity and evolution of gregarine apicomplexans

The work herein provides molecular and morphological insights to the evolutionary history and overall diversity of gregarine apicomplexans. Combined, this thesis describes 11 species using light and confocal microscopy, electron microscopy, as well as molecular phylogenetic data from SSU rDNA. This work also provides morphological, biogeographical, and phylogenetic data for other species that have been previously characterized in the literature.

The idea that marine gregarines are stem groups to other lineages of gregarines (Grassé 1953; Schrével 1971; Théodoridès 1984; Rueckert and Leander 2010) or other apicomplexans (Leander 2008) is not new. However, the work in this thesis was pivotal in being the first to place these hypotheses (e.g., archigregarines as a paraphyletic stem group to eugregarines) in a molecular phylogenetic context. One of the most important discoveries was *Veloxidium leptosynaptae* in Chapter 1 of this thesis. *Veloxidium* is unique because it has many characteristics of so-called archigregarines. For instance, the trophozoite bends and twists, and lacks longitudinal folds on its surface. Interestingly, in our phylogenetic analyses, *Veloxidium* branched as an early sister lineage to a clade of marine gregarines containing *Lecudina, Pterospora, Lankesteria* and a putative *Lithocystis* sp. This was the first concrete evidence from molecular data confirming the suspected paraphyly of archigregarines as a stem group to some lineages of marine gregarines (or eugregarines).

With the bizarre discovery of *Surculinium* in Chapter 5, I was able to further speculate on the origin of the eugregarine morphotype, specifically how it related to the evolution of terrestrial eugregarine and neogregarines. As part of the
phylogenetic analyses for this chapter, I found marine environmental sequences that were distributed among clades of terrestrial eugregarines and neogregarines. To say the least, the results from Chapter 5 were very unexpected. However, this finding is not inconsistent with other work (e.g., Veloxidium in Chapter 1 and Selenidium melitzanae in Chapter 6). In fact, the idea that the archigregarine morphotype is paraphyletic among gregarines seems fairly reasonable, when one considers all the discrepancies in morphological or life history characters (e.g., merogony) of archigregarines, eugregarines and neogregarines (Levine 1971, Leander 2006, 2007, 2008; Perkins et al. 2002; Schrével 1970, 1971a).

Chapter 6 in my thesis compared the morphology and ultrastructure of Selenidium terebellae and the newly described Selenidium melitzanae. This was the first study to thoroughly compare the ultrastructure of two sister species of gregarines conforming to both archigregarine (S. terebellae) and eugregarine (S. melitzanae) morphotypes. The study showed that S. terebellae conformed in many ways to the typical archigregarine morphotype (e.g., bending and twisting, seven folds on the surface, microtubules under the trilayer membrane). On the other hand, S. melitzanae stands out as an “atypical” eugregarine. Most notably we observed a tubulin-based fibril system underlying the ~30 helical folds forming the surface of the cell. Our TEM investigation of the mucron of S. melitzanae also revealed a putative feeding apparatus that was composed of microtubules and rhoptories. With the information provided from our phylogenetic analysis of S. terebellae and S. melitzanae, we confirmed the sister relationship of these species, and deepened our understanding of character gain and loss in relation to eugregarine and archigregarine concepts.

With these findings, it was concluded that convergence has played a role in the evolution of some marine lineages such as Lecudina, Paralecudina and Trichotokara, as well as a number of terrestrial species, including Gregarina. This is not the most parsimonious interpretation of morphological data, and to that
extent, it goes against the current classification of eugregarines (and potentially neogregarines) as natural groups. It could be that the diversity of archigregarine lineages is the result of the reversals to an ancestral state. However, phylogenies based on SSU rDNA consistently place all archigregarine-like lineages along the backbone of the tree, or as early-branching sister lineages to eugregarine clades, and not as deeply nested taxa within these clades. If, for instance, the latter were the case, then a possible explanation could be that a series of reversals has taken place during the evolutionary history of gregarines. However, this doesn't appear to be the case in the datasets, and an emerging phylogenetic framework based on HSP90, Actin and SSU rDNA data supports the conclusions favored here.

7.2 The use of SSU rDNA for delimiting species and inferring phylogenies in gregarine apicomplexans

The use of SSU rDNA isolated from single-cell isolates as a tool for delimiting species boundaries in marine gregarines was more broadly applied to three chapters in this thesis, namely chapters 3, 4 and 6. It was concluded here, as well as other studies looking at the diversity of marine gregarines, that molecular data can be used as an effective method for overcoming some of the challenges inherent with the study of gregarine apicomplexans. For example, gregarines have yet to be cultivated, and therefore sufficient amount of material may be hard to come by (e.g., some hosts are difficult to collect, or the infection rates within hosts are low). Some host species represent logistical problems with regard to sampling, that is, some samples are collected opportunistically or as "on-time opportunities" (e.g., deep sea dredges or SCUBA diving in remote areas). Regardless of the case, a simple, repeatable and accurate method (in this case, combing DNA isolated from single cells) is desirable for advancing our understanding of the overall diversity and evolutionary history of gregarines and apicomplexans as a whole.
My data was similar to previous work that showed 18S rDNA was useful in
delimiting closely related species. When single cells of *Polyplicarrium* were
compared at the molecular level, there was a clear "gap" in intra and
interspecificity that conformed to subtle morphological characteristics. For
example, intraspecific variation of 18S rDNA sequences ranged from 0.22% to
2.12% between observable morphotypes, while interspecific variation ranged
from 7.42% - 14.98% (Table 4.2). A similar result was observed with species and
*Selenidium* from tube-forming polychaetes (Table 3.2), and this method was
useful in helping us identify *Selenidium melitzanae* as a distinct lineage, and not
just a lifehistory stage of *S. terebellae*, the gregarine with which *S. melitzanae*
shares a host (Figure 6.12). These results represent exactly what has been
shown in previous studies using similar methods that used single cells, or
multiple clones from amplified DNA (Rueckert and Leander 2010; Rueckert et al.
2011b, Rueckert et al. 2010).

