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ORIGINAL ARTICLE

Molecular Phylogeny and Ultrastructure of *Caliculium glossobalani* n. gen. et sp. (Apicomplexa) from a Pacific *Glossobalanus minutus* (Hemichordata) Confounds the Relationships Between Marine and Terrestrial Gregarines

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ABSTRACT

Gregarines are a diverse group of apicomplexan parasites with a conspicuous extracellular feeding stage, called a "trophozoite", that infects the intestines and other body cavities of invertebrate hosts. Although the morphology of trophozoites is very diverse in gregarines as a whole, high degrees of intraspecific variation combined with relatively low degrees of interspecific variation make the delimitation of different species based on trophozoite morphology observed with light microscopy difficult. The coupling of molecular phylogenetic data with comparative morphology has shed considerable light onto the boundaries and interrelationships of different gregarine species. In this study, we isolated a novel marine gregarine from the hepatic region of a Pacific representative of the hemichordate *Glossobalanus minutus*, and report the first ultrastructural and molecular data from any gregarine infecting this distinctive group of hosts. Molecular phylogenetic analyses of an SSU rDNA sequence derived from two single-cell isolates of this marine gregarine demonstrated a strong and unexpected affiliation with a clade of terrestrial gregarines (e.g. *Gregarina*). This molecular phylogenetic data combined with a comparison of the morphological features in previous reports of gregarines collected from Atlantic representatives of *G. minutus* justified the establishment of a new binomial for the new isolate, namely *Caliculium glossobalani* n. gen. et sp. The molecular phylogenetic analyses demonstrated a clade of terrestrial gregarines associated with a sequence acquired from a marine species, which suggest that different groups of terrestrial/freshwater gregarines evolved independently from marine ancestors.

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 so 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 (Rueckert et al. 2010, 2011b; Wakeman and Leander 2012, 2013). 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 to

shed considerable light onto the boundaries and interrelationships of different species (Leander 2007, 2008; Leander et al. 2003; Rueckert and Leander 2008, 2009; Rueckert et al. 2010, 2011a, 2013; 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,b; 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). Our interpretation of *Selenidium* is consistent with a contemporary view that does not split the genus into two different "families" (Selenidioididae and Selenidiidae) based on the presence/absence of merogony (Levine 1971; Rueckert and Leander 2009).

The combination of inferred ancestral traits in archigregarines suggests that these species form a paraphyletic 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.

MATERIALS AND METHODS

Collection of host material and isolation of gregarine trophozoites

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 laboratory in buckets filled with seawater, and stored for no more than 24 h 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.

Light microscopy

Differential interference contrast (DIC) images of living trophozoites were taken with an Olympus BX51TF (Olympus Corporation), 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 h, washed in distilled water, and dehydrated with a graded series of ethanol washes (50%, 60%, and 70%), and mounted in glycerol on glass slides.

Transmission electron microscopy

Because trophozoites were infrequently encountered, single cells (SCs) of the trophozoite stage were collected for TEM imaging in Eppendorf tubes over a period of 3–4 h. The trophozoites were fixed in 2.5% glutaraldehyde in seawater on ice for 30 min. After being washed three times with cacodylate buffer (0.2 M pH 7.2), cells were fixed in 1% OsO_4 in cacodylate buffer for 90 min. 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 2–3 min 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). Agar blocks containing the cells were cutout 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 5 min each. The material was then placed in a 1:1 mixture of ethanol and acetone, and 100% acetone for 5 min. Cells were embedded in 1:1 acetone and Epon 812 resin for 30 min, and then transferred to 100% resin overnight. After changing the 100% resin one time, material was polymerized for 32 h at ~68 °C. Ultrathin sections through

five different trophozoites were cut using a diamond knife on a Leica EM UC6 ultramicrotome and double stained with 2% (w/v) uranyl acetate and lead citrate. Sections were observed using a Hitachi H7600 electron microscope.

