

Molecular Phylogeny and Surface Morphology of Marine Archigregarines (Apicomplexa), *Selenidium* spp., *Filipodium phascolosomae* n. sp., and *Platyproteum* n. g. and comb. from North-Eastern Pacific Peanut Worms (Sipuncula)

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ABSTRACT. The trophozoites of two novel archigregarines, *Selenidium pisinnus* n. sp. and *Filipodium phascolosomae* n. sp., were described from the sipunculid *Phascolosoma agassizii*. The trophozoites of *S. pisinnus* n. sp. were relatively small (64–100 µm long and 9–25 µm wide), had rounded ends, and had about 21 epicytic folds per side. The trophozoites of *F. phascolosomae* n. sp. were highly irregular in shape and possessed hair-like surface projections. The trophozoites of this species were 85–142 µm long and 40–72 µm wide and possessed a distinct longitudinal ridge that extended from the mucron to the posterior end of the cell. In addition to the small subunit (SSU) rDNA sequences of these two species, we also characterized the surface morphology and SSU rDNA sequence of *Selenidium orientale*, isolated from the sipunculid *Themiste pyroides*. Molecular phylogenetic analyses demonstrated that *S. pisinnus* n. sp. and *S. orientale* formed a strongly supported clade within other *Selenidium* and archigregarine-like environmental sequences. *Filipodium phascolosomae* n. sp. formed the nearest sister lineage to the dynamic, tape-like gregarine *Selenidium vivax*. Overall, these data enabled us to reassess the molecular systematics of archigregarines within sipunculid hosts and make the following revisions: (1) *Filipodium* was transferred from the Lecudinidae (eugregarines) to the Selenidiidae (archigregarines), and (2) *Platyproteum* n. g. was established for *Platyproteum vivax* n. comb. (ex. *S. vivax*) in order to account for the highly divergent morphological features and better resolved phylogenetic position of this lineage.

Key Words. Alveolata, Archigregarinorida, parasite, *Phascolosoma*, Selenidiidae, taxonomy, *Themiste*.

GREGARINE apicomplexans are obligate unicellular parasites infecting the intestines, reproductive vesicles, and coelomic cavities of invertebrates living in terrestrial, freshwater, and marine habitats. Since the 1950s, gregarines have been separated into three major groups: eugregarines, archigregarines, and neogregarines (Grassé 1953; Leander 2008a; Levine 1971). Even though these groups are widely used in the scientific literature, the identity and composition of each group is vague because of our poor understanding about the actual diversity and phylogenetic relationships of gregarines. Some authors have emphasized the absence of asexual merogony as the main criterion for assigning species to eugregarines (Levine 1971); moreover, archigregarines and neogregarines were once grouped together as “schizogregarines” based on the presence of merogony (Perkins et al. 2002). Because it is often difficult to provide evidence for the absence or presence of merogony, the use of this criterion in the systematics of gregarines is questionable at best and highly misleading at worst (e.g. Gunderson and Small 1986; Leander, Harper, and Keeling 2003b; Levine 1971; Ray 1930). Accordingly, we treat *Selenidioides*, erected by Levine (1971) for *Selenidium* species reported to undergo merogony, as a junior synonym of *Selenidium*. Characteristics, such as habitat, host range, and morphological features of the trophozoites in gregarine life cycles, can be used in combination with small subunit (SSU) rDNA sequences to more precisely evaluate the systematics of gregarines.

Archigregarines have retained several ancestral features for gregarines and perhaps apicomplexans as a whole: they are found exclusively in marine habitats in a wide range of marine invertebrate hosts and possess extracellular trophozoites that resemble the general morphology of the infective sporozoites (Leander 2008a; Schrével 1971a, b). Moreover, the life cycle of archigregarines involves just a single host, and some archigregarine trophozoites use a myzocytosis-based mode of feeding that is homologous to that of predatory colpodellid flagellates, the sister group of apicomplexans

(Barta and Thompson 2006; Grassé 1953; Leander 2008a; Leander and Keeling 2003; Schrével 1968, 1971a, b).

Although the trophozoites of archigregarines resemble the spindle-shaped morphology and bending behaviour of the sporozoites, they are bigger in size and sometimes flattened (Dyson, Grahame, and Evannett 1993, 1994; Kuvardina and Simdyanov 2002; Leander 2006; Ray 1930; Schrével 1968, 1970, 1971a, b; Simdyanov and Kuvardina 2007). Ultrastructural studies of the trophozoite surface have also shown that archigregarines possess longitudinal epicytic folds, albeit much fewer (<60) than in eugregarines (e.g. Leander 2006, 2008a). Overall, the combination of characteristics in archigregarines and available molecular phylogenetic data suggest that they are a paraphyletic stem group from which all other gregarines, and perhaps apicomplexans as a whole, evolved (Cavalier-Smith and Chao 2004; Cox 1994; Grassé 1953; Leander 2006, 2007, 2008a; Leander and Keeling 2003; Leander et al. 2003b, 2006; Théodoridès 1984; Vivier and Desportes 1990).

Although most archigregarine species share the general features described above, some lineages have become highly divergent in morphology and behaviour. For instance, the archigregarine *Selenidium vivax*, which inhabits the intestines of sipunculids, has extremely flattened trophozoites that are capable of dynamic, peristalsis-like changes in cell shape that differ greatly from the usual bending and coiling movements found in other archigregarines (Gunderson and Small 1986; Leander 2006). To date, nine *Selenidium* species have been described from sipunculid hosts, and at least six of these display a high degree of trophozoite plasticity that is akin to that described most comprehensively in *S. vivax* (Gunderson and Small 1986; Leander 2006).

In this study, we describe two new species of archigregarines isolated from the sipunculid *Phascolosoma agassizii*, namely *Selenidium pisinnus* n. sp. and *Filipodium phascolosomae* n. sp. We also report three new SSU rDNA sequences: one from each of the two new species and one from the previously described archigregarine *Selenidium orientale*, isolated from the intestines of the sipunculid *Themiste pyroides*. Our molecular phylogenetic analyses of these new data enabled us to re-evaluate the systematics of archigregarines within sipunculid hosts and make several revisions, including (1) the transfer of *Filipodium* from the Lecudinidae to the Selenidiidae and (2) the establishment of *Platyproteum* n. g. for the highly divergent archigregarine *Platyproteum vivax* n. comb. (ex. *S. vivax*).

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MATERIALS AND METHODS

Collection and isolation of organisms. Three gregarine species were collected from two different sipunculid hosts. *Selenidium orientale* Bogolepova, 1953 was collected from the burrowing peanut worm *T. pyroides* Chamberlain, 1920 at low tide from the tidal pools at Whiffen Spit Point in Sooke (48°21'22"N, 123°43'24"W), Vancouver Island, Canada in May 2008. *Selenidium pisinnus* n. sp. and *F. phascolosomae* n. sp. were isolated from Agassiz's peanut worm *P. agassizii* Keferstein, 1867, from the rocky pools of Grappler Inlet (48°50'17"N, 125°08'02"W) in the vicinity of the Bamfield Marine Science Centre, Vancouver Island, Canada in 2006 and 2007.

Trophozoites were released in seawater by teasing apart the intestines of the respective host with pointed forceps under a dissecting microscope (Leica MZ6, Wetzlar, Germany). The gut material was examined under an inverted microscope (Zeiss Axiovert 200, Carl-Zeiss, Göttingen, Germany) and parasites were isolated by micromanipulation and washed three times in filtered seawater before being examined under the light microscope or prepared for DNA extraction.

