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Molecular systematics of marine gregarines (Apicomplexa) from North-eastern Pacific polychaetes and nemerteans, with descriptions of three novel species: *Lecudina phyllochaetopteri* sp. nov., *Difficilina tubulani* sp. nov. and *Difficilina paranemertis* sp. nov.

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Most eugregarine apicomplexans infecting the intestines of marine invertebrates have been described within the family Lecudinidae and the type genus *Lecudina*. The diversity of these parasites is vast and poorly understood and only a tiny number of species has been characterized at the molecular phylogenetic level. DNA sequences coupled with high-resolution micrographs of trophozoites provide an efficient and precise approach for delimiting gregarine lineages from one another and also facilitate our overall understanding of gregarine biodiversity. In this study, phylogenetic analyses of small subunit (SSU) rDNA sequences from five (uncultivated) gregarines isolated from polychaetes and nemerteans in the North-eastern Pacific Ocean are presented. *Lecudina phyllochaetopteri* sp. nov. was isolated from the intestines of the parchment tubeworm *Phyllochaetopterus prolifica* (Polychaeta). *Lecudina longissima* and *Lecudina polymorpha* were both isolated from the intestines of *Lumbrineris japonica* (Polychaeta). *Difficilina tubulani* sp. nov. was isolated from the nemertean *Tubulanus polymorpha* and *Difficilina paranemertis* sp. nov. was isolated from the nemertean *Paranemertes peregrina*. This is the first report of molecular sequence data from gregarines that infect nemerteans. The two novel species of the genus *Difficilina* described in this study formed a strongly supported clade in the phylogenetic analyses. This *Difficilina* clade formed the sister group to a robust subclade of lecudinids consisting of *Lecudina longissima*, *Lecudina phyllochaetopteri* sp. nov. (which lacked epicytic folds), *Lecudina tuzetae*, species of the genus *Lankesteria* and several sequences derived from previous environmental DNA surveys of marine biodiversity.

INTRODUCTION

Apicomplexans form a large and diverse group of unicellular parasites containing at least 6000 described species. It has been estimated that probably well over a million more have yet to be discovered (Hausmann *et al.*, 2003; Adl *et al.*, 2007). Despite being infamous as intracellular pathogens of humans and livestock (e.g. *Plasmodium*, the causative agent of malaria, and *Cryptosporidium*), most of apicomplexan diversity is thriving in the world's oceans. Gregarines, for instance, are extracellular apicomplexan parasites that inhabit the intestines, coeloms and reproductive vesicles of marine, freshwater and terrestrial invertebrates. The general biology and overall diversity of these parasites is poorly understood, mainly because gregarines are difficult to collect and have no obvious medical or economic significance. In 1987, there were about 231 genera and 1624 species described within the subclass Gregarinasina Dufour, 1828

Abbreviations: DIC, differential interference contrast; GTR, general-time reversible; LM, light micrographs; ML, maximum-likelihood; SEM, scanning electron microscopy/micrographs; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the small subunit rDNA sequences of *Lecudina phyllochaetopteri* sp. nov., *Lecudina longissima*, *Lecudina polymorpha*, *Difficilina paranemertis* sp. nov. and *Difficilina tubulani* sp. nov. are FJ832156–FJ832160, respectively.

The GenBank accession numbers for the sequences used to construct the phylogenetic tree are available as a supplementary table with the online version of this paper.

(Levine, 1988). Gregarines are ubiquitous in marine ecosystems, have mostly monoxenous life cycles involving a single invertebrate host and possess extracellular feeding stages called 'trophozoites'. This combination of characteristics is inferred to have been retained from the most recent ancestor of all apicomplexans and has given gregarines the unwarranted reputation of being 'primitive', relative to the better-known intracellular apicomplexans with more complex life cycles involving vertebrate hosts (Leander, 2008).