DNA extraction, amplification, and sequencing

We extracted DNA from two SC isolates of the new gregarine collected in different months (October and November). 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 and RNA purification kit (Epicentre Biotechnologies, Madison, WI). However, the final elution step was lowered to 4 μl, to concentrate 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 with EconoTaq 2X Master Mix (Lucigen Corp., Middleton, WI). The following program was used on the thermocycler for the initial amplification: Initial denaturation at 94 °C for 2 min; 35 cycles of denature at 94 °C for 30 s, anneal at 52 °C for 30 s, extension at 72 °C for 1 min 50 s; final extension 72 °C for 9 min. Subsequently, internal primers F2 5′-GGTAGYGACAAGAAATAACAAC-3′ and R2 5′-GAYTACG ACGGTATCTGATCGTC-3′ were paired with outside primers in a nested PCR, using the following program on a thermocycler: Initial denaturation for 94 °C for 2 min; 25 cycles of denature at 94 °C for 30 s, anneal at 55 °C for 30 s, extension at 72 °C for 1 min 30 s; final extension at 72 °C for 9 min (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), and cloned into a pCR 2.1 vector using a StrataClone PCR cloning kit (Aligent Technologies, Santa Clara, CA). Sixteen clones from each cloning reaction were screened for size, digested with HAEIII restriction enzyme (Invitrogen, Frederick, MD), and sequenced using vector primers and ABI Big-dye reaction mix. A novel sequence (1,780 bp) was identified using the National Center for Biotechnology Information's (NCBI) BLAST tool and confirmed with molecular phylogenetic analyses. The SSU rDNA sequence from the October isolate was confirmed using a second (November) isolate, with the collection dates separated by 1 mo. The different isolates had identical SSU rDNA sequences (GenBank KC890798).

Molecular phylogenetic analyses

Two datasets were constructed to analyze the phylogenetic position of the new isolate among gregarines and other apicomplexans: (1) a 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 alignment. This dataset 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 = 0.624 080; character states = 4; 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 in a single run 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). One independent run was performed for each dataset. Covariation was not applied. We set our program for four Monte Carlo Markov Chains starting from a random tree (MCMC; default temperature = 0.2), a gamma distribution (character states = 4), and stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate). A total of 6,500,000 generations were run. Trees were sampled every 100 generations. Burnin was set to default (relburnin = yes, burninfrac = 0.25), majority-rule consensus trees were constructed; posterior probabilities correspond to the frequency at which a given node is found in the post-burnin trees.

RESULTS

General ultrastructure of the trophozoites

The trophozoites were crescent-shaped and on average 90-μm long and 11-μm wide (range = 60–110 × 10–14 μm, $n = 21$) (Fig. 1A–D). The nucleus was 10-μm wide, 14-μm long (range = 9–10 × 11–14 μm, $n = 21$), and positioned in the center of the cell; the nucleus also contained a large nucleolus (Fig. 1A–C). 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. Transverse TEM sections demonstrated 26 epicytic folds running along the longitudinal axis of the cell (Fig. 1D). TEM images also revealed distinct patches of mitochondria with tubular cristae underlying the epicytic folds (Fig. 2A, B, E). Amylopectin granules and lipid droplets were very common throughout the cytoplasm (Fig. 2A–D, 3A). Darkly stained organelles with a striated internal organization were also distributed throughout the cytoplasm and particularly numerous near the nuclear envelope (Fig. 2C, D, 3F).

Longitudinal TEM sections demonstrated a sucker-like mucron consisting of a sunken dome encircled by a