Light and scanning electron microscopy. Differential interference contrast (DIC) light micrographs of *S. orientale* and *S. pisinnus* n. sp. were produced by securing parasites under a cover slip with Vaseline and viewing them with an imaging microscope (Zeiss Axioplan 2, Carl-Zeiss, Göttingen, Germany) connected to a colour digital camera (Leica DC500, Wetzlar, Germany). DIC light micrographs of *F. phascolosomae* n. sp. were produced using an inverted microscope (Zeiss Axiovert 200, Carl-Zeiss, Göttingen, Germany) connected to a Pixelink Megapixel colour digital camera (PL-A662-KIT, Ottawa, Canada).

Fifty, 15, and 16 specimens of *S. orientale*, *S. pisinnus* n. sp., and *F. phascolosomae* n. sp., respectively, were prepared for scanning electron microscopy (SEM). Individuals were deposited directly into the threaded hole of a Swinnex filter holder, containing a 5- μ m polycarbonate membrane filter (Millipore Corp., Billerica, MA), which was submerged in 10 ml of seawater within a small canister (2 cm diam. and 3.5 cm tall). A piece of Whatman No. 1 filter paper was mounted on the inside base of a beaker (4 cm diam. and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4% (w/v) OsO₄ and the beaker was turned over the canister. The parasites were fixed by OsO₄ vapours for 30 min. Ten drops of 4% (w/v) OsO₄ were added directly to the seawater and the parasites were fixed for an additional 30 min. A 10-ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with CO₂. Filters were mounted on stubs, sputter coated with 5 nm gold, and viewed under a scanning electron microscope (Hitachi S4700, 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).

DNA isolation, polymerase chain reaction (PCR), cloning, and sequencing. Twenty to 136 individual trophozoites were isolated from the dissected hosts, washed 3 times in filtered seawater, and deposited into a 1.5-ml microfuge tube. DNA was extracted by using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Small subunit rDNA sequences were PCR amplified using a total volume of 25 μ l and puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada).

The SSU rDNA sequences from all three species were amplified in one fragment using universal eukaryotic PCR primers F1 5'-GCGCTACCTGGTTGATCCTGCC-3' and R1 5'-GATCCTTCTGCAGGTTACCTAC-3' (Leander, Clopton, and Keeling 2003a) and internal primers designed to match existing eukaryotic SSU sequences F2 5'-TGCGCTACCTGGTTGATCC-3', F3

5'-CATGTCATGGTAGAGTTTCAGA-3', R2 5'-GCCTYCGCACCATACTCC-3', and R3 5'-CCTACCGTCTAAAGCTGATAGGT-3'. Polymerase chain reaction products corresponding to the expected size were gel isolated using the UltraClean™ 15 DNA Purification kit (MO Bio, Carlsbad, CA) and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Eight cloned plasmids were digested with *Eco*RI and screened for size. Two clones for each species were sequenced with ABI big dye reaction mix using vector primers and internal primers oriented in both directions. The new SSU rDNA sequences were initially identified by BLAST analysis (GenBank Accession numbers FJ832161, FJ832162, and FJ832163).

Molecular phylogenetic analysis. The new sequences were aligned with 50 alveolate SSU rDNA sequences using MacClade 4 (Maddison and Maddison 2000) and visual fine tuning; gaps and ambiguously aligned bases were excluded from the 53-taxon alignment resulting in 1,177 unambiguous sites. PhyML (Guindon and Gascuel 2003; Guindon et al. 2005) was used to analyse the dataset (one heuristic search) with maximum-likelihood (ML) using a general-time reversible (GTR) model of base substitutions (Posada and Crandall 1998) that incorporated invariable sites and a discrete gamma distribution with eight rate categories (GTR+I+ Γ model). The GTR model was selected using the program MrAIC 1.4.3 with PhyML (<http://www.abc.se/~nylander/mraic/mraic.html>), and model parameters were estimated from the original dataset ($\alpha = 0.422$, proportion of invariable sites = 0.064). Maximum-likelihood bootstrap analyses were conducted with the same settings described above (100 pseudoreplicates; one heuristic search per pseudoreplicate).

We also examined the 53-taxon data set twice with Bayesian analysis using the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The program was set to operate with GTR, a gamma-distribution, and four Monte Carlo Markov chains starting from a random tree (MCMC; default temperature = 0.2). A total of 2,000,000 generations was calculated with trees sampled every 50 generations and with a prior burn-in of 200,000 generations (2,000 sampled trees were discarded; burn-in was checked manually). A majority-rule consensus tree was constructed from 38,001 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

GenBank accession numbers. (AF494059) *Adelina bambaroniae*, (AJ415519) *Amoebophrya* sp. ex. *Prorocentrum micans*, (DQ462456) *Ascogregarina culicis*, (DQ462455) *Ascogregarina taiwanensis*, (AY603402) *Babesia bigemina*, (AY078092) *Colpodella pontica*, (AF330214) *Colpodella tetrahymenae*, (L19068) *Cryptosporidium baileyi*, (AF093489) *Cryptosporidium parvum*, (AF093502) *Cryptosporidium serpentis*, (AF39993) *Cytauxoon felis*, (U67121) *Eimeria tenella*, (AB191437, AF372779, AF372780, AF372786, AY179975, AY179976, AY179977, AY179988) Environmental sequences, (FJ832163) *Filipodium phascolosomae*, (AF129882) *Gregarina niphandrodes*, (FJ832159) Gregarine from *Paranemertes peregrina*, (FJ832156) Gregarine from *Phyllochaetopterus prolifica*, (FJ832160) Gregarine from *Tubulanus polymorpha*, (AF022194) *Gymnodinium fuscum*, (AF286023) *Hematodinium* sp., (AF130361) *Hepatozoon catesbianae*, (DQ093796) *Lankesteria abbotti*, (EU670240) *Lankesteria chelyosomae*, (EU670241) *Lankesteria cystodytae*, (AF080611) *Lankesterella minima*, (FJ832157) *Lecudina longissima*, (AF457128) *Lecudina tuzetae*, (AF457130) *Leidyana migrator*, (DQ093795) *Lithocystis* sp., (AB000912) Marine parasite from *Tridacna crocea*, (AY334568) *Mattesia geminata*, (AF457127) *Monocystis agilis*, (AJ271354) *Neospora caninum*, (AF129883) *Ophryocystis elektroscirrha*, (AY196708) *Platyproteum vivax* ex. *Selenidium vivax*, (DQ093794) *Pterospira floridiensis*, (DQ093793) *Pterospira schizosoma*, (DQ273988)

Rhytidocystis polygordiae, (M64244) *Sarcocystis muris*, (FJ832161) *Selenidium orientale*, (FJ832162) *Selenidium pisinnus*, (DQ683562) *Selenidium serpulae*, (AY196709) *Selenidium terebellae*, (DQ176427) *Syncystis mirabilis*, (AF013418) *Theileria parva*, (M97703) *Toxoplasma gondii*.

RESULTS

***Selenidium orientale*.** Trophozoites were isolated from the sipunculid *T. pyroides* and conformed in morphology to the original description of *S. orientale* from *Phascolosoma japonicum* (Bogolepova 1953). The cells were compressed (Fig. 5) with a very narrowly fusiform to very narrowly lomentiform cell shape [length = 181 (120–300, SD = 50.8) μm , width = 26 (15–40, SD = 6.5) μm at the widest part of the cell, $n = 11$] (terms after Clopton 2004). All trophozoites were brownish in colour under the light microscope, reflecting an accumulation of amylopectin granules within the cytoplasm. The anterior and posterior ends were both pointed; the mucron was free of amylopectin granules and was more slender compared with the posterior end (Fig. 1–5). Some trophozoites showed a bulge-like widening near the anterior part of the trophozoite (Fig. 3, 6). The ellipsoidal nucleus [10 (8–15, SD = 2.9) \times 18 (10–25, SD = 4.2) μm , $n = 11$] was situated in the middle of the trophozoite (Fig. 1–4). Some trophozoites had lateral depressions at the level of the nucleus (Fig. 4). Nine to 10 longitudinal epicytic folds (Fig. 5, 7) were present on each flattened side of the trophozoites, and the tips of the cells were free of folds (Fig. 6). Some of the folds were slightly concave along the mid-longitudinal axis (Fig. 6). The cells were capable of bending and twisting movements, comparable to those made by some nematodes.