Other methods used to study gregarines are based solely on morphological or
morphometric data, and require a large amount of sample material that
represents various stages of a particular species (Clopton 2009, 2012).
Moreover, morphology alone may be deceptive with regard to differentiating
species. For example, gregarines species can have high degrees morphological
plasticity within a species (e.g., *Lecudina tuzetae*) (Rueckert et al. 2011b). The
opposite has also been shown, that is, some species that are indistinguishable at
the morphological level are highly distinct with regard to host specificity and
molecular phylogenetic relationships (e.g., *Difficilina* spp. from nemerteans)
(Rueckert et al. 2010).

Morphometrics alone has been used in a number of studies describing
gregarines from terrestrial invertebrates (Clopton 2012, and references therein).
However, this method has an inherent flaw in distinguishing between a life-stage
and something that may represent different species. Our study of *S. melitzanae*
and *Selenidium terebellae* was a prime example of the inherent problem with
morphometric data. It is easy to see how, even with TEM data, *M. thelepa* could be confused for an early life stage of *S. terebellae*. Early gamont stages of *Selenidium*-like gregarines are in fact very similar in shape to *S. meliztanae* (Schrével 1971a). The use of molecular data simply avoids this confusion.

However, It should be mentioned that while there are many advantages for using SSU rDNA for closely separating closely related species, this particular marker was unable to resolve deeper nodes in my trees. Sequences from some gregarines appear to be relatively quickly evolving compared to other gregarines, and especially other "core" apicomplexans (i.e., coccidians and piroplasmids). For example, the SSU rDNA from gregarines isolated from crustaceans and paralecudinids (i.e., *Trichotokara*) are probably some of the longest branches on the eukaryotic tree of life. Still, other lineages, such as *Selenidium terebellae* and *Polyplicarium* appear to be on relatively short branches compared to other gregarines. Perhaps targeting these short-branching lineages for high-throughput sequencing and genome projects will shed more light on the modifications that have taken place, at least at the molecular level, in gregarines. In the end, 18S rDNA is a good starting point for understanding the general phylogenetic position of a species compared to close relatives. However, a more robust framework will be needed to tease apart those lineages that are more distantly related.

### 7.3 Future directions

One of the future projects that is already underway is collecting protein coding genes, specifically, HSP90 and Actin (Figure 7.1). The goal of this work is to construct more in depth phylogenetic analyses of gregarines and other apicomplexans. Preliminary results from this project corroborate the phylogenies based on SSU rDNA. Nevertheless, it is clear that a more elaborate datasets that take into account a greater number of genes will be required to build a more complete picture of the evolutionary relationships between gregarines, and other apicomplexans groups. While conducting this project, I found I could successfully
use RT-PCR from RNA isolated from single cells, in order to amplify protein-coding genes. As molecular work is becoming more commonplace among microeukaryotes, these methods are going to be helpful in guiding the study of gregarines, and other protistan lineages. Still, there remains many lineages with an abnormal and poorly understood affiliation to what we currently know. Understanding these lineages might very well hold a key to comprehending the complex parasitism of apicomplexans and their evolution (Fig 7.2).
Figure 7.1 Phylogeny of gregarines inferred from HSP90, Actin and 18S rRNA. Maximum likelihood tree (-lnL = 4,342.24699) based on concatenated HSP90 and Actin amino acid sequences, as well as 18S SSU rDNA. Black dots on branches represent bootstrap and Bayesian support of 95 and 0.95, respectively. Bootstrap and Bayesian support lower than 55 and 0.95 were left off the tree. Some long branches were shortend according to the length of the substitutions/site scale bar (i.e., X4). The 14 new sequences generated for this analyses are in bold.
Figure 7.2 Interpretation of the relationships between gregarine lineages. Unrooted tree based on combined information from HSP90, Actin and 18S rRNA datasets. Dashed lines in the middle of the figure represent phylogenetic uncertainty at deeper nodes in the tree. Trophozoite stages at the tips of the branches are labeled a to zz and represent *Trichotokara eunicae* (a), *T. northriae* (b), *Paralecudina polymorpha* (c), *Lecudina tuzetae* (d), *Pterospora schizosoma* (e), *Veloxidium leptosynaptae* (f), *Cephaloidophora* sp. (g), undescribed lecudinid sp. (h), *Surculinium metchnikowi* (i), *Gregarina polymorpha* (j), *Gregarina*
blattarum (k), Monocystis agilis (l), Xiphocephalus sp. (m), Mattesia geminata (n), Prismatospora sp. (o), Hoplorhynchus sp. (p), Polypicularium lacrimae (q), P. curvarae (r), P. translucidae (s), P. citrusae (t), Selenidium terebellae (u), Melitzana thelepae (v), Selenidium orientale (w), S. pisinnus (x), S. neosabellariae (y), Platyproteum vivax (z) and Filipodium phascolosomae (zz).

Marine environmental sequences are indicated by the abbreviation "MES". Lineages conforming to an archigregarine morphotype are found at positions f, i, u, w, x and y. Letters j to p represent terrestrial and freshwater lineages. All other letters represent marine lineages.
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