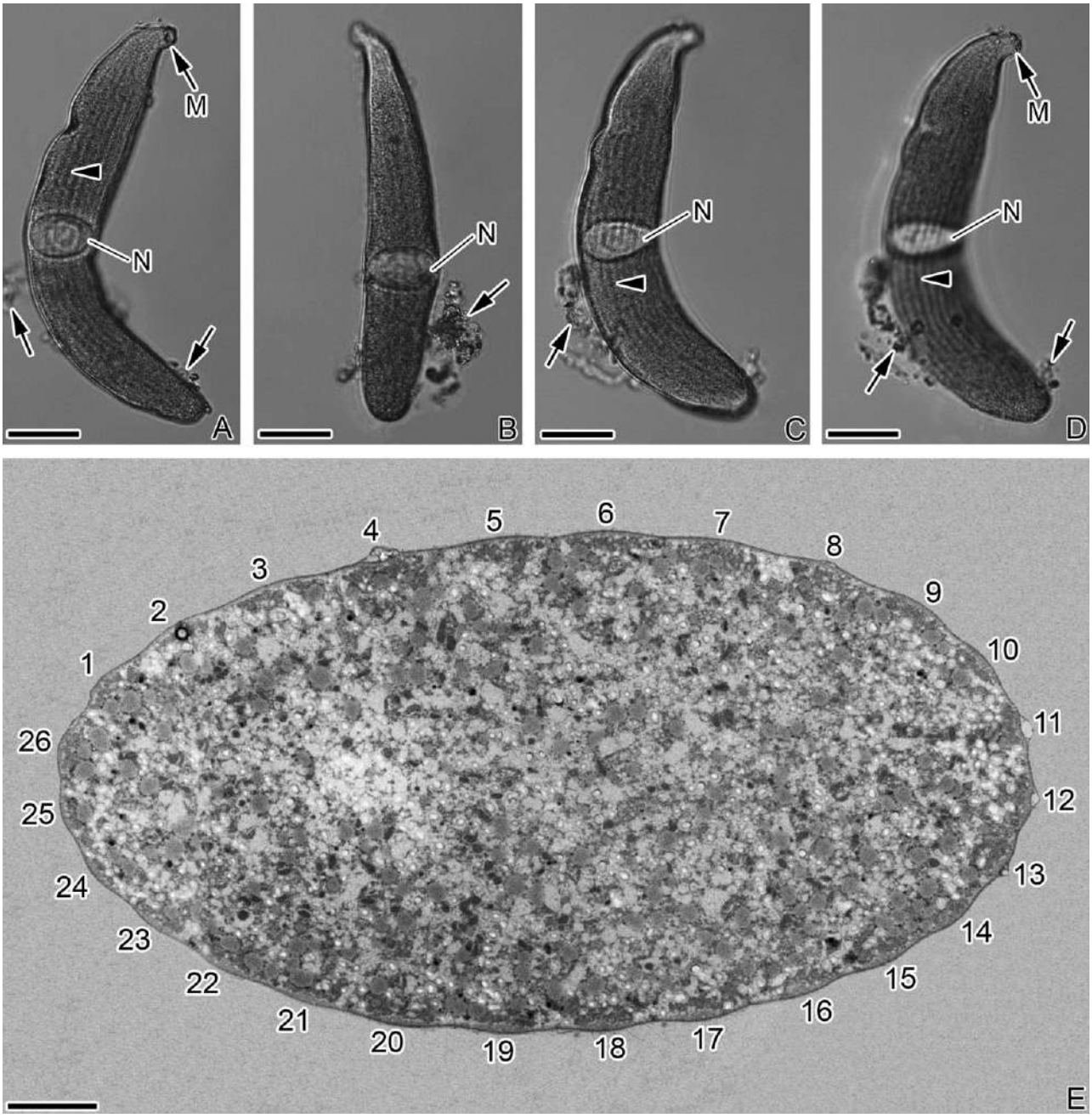

Figure 1 Micrographs of the trophozoite stage of *Caliculium glossobalani* n. gen. et sp. **A–D.** Differential interference contrast light micrographs showing the sucker-like mucron (M), ovoid nucleus (N), and longitudinal epicytic folds (arrowhead). Sticky host material (arrow) covers the surface of the cell. **E.** Transverse TEM images showing 26 epicytic folds on the surface of the trophozoite and a low magnification view of the cell contents. Scale bars: A–D = 15 μm; E = 500 nm.

raised lip (Fig. 3A–C). The base of the mucron was also defined by 3–4 transverse striations (Fig. 3B, C). Tangential TEM sections near the base of the mucron passed through the bases of the grooves between the transverse folds (Fig. 3D). Pinocytotic whorled vesicles, formed from invaginations of the trilayered membrane complex, were common within the mucron region (Fig. 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. 3C, E). We did not observe rhoptories, micronemes, a conoid, or microtubules in any of our sections through the cell surface or mucron region (Fig. 4A, B).