***Selenidium pisinnus* n. sp.** Trophozoites isolated from the intestines of *P. agassizii* were very narrowly oblong to very narrowly elliptoid. Trophozoites were 64–100 μm long (mean length = 78 μm , SD = 11.8 μm , $n = 11$) and 9–25 μm wide (mean width = 16 μm , SD = 5.4 μm , $n = 11$). The mucron was rounded and free of amylopectin, while the posterior end of the cell was more pointed (Fig. 8–10, 12). The ellipsoidal nucleus [5 (SD = 0.5) \times 11 (SD = 0.4) μm , $n = 5$] was situated in the anterior half of the trophozoite (Fig. 8, 9). The trophozoite surface was sulcate due to around 21 epicytic folds per side (range = 20–22; $n = 17$) (Fig. 10–12). Some of the cells prepared for SEM were covered in the host's sperm (Fig. 12), and others had bleb-like material sticking to their surfaces (Fig. 11). The trophozoites were able to bend and twist.

***Filipodium phascolosomae* n. sp.** The trophozoites of this species were relatively less abundant within the intestines of *P. agassizii*. The extracellular trophozoites were very active and capable of cellular deformations. They were often crudely triangular in shape, with one short (anterior end) side and two longer sides (Fig. 13, 17); the trophozoites were sometimes rounded up into an elliptoid shape (Fig. 18–20). Trophozoites were about 114 (85–142, SD = 16.5) μm long and 50 (40–72, SD = 10.2) μm wide ($n = 9$). An edge-like anterior end measured up to 72 μm in width when expanded (AE, Fig. 17), but was very plastic in shape and size. In some cells, the anterior mucron was more pointed in shape (Fig. 14, 16, 18). The spherical nucleus was large [23.6 (20–30, SD = 3.7) μm diam., $n = 5$] and situated near the anterior end of the cell (Fig. 13–16). A distinct

longitudinal ridge was observed on all cells under the SEM and could also be seen under the LM. The longitudinal ridge was oriented on the ventral side and extended from the posterior end of the cell to the mucron (Fig. 17, 18, 20). The bending and twisting movements were mostly in the dorsoventral direction. The entire trophozoite surface was covered with numerous hair-like projections about 1.6 μm long by 0.16 μm wide (Fig. 14, 17–19, 21, 22). Because of the density of the hair-like projections, it was impossible to observe the underlying surface structure of the trophozoites (e.g. the possible presence of longitudinal epicytic folds) (Fig. 21, 22).

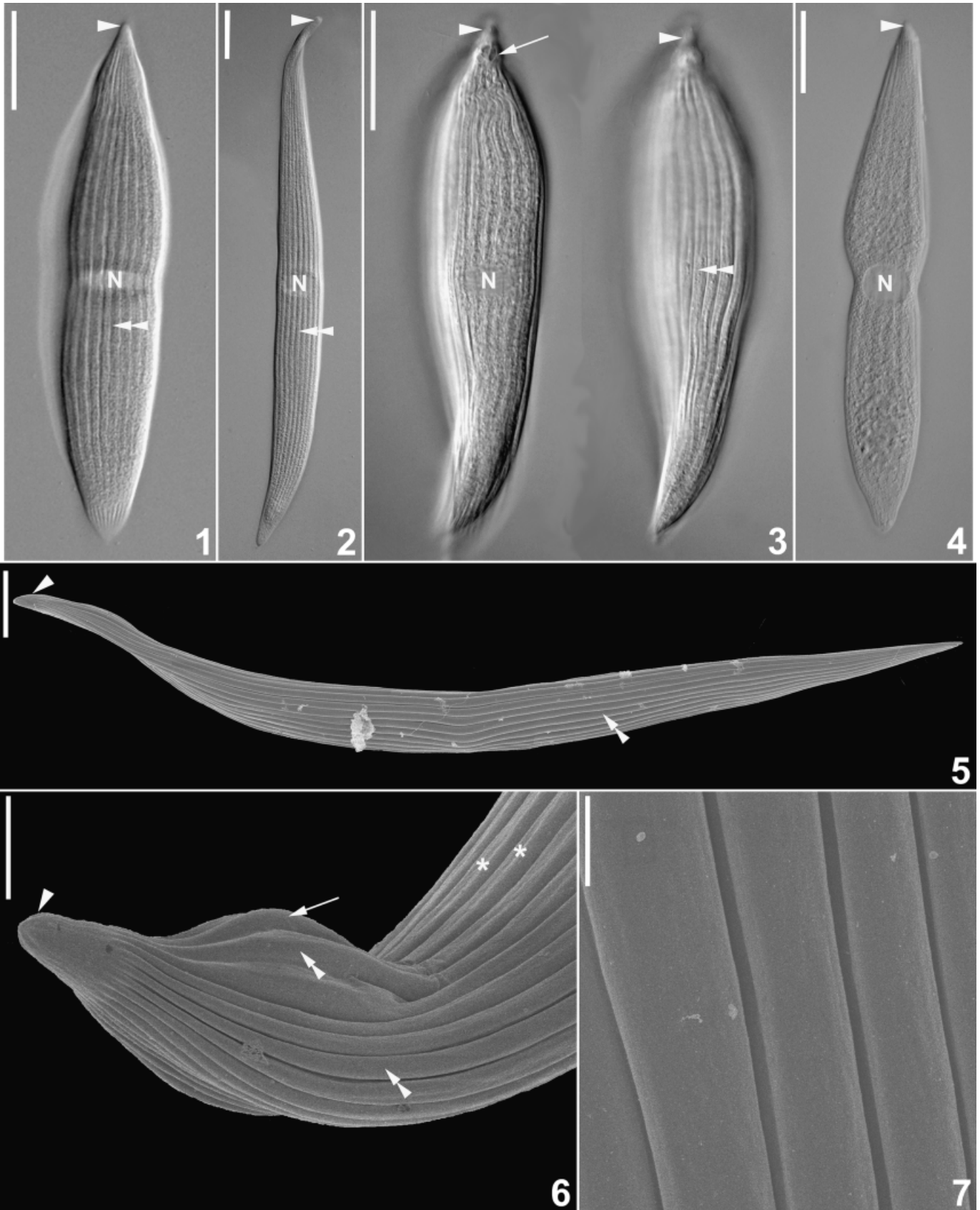
Molecular phylogenetic analyses. Phylogenetic analyses of the 53-taxon data set resulted in a backbone that gave rise to a strongly supported clade of dinoflagellates (outgroup), a moderately supported clade of colpodellid lineages, and a weakly supported clade of apicomplexans. The apicomplexan backbone gave rise to three moderately supported clades: (1) a group consisting of coccidians and piroplasmids, (2) a marine group consisting of rhytidocystids, and (3) a terrestrial group consisting of cryptosporidians, neogregarines, and monocystid eugregarines (Fig. 23). The sequences from (marine) archigregarines, including several environmental sequences, diverged as a paraphyletic assemblage that gave rise to a moderately supported clade consisting of septate eugregarines and marine eugregarines (Bayesian posterior probability = 0.99, ML bootstrap percentage = 78) (Fig. 23). Two strongly supported clades branched within the archigregarine assemblage: (1) a group consisting of *S. orientale* and *S. pisinnus* n. sp. and (2) a group consisting of *P. vivax* n. g. and comb. (ex. *S. vivax*) and *F. phascolosomae* n. sp. *Selenidium serpulae* formed the nearest sister lineage to the clade consisting of *P. vivax* n. g. and comb. and *F. phascolosomae* n. sp., albeit with weak statistical support.