Historically, gregarines have been conveniently separated into three major groups (Grassé, 1953): (a) archigregarines occur exclusively in marine habitats and have trophozoite stages that resemble the general morphology of infective sporozoite stages; (b) eugregarines occur in marine, freshwater and terrestrial habitats and have intestinal trophozoites that are significantly different in morphology and behaviour from sporozoites; (c) neogregarines exclusively infect insects, have reduced trophozoite stages and associate with host tissues other than the intestines per se. The majority of known gregarine species are eugregarines, primarily because eugregarines are commonly encountered within the intestines of insects, which have garnered more attention from the research community. The order Eugregarinorida Léger, 1900 comprises both aseptate and septate gregarines; the latter designation refers to the presence of a transverse superficial septum that demarcates the trophozoite cell into two pseudo-compartments (compare Levine, 1976; Leander, 2008). Eugregarines have been further classified into 27 families and around 244 genera, 14 of which have more than 25 species each (Levine, 1988; Clopton, 2000). The majority of eugregarine species that inhabit marine invertebrates have been classified within the poorly circumscribed family Lecudinidae Kamm, 1922 (25 genera) and the genus *Lecudina* Mingazzini, 1899, which consists of about 45 species (Levine, 1988). It has been generally thought that gregarines are relatively host-specific and, for that reason, differences in host taxa have been used in the past to differentiate some gregarine species and genera (Levine, 1979; Perkins *et al.*, 2000; Rueckert & Leander, 2008).

In this study of gregarine diversity, we used microscopy and molecular phylogenetic analyses of small subunit (SSU) rDNA sequences to describe three novel species from marine invertebrate hosts collected from the western Pacific coast of Canada: *Lecudina phyllochaetopteri* sp. nov. isolated from the parchment tubeworm *Phyllochaetopterus prolifica* Hering, 1949, *Difficilina tubulani* sp. nov. isolated from the nemertean *Tubulanus polymorphus* Renier, 1804 and *Difficilina paranemertis* sp. nov. isolated from the nemertean *Paranemertes peregrina* Coe, 1901. We also provide molecular phylogenetic data from *Lecudina longissima* Hoshide, 1944 and a morphotype of *Lecudina polymorpha* Schrével, 1963, both isolated from the intestines of the polychaete *Lumbrineris japonica* Marenzeller, 1879.

METHODS

Collection and isolation of organisms. The nemertean *T. polymorphus* and the polychaete *Lumbrineris japonica* were collected from the rocky pools of Grappler Inlet (48° 50′ 17″ N, 125° 08′ 02″ W) in June 2006 near Bamfield, British Columbia, Canada. A dredge haul was conducted at Wizard Islet (48° 51′ 6″ N, 125° 09′ 4″ W) in July 2007 at a depth of 20 m during a trip on the research vessel *MV Alta* from the Bamfield Marine Science Centre, British Columbia, Canada. The parchment tubeworm *Phyllochaetopterus prolifica* was collected from these samples. The nemertean *Paranemertes peregrina* was collected in August 2008 on the shores of English Bay Beach (49° 17′ 18″ N, 123° 08′ 37″ W), Vancouver, British Columbia, Canada.

Gregarine trophozoites were released from host tissue by teasing apart the intestines with fine-tipped forceps under a low magnification stereomicroscope (MZ6; Leica). Gut contents containing gregarines were examined with an inverted compound microscope (Axiovert 200, Zeiss or DM IL, Leica) and individual trophozoites were isolated by micromanipulation and washed in filtered seawater before being prepared for microscopy and DNA extraction.

Light and scanning electron microscopy. Most of the light micrographs (LM) were produced using a portable field-based microscopy system consisting of a Leica DM IL inverted microscope configured with Hoffman modulation contrast and connected to a PixeLink Megapixel colour digital camera. Some LM were taken with differential interference contrast (DIC) optics using either a Zeiss Axiovert 200 inverted microscope connected to the PixeLink camera or an upright Zeiss Axioplan 2 microscope connected to a Leica DC500 colour digital camera.

Individual trophozoites of *Lecudina phyllochaetopteri* sp. nov. (n=25), *D. paranemertis* sp. nov. (n=2) and *D. tubulani* sp. nov. (n=20) were prepared for scanning electron microscopy (SEM). The sticky and viscous consistency of the nemertean host tissue limited the number of *D. paranemertis* sp. nov. trophozoites that could be isolated for SEM. Nonetheless, isolated cells of both gregarine species were deposited directly into the threaded hole of separate Swinnex filter holders containing a 5 μm polycarbonate membrane filter (Millipore Corp.). The filter was submerged in 10 ml seawater within a small canister (2 cm diameter and 3.5 cm tall). A piece of Whatman filter paper was mounted on the inside base of a beaker (4 cm diameter and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4 % OsO_4 and the beaker was turned over the canister. The parasites were fixed by OsO_4 vapours for 30 min. Ten drops of 4 % OsO_4 were added directly to the seawater and the parasites were fixed for an additional 30 min on ice. 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 and then dehydrated with a graded series of ethyl alcohol and critical-point-dried with CO_2. Filters were mounted on stubs, sputter-coated with 5 nm gold and viewed under a SEM (Hitachi S4700). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems).