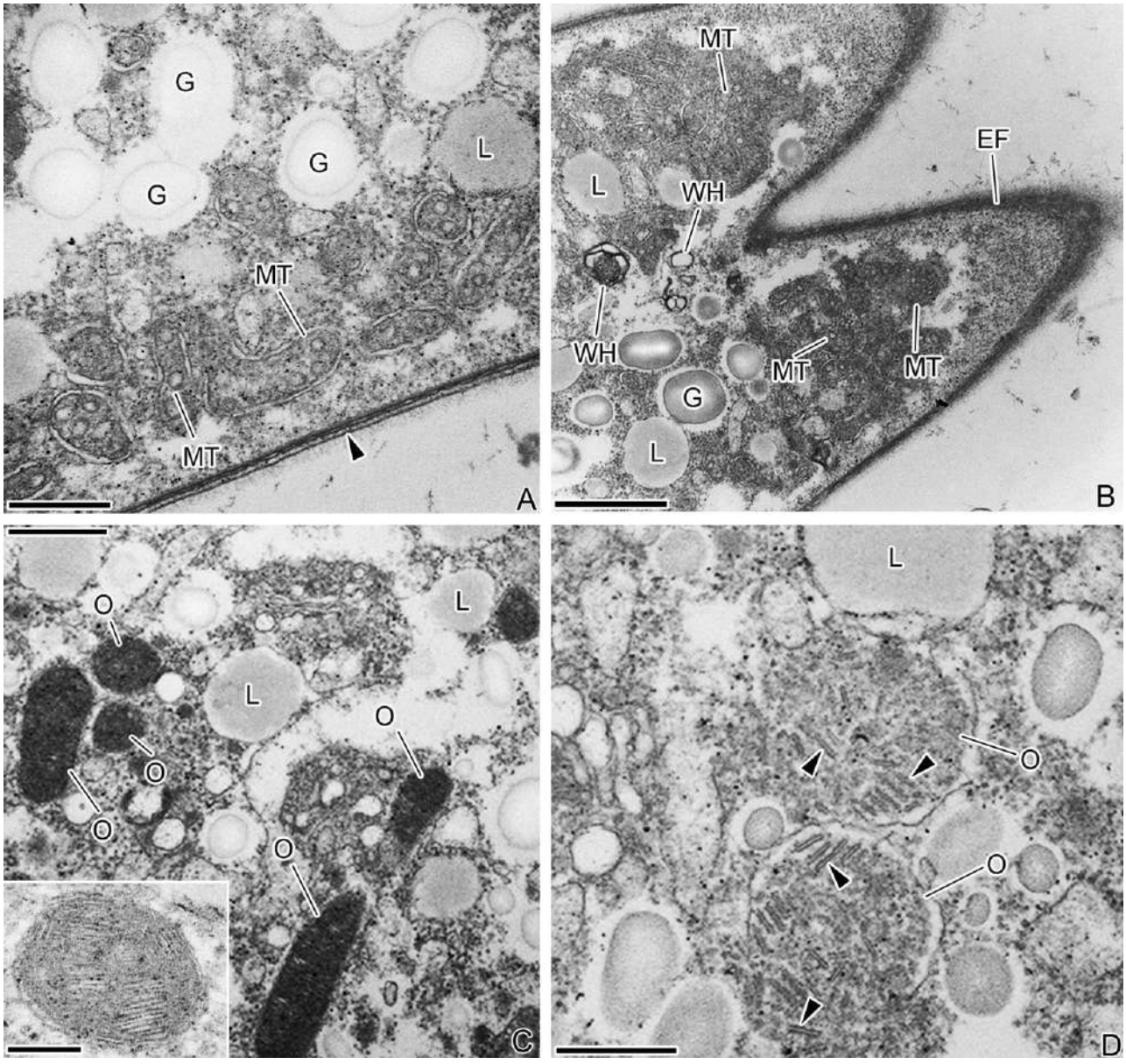

Figure 2 Transmission electron microscope (TEM) images of the trophozoites stage of *Caliculium glossobalani* n. gen. et sp. showing dense patches of mitochondria (MT) beneath the plasma membrane (arrowhead) and inner membrane complex. The TEM images also show epicytic folds (EF), amylopectin granules (G), lipids (L), pinocytotic whorled vesicles (WH), electron-dense organelles (O), the nucleus (N), and the nucleolus (NO). **C** (inset). High-magnification TEM image of these electron-dense organelles with a striated appearance. Scale bars: A = 500 nm; B = 2 μm; C = 500 nm (inset = 100 nm); D = 500 nm.