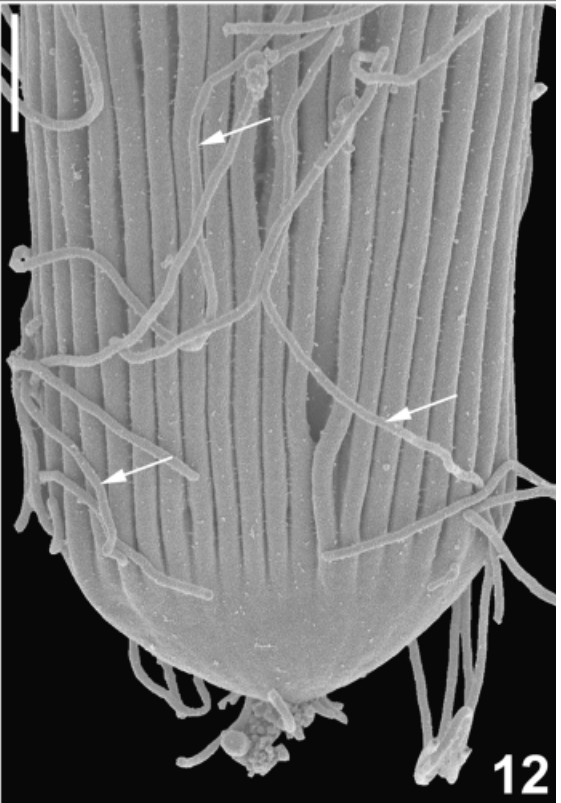
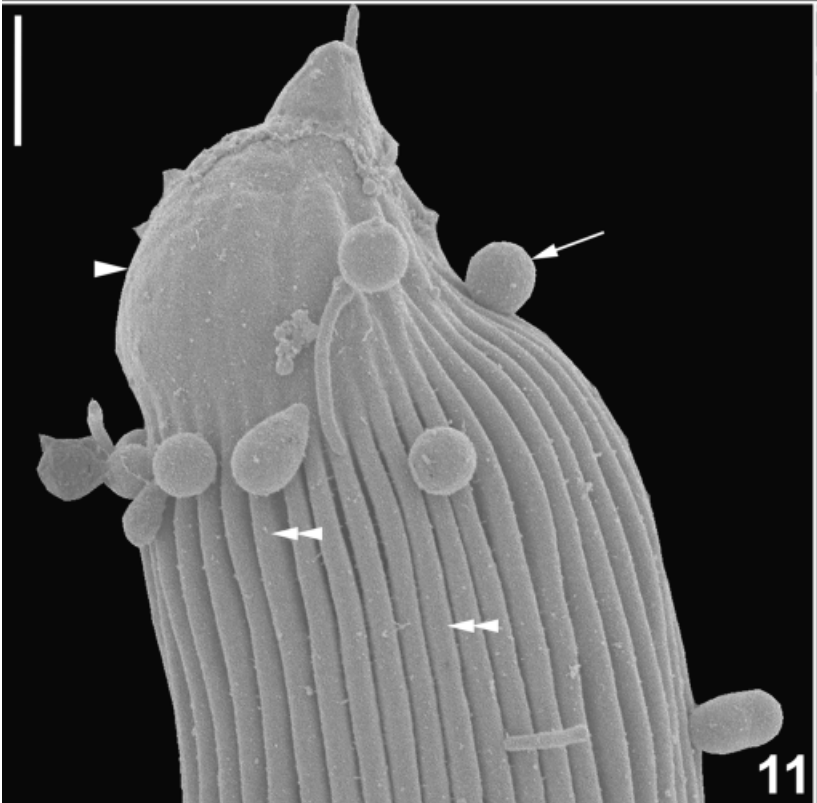
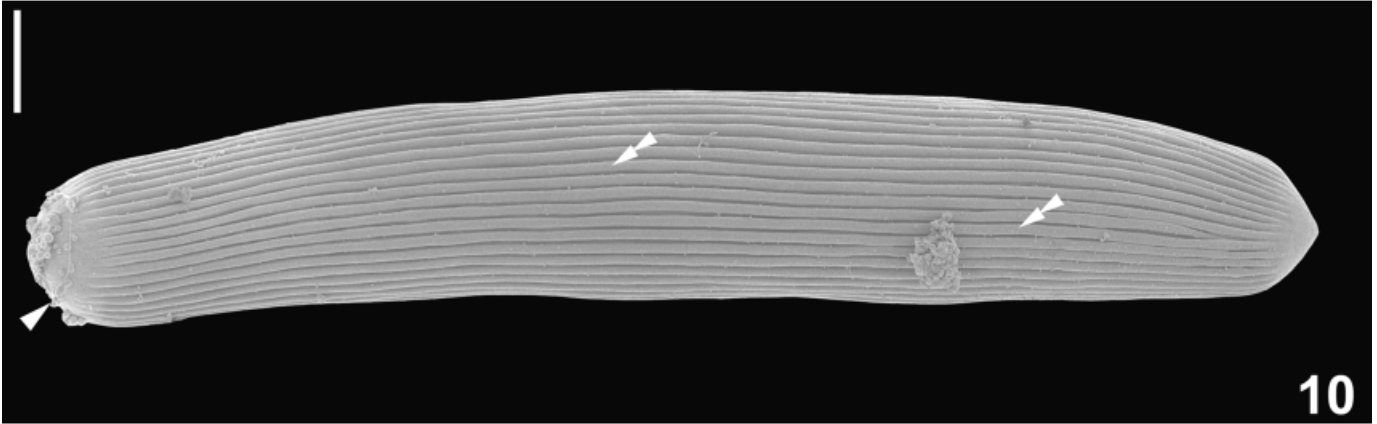
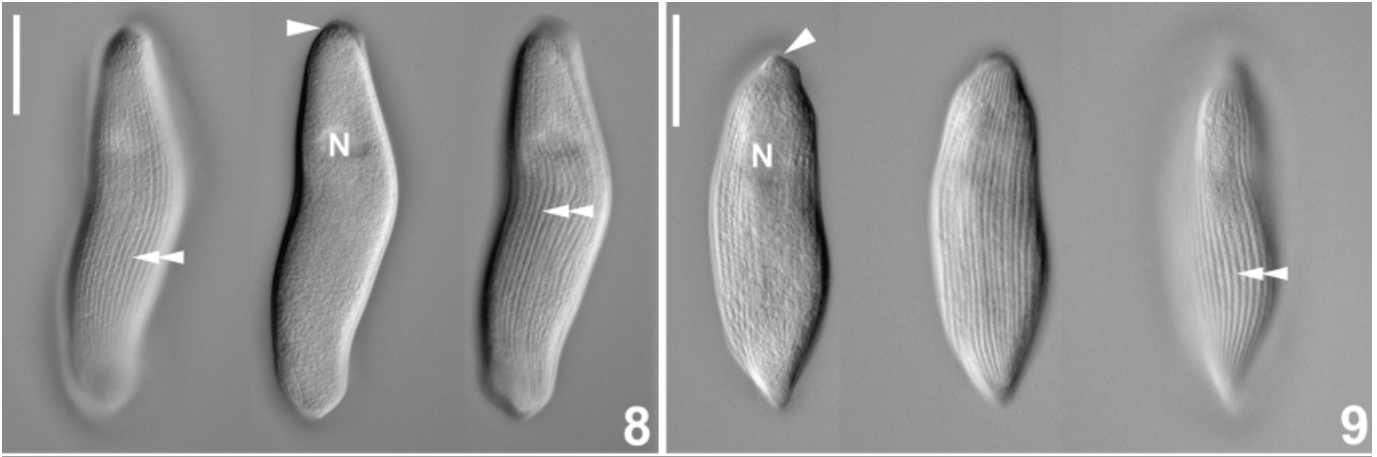
DISCUSSION