DNA isolation, PCR amplification, cloning, and sequencing. Eight to 68 individual trophozoites, depending on the species, were manually isolated from dissected hosts, washed three times in filtered seawater and deposited into a 1.5 ml Eppendorf tube. Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification kit (EPICENTRE Biotechnologies). Small subunit rDNA sequences were PCR-amplified using puRe*Taq* Ready-to-go PCR beads (GE Healthcare) and the following eukaryotic PCR primers: F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander *et al.*, 2003a). The following internal primers, designed to match existing eukaryotic SSU sequences, were used for nested PCR: F2 5′-AAGTCTGGTGCC-AGCAGCC-3′, F3 5′-TGCGCTACCTGGTTGATCC-3′, R2 5′-TTTA-AGTTTCAGCCTTGCCG-3′, R3 5′-GCCTYGCGACCATACTCC-3′

and R4 5′-CGGCCATGCACCACCACCCATAAAATC-3′. PCR products corresponding to the expected size were gel-isolated and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen). Eight cloned plasmids, for each PCR product, were digested with *Eco*RI, and inserts were screened for size using gel electrophoresis. Two clones were sequenced with ABI Big-dye reaction mix using vector primers oriented in both directions. The SSU rDNA sequences (lengths of sequences: *Lecudina phyllochaetopteri* 1753 bp; *Lecudina longissima* 1751 bp; *Lecudina polymorpha* 1754 bp; *D. paranemertis* 1751 bp; *D. tubulani* 1742 bp) were identified by BLAST analysis and molecular phylogenetic analyses.

Molecular phylogenetic analysis. The five new SSU rDNA sequences were aligned with 48 additional alveolate sequences using MacClade 4 (Maddison & Maddison, 2000) and visual fine-tuning. The PhyML program (Guindon & Gascuel, 2003; Guindon *et al.*, 2005) was used to analyse the 53-sequence alignment (1175 unambiguously aligned positions; gaps excluded) with maximum-likelihood (ML) using a general-time reversible (GTR) model of nucleotide substitutions (Posada & Crandall, 1998) that incorporated invariable sites and a discrete gamma distribution (eight categories) (GTR+I+Γ+8 model: α=0.417 and fraction of invariable sites=0.051). ML bootstrap analyses were performed on 100 resampled datasets using the same program set to the GTR model+Γ+8 rate categories+invariable sites. Bayesian analysis of the 53-sequence dataset was performed using the program MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). The program was set to operate with GTR, a gamma-distribution and four Monte Carlo Markov chains (MCMC; default temperature=0.2). A total of 1 000 000 generations was calculated with trees sampled every 50 generations and with a prior burn-in of 100 000 generations (2000 sampled trees were discarded; burn-in was checked manually). A majority rule consensus tree was constructed from 18 001 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees. Independent Bayesian runs on each alignment yielded the same results.

GenBank accession numbers. Details of the GenBank accession numbers used for the phylogenetic analyses are given in Supplementary Table S1 (available in IJSEM Online).

RESULTS

Trophozoite morphology

Descriptions of general cell shapes follow the nomenclatural system established by Clopton (2004). The trophozoites of all five species described below contained brownish amylopectin granules within the cytoplasm.

***Lecudina phyllochaetopteri* sp. nov.** The trophozoites were elliptoid in shape (length=24–32 μm, width=11–15 μm, n=7) with rounded anterior and posterior ends (Figs 1a, b). The anterior end was distinguished by a nipple-like mucron that was free of amylopectin granules (Figs 1a–c). A spherical nucleus (8–9 μm diameter) was positioned within the posterior half of the cell. Trophozoites that were photographed immediately after the collection of the hosts (i.e. within 3 h) had smooth cell margins and lacked notch-like depressions (Figs 1a, b). Trophozoites that were photographed 2–5 days after the collection of the host worms possessed notch-like depressions when examined with both LM and SEM (Fig. 1c). SEM demonstrated that the trophozoites completely lacked epicytic folds and were covered with a network and little knob-like structures of mucilaginous material extruded from the cell (Figs 1c–e). The trophozoites did not show any evidence of gliding motility or cellular plasticity.