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). 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). This result was consistent with the phylogenetic analyses of the 61-taxon dataset, which included a more

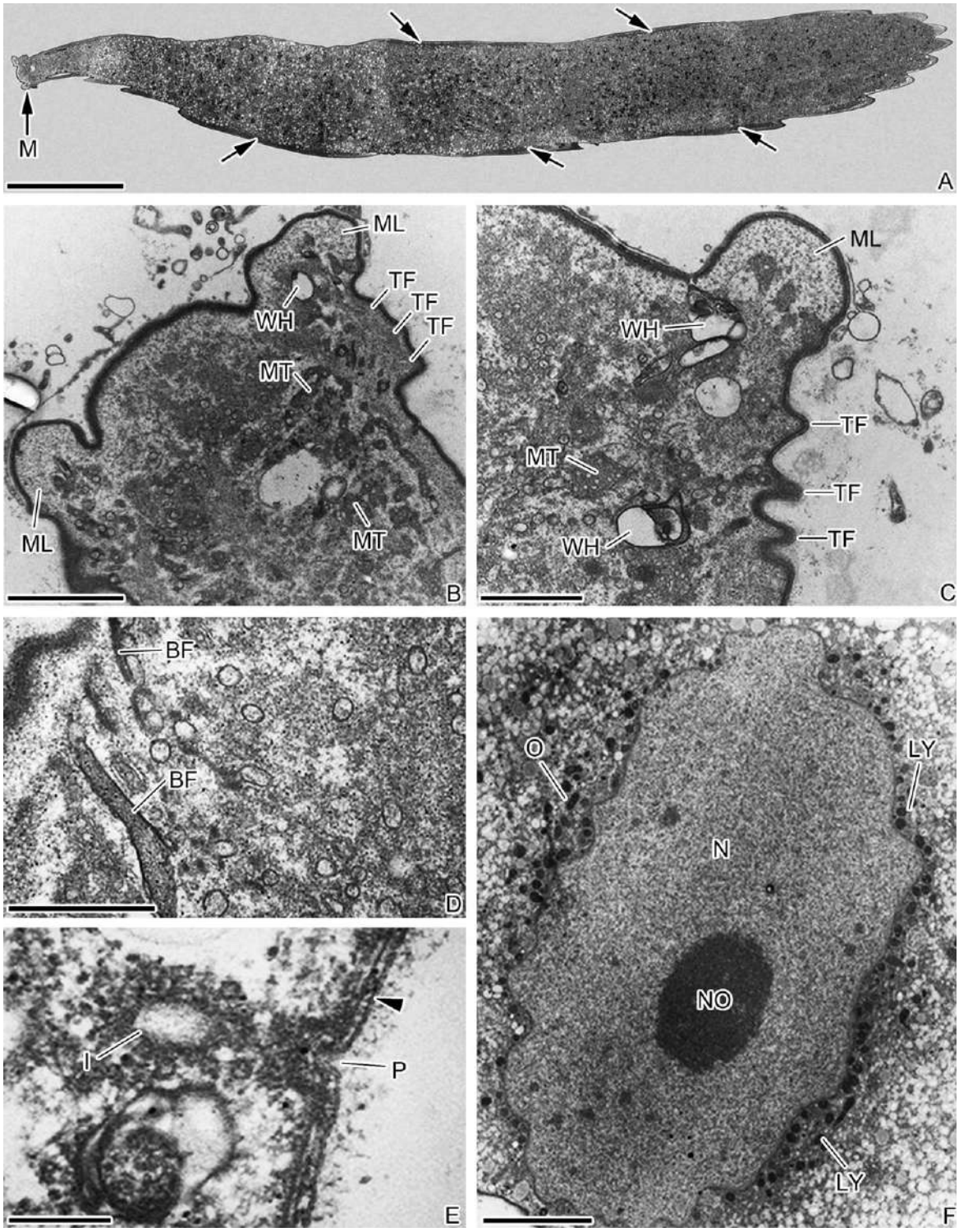

Figure 3 Transmission electron microscope (TEM) images of the trophozoite stage of *Caliculium glossobalani* n. gen. et sp. **A**. Longitudinal TEM images showing the mucron (M) and dense patches of mitochondria (arrows) beneath the surface of the epicytic folds. **B, C**. Longitudinal TEM images showing 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 organelles (O) surrounding the nuclear envelope. Scale bars: A = 20 μm; B = 2 μm; C = 1 μm; D, E = 500 nm; F = 5 μm.