The Archigregarinorida is a tenuously defined group containing just eight described genera that fall within either the Selenidiidae or the Exoschizonidae. There are approximately 65 described species, and most of them fall within the genus *Selenidium* (Leander 2007; Schrével 1971a, b). To date, ultrastructural data are available for 13 archigregarine species (Dyson et al. 1993, 1994; Kuvardina and Simdyanov 2002; Leander 2006, 2007; Leander et al. 2003b, Macgregor and Thomasson 1965; Schrével 1968, 1971a, b; Simdyanov 1992; Vivier and Schrével 1964, 1966), and molecular sequence data are only available for three species (Leander 2007; Leander et al. 2003b). Although several archigregarines (e.g. *S. terebellae*) have retained a large number of ancestral features, such as their restriction to marine habitats, monoxenous life cycles and extracellular feeding stages that resemble the morphology of infective sporozoite stages, the highly derived trophozoites in some lineages (*S. vivax*) demonstrate a more extensive diversification of archigregarines than is usually appreciated (Leander 2008a).

***Selenidium*.** The genus *Selenidium* contains 56 species, most of them occurring in polychaetes (compare Levine 1971). The type species is *S. pendula* Giard, 1848 from the polychaete *Nerine cirratulus* (Delle Chiaje, 1831). The main characteristic of the genus that sets *Selenidium* apart from Lecudinidae and Urospor-

Fig. 1–7. Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of the gregarine *Selenidium orientale*. 1–4. DIC images of trophozoites showing different cell shapes. The nucleus (N) is oval (Fig. 1) to round (Fig. 4). The mucron area (arrowhead) is free of amylopectin and no epicytic folds are visible in that area or at the posterior end. The rest of the cell is covered by longitudinal epicytic folds (double arrowheads). Some cells show a bulge-like widening near the mucron (arrow, Fig. 3). 5. SEM of an elongated trophozoite with a pointed mucron (arrowhead). The cell is dorso-ventrally compressed and possesses 9–10 longitudinal epicytic folds (double arrowheads) per flattened side. 6. SEM showing the anterior end of a trophozoite with a mucron (arrowhead) free of epicytic folds and a bulge like widening (arrow) on the dorsal side. The single epicytic folds (double arrowhead) are still distinguishable but expanded. The asterisks indicate slightly concave shaped epicytic folds. 7. High-magnification SEM of epicytic folds. Scale bars: Fig. 1, 4 = 27 μm ; Fig. 2, 3 = 30 μm ; Fig. 5 = 13 μm ; Fig. 6 = 5 μm ; Fig. 7 = 2 μm .





idae is the pendulum-like motility of the trophozoites (Schrével 1970). The spindle-shaped trophozoites of *Selenidium* have only a few epicytic folds (<60) and are able to actively bend, coil, and twist in a manner that is reminiscent of nematodes. Ultrastructural studies have shown that archigregarine trophozoites possess a robust trilayered inner-membrane system subtended by a sheet(s) of longitudinal microtubules; each microtubule is surrounded by an electron-transparent sheath or sleeve (Leander 2007; Schrével 1971a; Stebbings, Boe, and Garlick 1974; Vivier and Schrével 1964). This stands in contrast to intestinal eugregarines (e.g. *Lecudina*), which possess hundreds of epicytic folds resulting in increased cell rigidity and a limited ability of cellular deformation (Leander 2008a). The trophozoites of eugregarines lack layers of parallel microtubules beneath the inner membrane complex and instead utilize an actinomyosin-based system that enables the trophozoites to glide along substrates (Heintzelmann 2004; Mellor and Stebbings 1980; Walker et al. 1979).

Thirteen gregarine species have been described from the intestines of sipunculids before this study, nine of which fall within *Selenidium*. *Selenidium pisinnus* n. sp., isolated from *P. agassizii*, has relatively small trophozoites. With a mean length of 78 µm, a mean width of 16 µm and around 42 epicytic folds the newly described species differs from all *Selenidium* species found in sipunculids so far. *Phascolosoma agassizii*, however, is also parasitized by two other gregarines with much larger and more dynamic trophozoites, namely *S. vivax* and *F. phascolosomae* n. sp. (see Table 1). Before this study, there was the formal possibility that the small cells representing *S. pisinnus* n. sp. were actually early developmental stages of one of these two larger species, rather than the trophozoites of a different species altogether. However, our phylogenetic analyses of the new SSU rDNA sequences demonstrated the validity of *S. pisinnus* n. sp. and that the sequence from this species clusters strongly with the newly obtained sequence from *S. orientale*, isolated from the sipunculid *T. pyroides*, rather than the two other species from *P. agassizii*.

A comparison of the morphology and behaviour of gregarine species within sipunculid hosts indicates that six of the now 10 described species of *Selenidium* display a relatively higher degree of cell motility than the bending and coiling movements found in the *Selenidium* type species *S. pendula*. For instance, *S. vivax* contains extremely large and flattened trophozoites capable of periodic cell deformations that are perhaps best described as peristalsis-like motility (e.g. Gunderson and Small 1986; Leander 2006). These features are significantly different from the spindle-shaped trophozoites of the type species *S. pendula* as well as *S. pisinnus* n. sp., *S. orientale*, and several other *Selenidium* species that inhabit polychaete hosts (e.g. *S. serpulae* and *S. terebellae*). These morphological differences are also consistent with our molecular phylogenetic analyses: *S. vivax* branched separately from the clade consisting of *S. pisinnus* n. sp. and *S. orientale* and instead clustered strongly with *F. phascolosomae* n. sp. The clade consisting of *S. vivax* and *F. phascolosomae* n. sp. was most closely related to *S. serpulae* from northeastern Pacific calcareous tubeworms, albeit with only modest statistical support. Accordingly, future studies will be required to determine whether other described species of *Selenidium* within sipunculid hosts (i.e. *S. cantoui*, *S. franciana*, *S. stellatum* and *S. sipunculi*) will cluster most