***Lecudina polymorpha*.** Two morphotypes of *Lecudina polymorpha* have been described previously from '*Lumbrineris* sp.' (Leander *et al.*, 2003b) and the trophozoites described here conformed most closely to the morphology of *Lecudina polymorpha* morphotype 2 (Figs 2a, b). These trophozoites were isolated from the intestines of *Lumbrineris japonica*, which provides a more precise host species identification for this gregarine species. The trophozoites were narrowly obdeltoid (330–1120 μm long, 25–60 μm wide, n=6) with a prominent bulge in between the anterior end of the cell and the nucleus; the width of the bulge measured 70–130 μm (Fig. 2a). The mucron was situated at the tip of the anterior end and was free of amylopectin granules. An oval nucleus (35–60 μm long and 25–35 μm wide) containing a large spherical nucleolus (20 μm diameter) was located in the anterior half of the cell (Figs 2a, b). The trophozoites were rigid, except in the mucron area, and were capable of gliding motility.

***Lecudina longissima*.** The trophozoites were collected from the intestines of *Lumbrineris japonica*, were linearly elliptoid and conformed to the original description of *Lecudina longissima* in both morphology and host specificity (compare Hoshide, 1958; Levine, 1974) (Fig. 2c). Trophozoites were 300–540 μm long and 40–55 μm wide (n=6). The anterior mucron was rounded and free of amylopectin; the posterior tip of the cell was more pointed (Fig. 2c). The spherical or ellipsoidal nucleus (20–32 × 25–30 μm) was situated in the anterior third of the trophozoite. The cells were rigid and capable of gliding motility.

***Difficilina paranemertis* sp. nov.** The trophozoites were isolated from the intestines of the nemertean *Paranemertes peregrina* and were narrowly elliptoid to narrowly spatulate. At the level of the nucleus, cells were 240–480 μm long and 40–55 μm wide (n=14) (Figs 3a, b). The rounded mucron was free of amylopectin and situated at the anterior tip of the cell (Figs 3a, b). Some trophozoites possessed a distinctive nipple-like structure at the tip of the mucron (Fig. 3b). The nucleus was spherical to elliptoid (20–25 μm diameter) and positioned either in the middle or in the anterior half of the trophozoite. Fine longitudinal striations were observed with LM, suggesting that the surface was covered with epicytic folds. Trophozoites were relatively rigid and capable of gliding motility.

***Difficilina tubulani* sp. nov.** Trophozoites were collected from the intestines of the nemertean *T. polymorphus* and were narrowly obdeltoid and distinctly crescent-shaped. At the position of the nucleus, the cells were 270–350 μm long and 35–40 μm wide (n=14). A noticeable neck-like constriction was visible in between the anterior end of