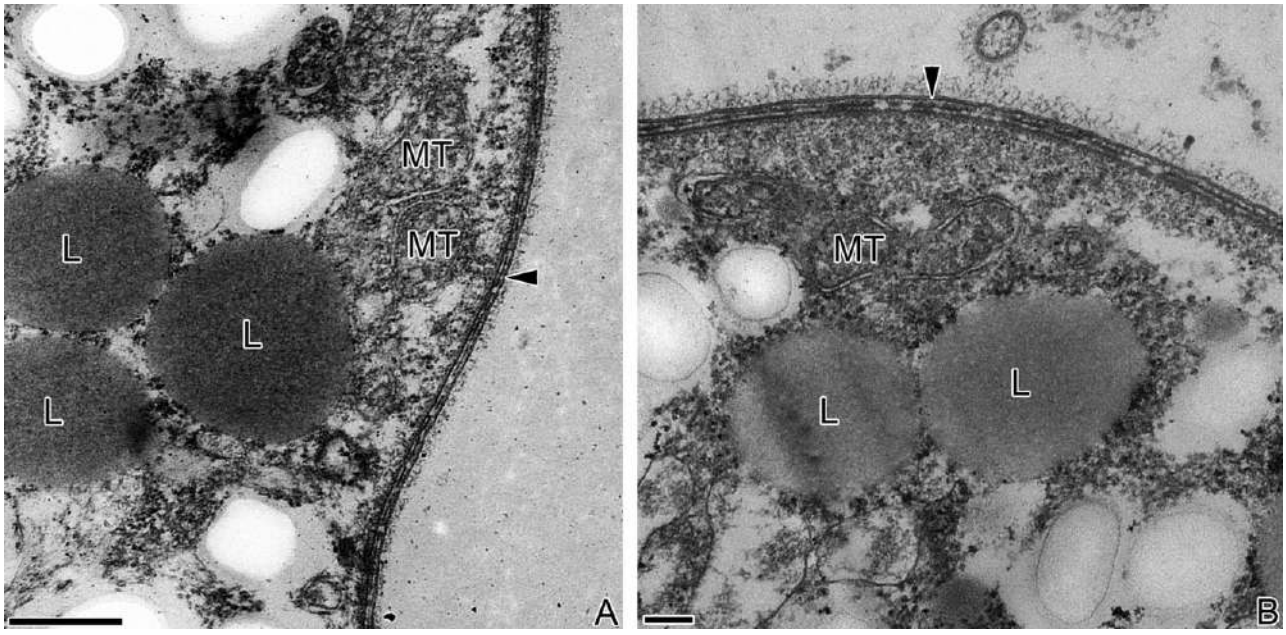

Figure 4 High-magnification transmission electron microscope (TEM) images of the trophozoite cortex of *Caliculium glossobalani* n. gen. et sp. **A.** Longitudinal section showing lipid droplets (L), mitochondria (MT), and a trilayer membrane complex (arrowhead). **B.** Transverse section showing lipid droplets (L), mitochondria (MT), and a trilayer membrane complex (arrowhead). Scale bars: A = 0.5 μm; B = 0.1 μm.

comprehensive set of sequences from terrestrial gregarines (data not shown).

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 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 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 most consistent with *S. grassei* (Table 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 1). Moreover, our study is the first to perform TEM on individual trophozoites isolated from hemichordates, 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 *Caliculium glossobalani* n. gen. et sp. also encountered conspicuous, electron-dense structures that were distributed throughout the cytoplasm of the cell (Fig. 2C, D). One interpretation of this data is that these structures are mitochondria with densely packed cristae like those reported in *Pterospora* (Landers 2002). However, unlike mitochondria, two membranes were not present around the electron-dense structures (Fig. 2C); canonical mitochondria with tubular cristae were abundant under the epicytic folds (Fig. 2A). Further work will be needed to identify and better understand these abundant and enigmatic organelles.