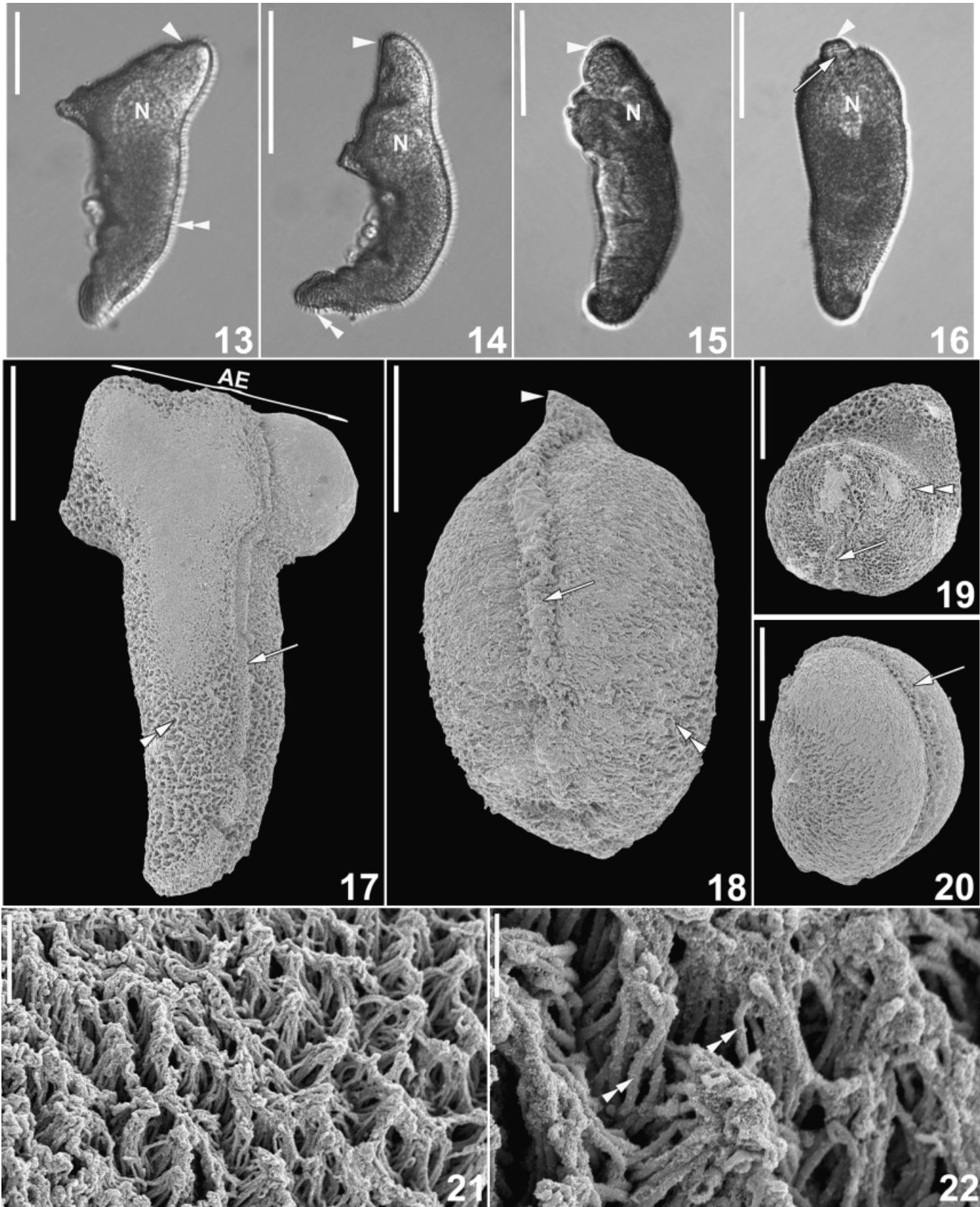
closely with the clade consisting of *S. pisinnus* n. sp. and *S. orientale* or the clade consisting of *F. phascolosomae* n. sp. and *S. vivax*.

Platyproteum n. g. Trophozoites of *S. vivax* are large (120–500 µm long) (Leander 2006) compared with the trophozoites of the *Selenidium* type species *S. pendula*, *S. orientale*, *S. pisinnus* n. sp., and the three *Filipodium* species (Table 1). *Selenidium vivax* is not spindle-shaped or ellipsoid, but extremely flattened and therefore, tape-like (Gunderson and Small 1986; Leander 2006, 2007). The pendulum-like motility, which is characteristic for the genus *Selenidium*, is very different from the dynamic peristaltic motility found in *S. vivax*; the trophozoites of this species are able to fold, twist, stretch, and contract different parts of the cell (compare Gunderson and Small 1986; Leander 2006, 2007). The longitudinal folds differ from those of *S. pendula* (type species) and other *Selenidium* species as they are subtle and transient, depending on the stage of cellular deformation (Gunderson and Small 1986; Leander 2006, 2007; Leander et al. 2003b). *Selenidium vivax* also possesses narrow, but prominent transverse striations that are derived from organized folds in the plasma membrane and the underlying inner membrane complex (Leander 2006). Transverse striations like those in *S. vivax* are also known in other described species of archigregarines (Dyson et al. 1993; Leander 2006, 2007; MacKinnon and Ray 1933; Ray 1930; Schrével 1970).

The close phylogenetic relationship between *S. vivax* and *F. phascolosomae* n. sp. is congruent with distinctive morphological features shared by both lineages, such as an edge-like mucron and a dynamic pattern of cellular deformations (Table 1). Accordingly, the highly divergent morphological features of *S. vivax* combined with its better-resolved phylogenetic position with *Filipodium* prompted us to establish a new genus name for this lineage within the Selenidiidae, namely *Platyproteum* n. g. Moreover, the sister relationship between *S. serpulae* and the clade consisting of *P. vivax* n. g. and n. comb. and *F. phascolosomae* n. sp. suggests that the highly derived trophozoites of the latter clade evolved from an ancestor with spindle-shaped trophozoites comparable to that shared by *Selenidium* species in general (e.g. *S. terebellae*, *S. serpulae*, *S. pisinnus* n. sp., and *S. orientale*) (Leander 2007, 2008a).

Filipodium. Until this study, there were only two *Filipodium* species described: *Filipodium ozakii* Hukui, 1939, the type species, and *Filipodium aspidosiphoni* Tuzet and Ormières, 1965. Both species occur in sipunculids; *F. ozakii* was described from *Siphonosoma cumanense* (Keferstein, 1867) in Japan (Hoshide and Todd 1996; Hukui 1939) and *F. aspidosiphoni* from *Aspidosiphon clavatus* Diesing, 1851 in France (Tuzet and Ormières 1965). One of the unifying features of *Filipodium* species is the presence of hair-like projections that cover the entire cell, which gives them a very distinctive appearance (Table 1). Trophozoites of *F. ozakii* are either elongated, cylindrical, or flattened and around 350 µm long and 100 µm wide, with a light yellowish-brown colour. The trophozoites of this species also have a prominent irregular mucron, a lens-shaped nucleus in the middle of the cell, and a series of longitudinal epicytic folds beneath the hair-like projections (compare Hoshide and Todd 1992, 1996; Hukui 1939). Hukui (1939) described the hair-like projections as protoplasmic projections capable of being drawn in and out. This

Fig. 8–12. Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of the gregarine *Selenidium pisinnus* n. sp. 8–9. DIC images showing trophozoites in different focal planes. The anterior mucron (arrowhead) is more rounded than the posterior end. An oval to spherical nucleus (N) is situated in the anterior half of the cell. The trophozoites are covered with longitudinal epicytic folds (double arrowheads). 10. SEM of a trophozoite showing that the longitudinal epicytic folds (double arrowheads) terminate before reaching the tips of the cell; the mucron (arrowhead) is more rounded than the posterior end. 11. High-magnification SEM of the mucron (arrowhead) showing 21 epicytic folds (double arrowheads) per side and material excreted from the cell (arrow). 12. High-magnification SEM of the posterior end of the trophozoite showing the presence of host sperm cells (arrows). Scale bars: Fig. 8, 9 = 25 µm; Fig. 10 = 7 µm; Fig. 11 = 2 µm; Fig. 12 = 2 µm.



behaviour was not observed by Hoshide and Todd (1996) or in the present study. The mucron in *Filipodium* species is edge-like and lobular and 2–3 times wider than the rest of the cell (Hoshide and Todd 1996). The lobules apparently function in pinocytosis and play a role in maintaining the position of *F. ozakii* in the hosts' intestine (Hoshide and Todd 1996).