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 *Selenidi-*

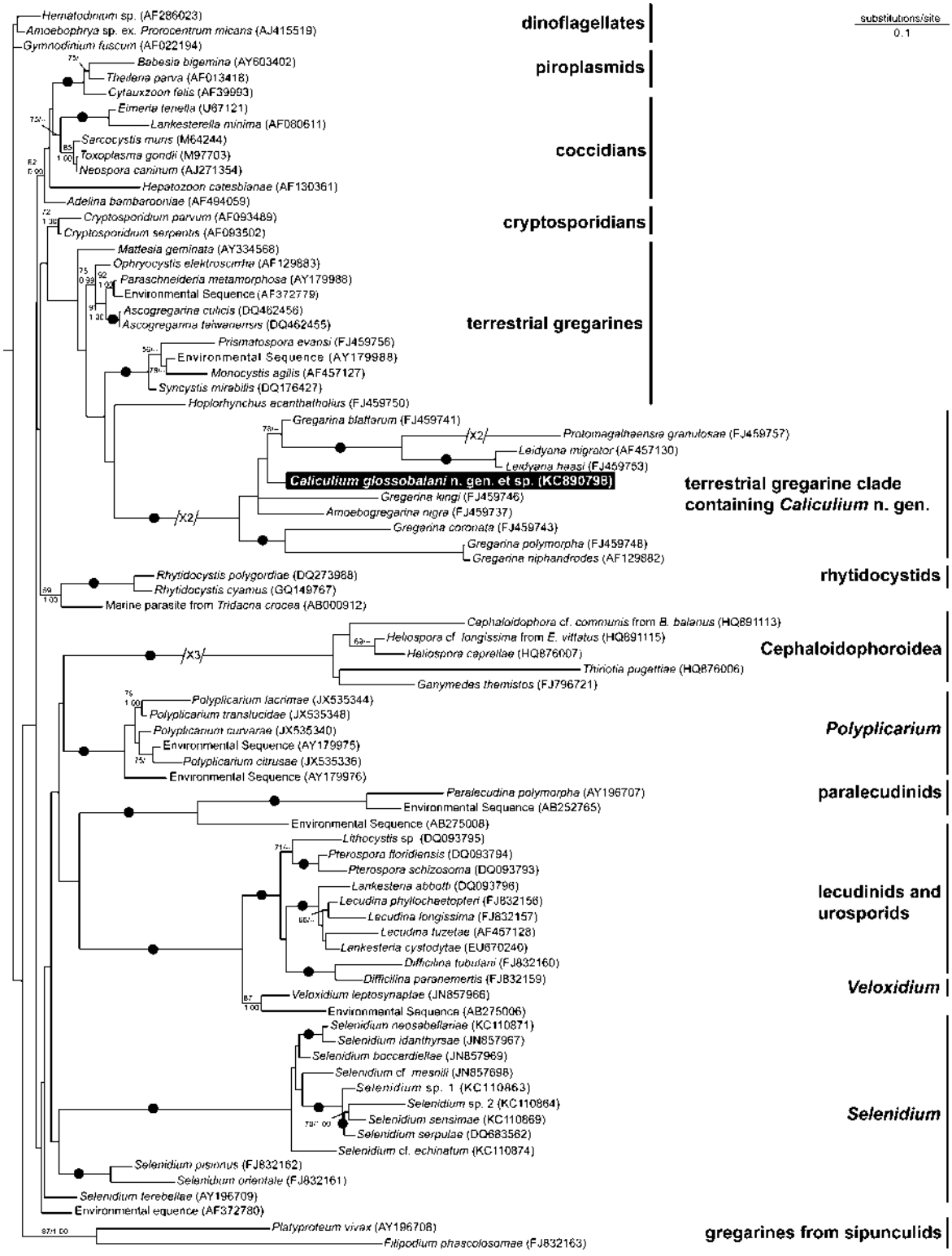

Figure 5 Maximum likelihood (ML) tree derived from phylogenetic analysis of the 80-taxon dataset (1,054 unambiguously aligned sites) of small subunit (SSU) rDNA sequences. This tree was inferred using the GTR + I + Γ substitution model (−ln *L* = 19,526.46 146 gamma shape = 0.6240, proportion of invariable 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 *Caliculium glossobalani* n. gen. et sp. is highlighted in a black box. Long branches were shortened by multiple lengths of the substitutions/site scale bar.

Table 1. Comparisons of traits in known species of marine gregarines isolated from hemichordates

	Selenidium metchnikovi	*Selenidium grassei*	"Selenidium sp."	***Caliculium glossobalani* n. gen. et sp.**
Host	Glossobalanus minutus	*Balanoglossus clavigerus*	*Glossobalanus minutus*	***Glossobalanus minutus***
Host tissue	Hepatic region	Hepatic and reproductive region	Hepatic region	**Hepatic region**
Locality	E. Atlantic Ocean	E. Atlantic Ocean	E. Atlantic Ocean	**W. Pacific Ocean**
Trophozoite shape	Vermiform	Vermiform	Vermiform	**Vermiform**
Tophozoite size (L × W, μm)	30–34 (W not reported)	60–120 × 10–12	120–125 × 12–14	**60–110 × 10–14**
Nucleus shape	Oval	Oval	Oval	**Oval**
Position of nucleus	Middle	Middle	Middle	**Middle**
Shape of posterior end	Rounded	Rounded	Rounded	**Rounded**
Number of long epicytic folds	5	7–8	7–8	**26**
Transverse surface folds	Unknown	Unknown	Unknown	**Yes**
Shape of mucron	Undescribed	Narrowed; Sucker-like	Narrowed; Sucker-like	**Narrowed; Sucker-like**
Merogony	Present	Not observed	Present	**Not observed**
Motility	No	Not reported	Not reported	**No**
Literature	Léger and Duboscq (1917)	Théodoridès and Desportes (1968)	Fernandez (1982)	**This study**

The new species described here is highlighted in bold.

um species currently available for molecular phylogenetic analyses (Rueckert and Leander 2009; Wakeman and Leander 2012, 2013). An SSU rDNA sequence from the type species of *Selenidium*, *S. pendula*, is currently unavailable. Acquiring this 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 SC isolates of the gregarine from two hosts, each collected on different trips separated by over 4 wk. 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 and freshwater gregarines are polyphyletic and represent more than one clade that independently descended from marine ancestors. 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 *C. glossobalani* n. gen. et sp. from hemichordates from distinct geographical 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 apical complex elements, conspicuous 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, *C. glossobalani* n. gen. et sp.

TAXONOMIC SUMMARY

Phylum Apicomplexa Levine, 1970.
Class Conoidasida Levine, 1988
Subclass Gregarinasina Dufour, 1828

Caliculium n. gen. Wakeman, Reimer, Jenke-Kodama et Leander 2014

Description. Trophozoites nonmotile, 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 absent/inconspicuous; conoid absent.
Type species. *Caliculium glossobalani* n. sp. Wakeman, Reimer, Jenke-Kodama et Leander 2014
Etymology. The genus name is taken from "*Caliculus*" (Latin) = "sucker" and refers to the sucker-like mucron of the trophozoites.

Caliculium glossobalani n. sp. Wakeman, Reimer, Jenke-Kodama et Leander 2014

Description. Trophozoites are crescent-shaped, mean length = 90 μm (range = 60–110 μm) and mean width = 11 μm (range = 10–14 μm). An oval nucleus (10 × 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.
DNA 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 habitat. Marine.
Type host. *Glossobalanus minutus* (Kowalevsky, 1866) (Metazoa, Hemichordata, Enteropneusta, Ptychoderidae).
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). The holotype is the cell shown in Fig.2C; it is fixed in glutaraldehyde and embedded in glycerol on a slide (labeled MI-PR126) in a desiccation chamber, such that it can be found among the rest of the type material.
Etymology. The specific epithet is named for the genus of the hemichordate host in which this species was found.

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