The trophozoites of the new species described here from the sipunculid *P. agassizii*, namely *F. phascolosomae* n. sp., also possess hair-like projections and were comparable in general cell shape to the two previously described species of *Filipodium* (Table 1). The trophozoites of *F. phascolosomae* n. sp. are smaller than those of the type species *F. ozakii*, but bigger than those of *F. aspidosiphoni*. The crude triangular shape of the trophozoites in *F. phascolosomae* n. sp. differs from that of the type species but is very similar to that of *F. aspidosiphoni* (Table 1). All three species were isolated from different hosts and different localities (Table 1). The best synapomorphy for *Filipodium* is the conspicuous hair-like projections that cover the entire trophozoite cell. Although the hair-like projections resemble cilia or flagella, they are stiff, non-motile, and lack a 9+2 configuration of microtubules (Hoshide and Todd 1996). The functional significance of the hair-like projections is unclear; however, they presumably increase surface area for surface-mediated nutrition in a manner that is comparable to the microtrichs and thecal barbs of cestodes and haplozoon dinoflagellates, respectively (Leander 2008b; Rueckert and Leander 2008). Nonetheless, *F. phascolosomae* n. sp. is a rarely encountered gregarine; only about one out of 10 examined hosts was infected, and no more than five cells could be isolated from a single host organism. A relatively rare occurrence was also reported for *F. aspidosiphoni* (Tuzet and Ormières 1965).

The genus *Filipodium* was originally placed within the eugregarines and the family Dactylophoridae by Hukui (1939). Grassé (1953) changed the assignment to the Lecudinidae because *Filipodium* lacks a transverse septum (Levine 1977). Based on the general ultrastructure of the cell cortex, Hoshide and Todd (1996) suggested that *Filipodium* might have a closer affinity with *Selenidium* species. Simdyanov (2007) subsequently removed *Filipodium* from the family Lecudinidae and considered the lineage as gregarine of uncertain phylogenetic position. Our molecular phylogenetic analyses of SSU rDNA sequences reinforce the interpretations made by Hoshide and Todd (1996) and Simdyanov (2007), and indicate that the taxonomic position of *Filipodium* should be revised. Because the sequence from *F. phascolosomae* n. sp. clustered strongly with *P. vivax* n. g., forming the sister clade to *S. serpuluae*, we have transferred *Filipodium* and its three described species from sipunculid hosts to the archigregarine family Selenidiidae.

TAXONOMIC SUMMARY

Phylum Apicomplexa Levine, 1970
 Class Conoidasida Levine, 1988
 Subclass Gregarinasina Dufour, 1828
 Order Archigregarinorida Grassé, 1953
 Family Selenidiidae Brasil, 1907

Genus *Selenidium* Giard, 1884

Selenidium pisinnus n. sp.

Diagnosis. Trophozoites are very narrowly oblong to very narrowly elliptoid and 64–100 µm long (mean length 78 µm) and 9–25 µm wide (mean width 16 µm). They appear brownish under the light microscope due to amylopectin granules in the cytoplasm. The anterior end bearing the amylopectin free mucron is rounded, while the posterior tip of the cell is more pointed. The nucleus is ellipsoidal (5 µm × 11 µm) and situated in the anterior half of the trophozoite. The trophozoite surface possesses 21 epicytic folds per side (20–22). Trophozoites are able to bend and twist.

Gene sequence. A sequence of the 18S rDNA is deposited as GenBank accession No. FJ832162.

Type locality. Grappler Inlet near Bamfield Marine Sciences Centre, Vancouver Island, Canada (48°50'17"N, 125°08'02"W).

Type habitat. Marine.

Type host. *Phascolosoma agassizii* Keferstein, 1866 (Metazoa, Sipuncula, Sipunculida, Phascolosomatidae).

Location in host. Intestinal lumen.

Holotype. Fig. 10. 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) at the University of British Columbia, Vancouver, Canada.

Paratype. Fig. 8, 9.

Etymology. The species name *pisinnus* (Greek) means very little, which refers to its relatively small size.

Genus *Filipodium* Giard, 1884

Filipodium phascolosomae n. sp.

Diagnosis. Trophozoites are very active, plastic, and capable of cellular deformations. They are often triangular shaped with one short (anterior end) and two longer sides; sometimes rounded up to an elliptoid shape. They are about 114 (85–142) µm long and 50 (40–72) µm wide. The anterior end can be up to 72 µm wide when expanded and showed a wide range of plasticity. Most cells possess a pointed mucron regardless of the cell shape. A spherical nucleus (23.6 (20–30) µm in diam.) is situated near the anterior end. A distinct longitudinal ridge extends on the ventral side from the posterior end of the trophozoite to the mucron. Bending and twisting movements are in the ventral–dorsal plane. The whole trophozoite is covered with numerous hair-like projections (about 1.6 µm long and 0.16 µm wide).

Gene sequence. A sequence of the 18S rDNA is deposited as GenBank accession No. FJ832163.

Type locality. Grappler Inlet near Bamfield Marine Sciences Centre, Vancouver Island, Canada (48°50'17"N, 125°08'02"W).

Type habitat. Marine.

Type host. *Phascolosoma agassizii* Keferstein, 1866 (Metazoa, Sipuncula, Sipunculida, Phascolosomatidae).

Location in host. Intestinal lumen.

Holotype. Fig. 17. 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) at the University of British Columbia, Vancouver, Canada.

Fig. 13–22. Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of the gregarine *Filipodium phascolosomae* n. sp. 13–16. DIC micrographs showing the variable shapes of the trophozoites. The mucron (arrowhead) can be rounded (Fig. 16) or expanded as a linear edge (Fig. 13). The big spherical nucleus (N) is situated at the anterior end of the cell. Numerous hair-like projections (double arrowheads) were visible on the surface of the trophozoites. 17. SEM of a trophozoite with an expanded anterior end (AE). A distinct ridge (arrow) runs longitudinally along the trophozoite from the mucron to the posterior end. The entire surface is covered in hair-like projections. 18–20. SEMs showing trophozoites with an elliptoid cell shape, a pointed mucron (arrowhead), hair-like projections (double arrowheads), and a prominent longitudinal ridge (arrow). 21–22. High-magnification SEM showing the hair-like projections (double arrowheads) covering the surface of the trophozoites. Scale bars: Fig. 13 = 30 µm; Fig. 14 = 52 µm; Fig. 15 = 40 µm; Fig. 16, 17 = 43 µm; Fig. 18 = 31 µm; Fig. 19 = 47 µm; Fig. 20 = 32 µm; Fig. 21 = 4 µm; Fig. 22 = 1 µm.

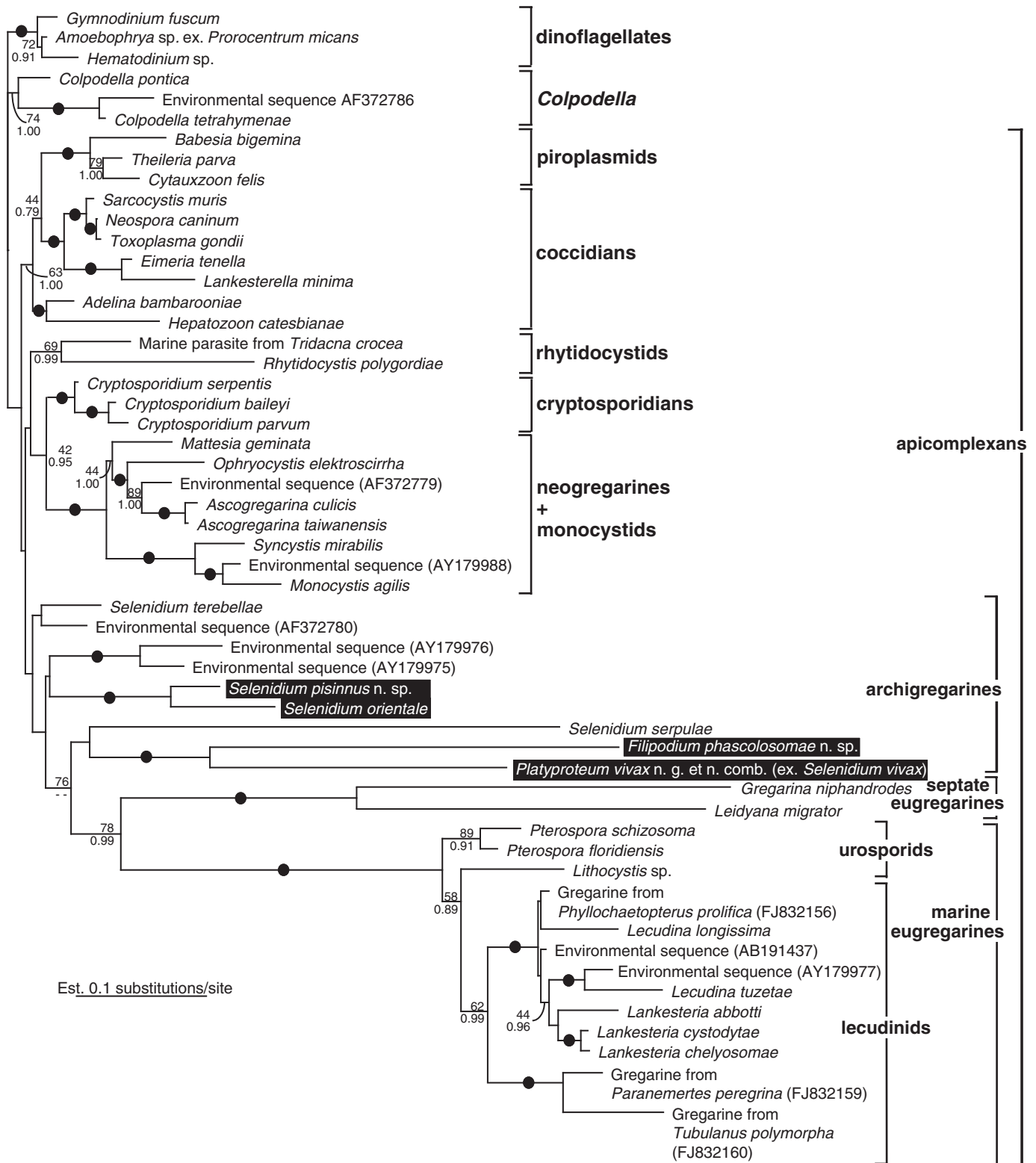


Fig. 23. Maximum likelihood (ML) tree of apicomplexans inferred using the GTR+I+ Γ model of substitution on an alignment of 53 small subunit (SSU) rDNA sequences and 1,177 unambiguously aligned sites ($-\ln L = 15594.52859$, $\alpha = 0.422$, proportion of invariable sites = 0.064, eight rate categories). Numbers at the branches denote ML bootstrap percentage (top) and Bayesian posterior probabilities (bottom); values provided when at least one is higher than 65% (i.e. the absence of values reflect statistical support below 65%). Black dots on branches denote Bayesian posterior probabilities and bootstrap percentages of 90% or higher. The three sequences derived from this study are highlighted with black boxes; the sequence from *Platypoteum vivax* n. g. and comb. is also highlighted to facilitate the taxonomic discussion.

Table 1. Morphological comparison of *Selenidium pendula* (type species) and relevant archigregarines described from sipunculid hosts: *S. orientale*, *S. pisinnus* n. sp., *Platyproteum vivax* n. g. and comb. (ex. *S. vivax*), *Filipodium phascolosomae* n. sp., *F. ozakii* and *F. aspidosiphoni*.

	<i>S. pendula</i> (type species)		<i>S. orientale</i>		<i>S. pisinnus</i> n. sp.		<i>P. vivax</i> n. g. and comb.		<i>F. phascolosomae</i> n. sp.		<i>F. ozakii</i> (type species)		<i>F. aspidosiphoni</i>	
Host	<i>Nerine cirratulus</i>		<i>Themiste pyroides</i>		<i>Phascosoma agassizii</i>		<i>Phascosoma agassizii</i>		<i>Phascosoma agassizii</i>		<i>Siphonosoma cumanense</i>		<i>Aspidosiphon clavatus</i>	
Host tissue	Intestine		Intestine		Intestine		Intestine		Intestine		Intestine		Intestine	
Locality	E. Atlantic		E. and W. Pacific		E. Pacific		E. Pacific		E. Pacific		W. Pacific		Mediterranean	
Trophozoites	Spindle-shaped		Spindle-shaped, flattened		Oblong to elliptoid		Tape-like		Triangular to elliptoid		Cylindrical & flattened		Triangular	
Size (<i>L</i> × <i>W</i> , μm)	180 × 30–40		120–300 × 15–40		64–100 × 9–25		120–500 × 15–80		85–142 × 40–72		350 × 30–105		60	
Nucleus	Round to oval		Ellipsoidal		Ellipsoidal		Round to oval		Spherical		Lens-shaped		Oval	
Shape	18–33 × 13–32		8–15 × 10–25		5 × 11		7–36		20–30					
Size (<i>L</i> × <i>W</i> or $\bar{\phi}$, μm)	Middle		Middle		Anterior half		Middle		Anterior		Middle		Anterior	
Position	Bending, twisting, pendulum-like		Bending, twisting, pendulum-like		Bending, twisting, pendulum-like		Folding, twisting, peristalsis		Bending, twisting, stretching		Bending		“amoeboid”	
Motility	Yes		Yes		Yes		No		Unknown		Yes		Unknown	
Longitudinal epicytic folds	20–30		18–20		40–44		0		Unknown		Many		Unknown	
Total number	Unknown		No		No		Yes		Unknown		No		Unknown	
Transverse surface folds	No		No		No		No		Yes		Yes (retractable?)		Yes	
Hair-like projections	Pointed		Pointed		Pointed		Edge-like		Edge-like		Edge-like		Edge-like	
Shape of mucron	Levine (1971); Schrével (1970)		Present study; Simdyanov and Kuvardina (2007)		Present study		Gunderson and Small (1986); Leander (2006)		Present study		Hoshida and Todd (1992, 1996); Hukui (1939)		Tuzet and Ormieres (1965)	

Paratype. Fig. 13, 14, 18–20.

Etiology. Named after the type host in which this species was found.

Phylum Apicomplexa Levine, 1970
Class Conoidasida Levine, 1988
Subclass Gregarinasina Dufour, 1828
Order Archigregarinorida Grassé, 1953
Family Selenidiidae Brasil, 1907

Genus *Platyproteum* n. g.

Diagnosis. Trophozoites tape-like with fine transverse striations; without epicytic folds; with dynamic bending and peristalsis-like changes in cell shape; sporozoites and oocysts unknown; in the intestines of sipunculids.

Type species. *Platyproteum vivax* n. comb. (basionym *S. vivax* Gunderson & Small, 1986)

Etiology. The generic name is a composite of the root names *platy* (Greek), which means flat and the name *proteus* (Greek), within the neuter form *proteum*, a sea god in Greek mythology who was able to change his shape at will. The gender of the name is neuter. The name refers to flattened trophozoites that show a high degree of cell plasticity.

Remarks. The new genus is established for the formerly described gregarine *S. vivax* Gunderson & Small, 1986 found in the intestine of *P. agassizii*. The phylogenetic relationship of this lineage to *Filipodium* as inferred from SSU rDNA and the distinctiveness of the cell shape and behaviour justify the establishment of the new genus.

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