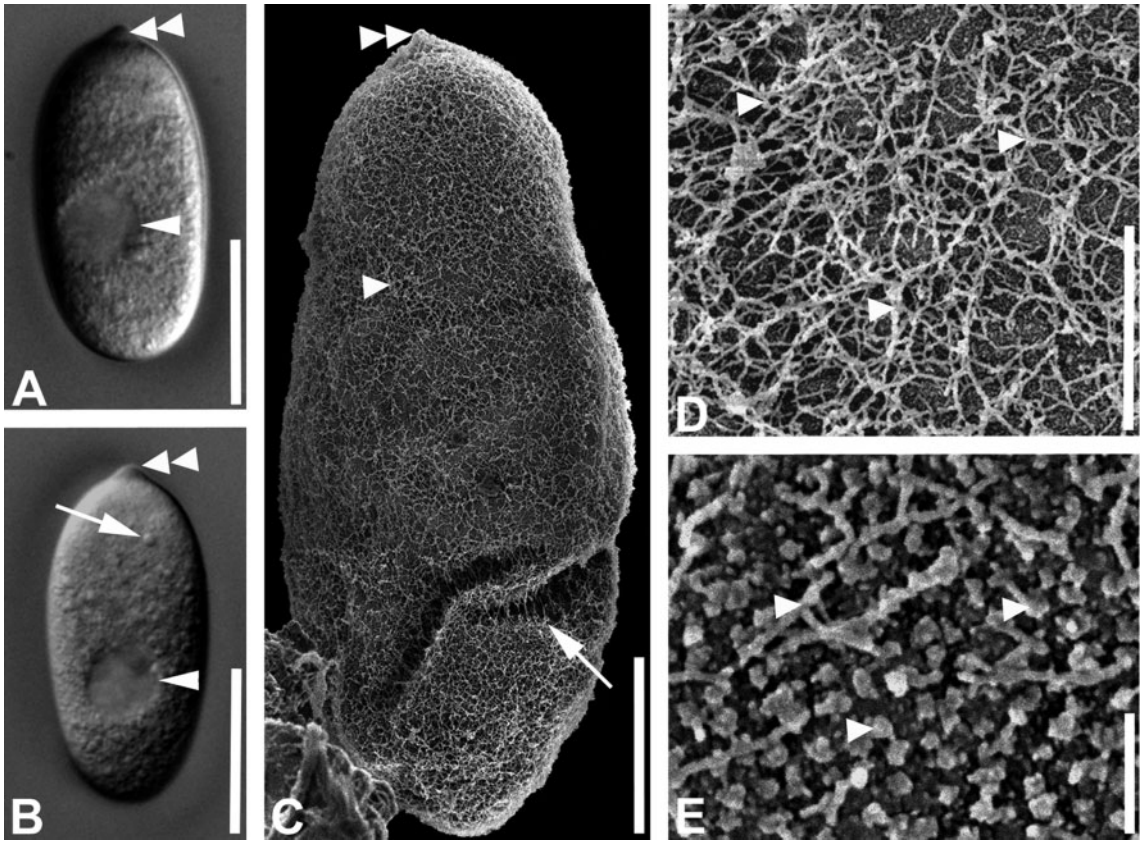

Fig. 1. DIC LM and SEM showing general trophozoite morphology and surface ultrastructure of *Lecudina phyllochaetopteri* sp. nov. Small subunit rDNA sequences were obtained from trophozoites with this morphology. (a, b) Trophozoite in different focal planes showing a large spherical nucleus (single arrowhead) in the posterior half of the cell, the nipple-like mucron (double arrowhead) and amylopectin (arrow). Bars, 15 μm. (c) SEM of a trophozoite with a nipple-like mucron (double arrowhead) showing a network of extruded mucilaginous material (single arrowhead). The arrow indicates one of the notches in the cell cortex that reflect the state of trophozoites within hosts that were dissected two or more days after collection. Bar, 6 μm. (d) Higher magnification view of the cell surface and the extruded material (single arrowheads). Longitudinal epicytic folds were absent. Bar, 1 μm. (e) High magnification view of the extruded material (single arrowheads). Bar, 250 nm.

the cell and the nucleus (Fig. 3c) in most trophozoites. The mucron was rounded and free of amylopectin and the posterior end was sharply pointed (Figs 3c, d). A spherical nucleus was situated in the anterior half of the cell (20–25 μm diameter). Fine longitudinal striations were visible with LM and SEM and confirmed the presence of longitudinally oriented epicytic folds over the entire cell surface, except the mucron (Fig. 3e). The cells were rigid and capable of gliding motility.

Molecular phylogenetic analyses of SSU rDNA sequences

Molecular phylogenetic analyses of the 53-sequence dataset produced a tree topology with an unresolved backbone. The analyses demonstrated a strongly supported clade of dinoflagellates (outgroup), a moderately supported clade of *Colpodella* species and a weakly supported clade of apicomplexan sequences (Fig. 4). The apicomplexan clade contained a strongly supported piroplasmid clade nested within a weakly supported (paraphyletic) group of coccidian sequences. The apicomplexan backbone also gave rise to several other clades, including rhytidocystids, cryptosporidians and a strongly supported clade consisting of terrestrial neogregarines and monocystid gregarines (Fig. 4). The sequences from species of the genus *Selenidium* (i.e. 'archigregarines'), including some environmental sequences, branched weakly and paraphyletically near the origin of a moderately supported clade consisting of (terrestrial) septate eugregarines and a large clade of marine eugregarines (Fig. 4).

The new sequences from *D. tubulani* sp. nov. and *D. paranemertis* sp. nov. formed a strongly supported clade within the clade of marine intestinal eugregarines (Fig. 4). The sister group to the *Difficilina* clade was a strongly supported clade consisting of species of the genera *Lecudina* and *Lankesteria* as well as two environmental

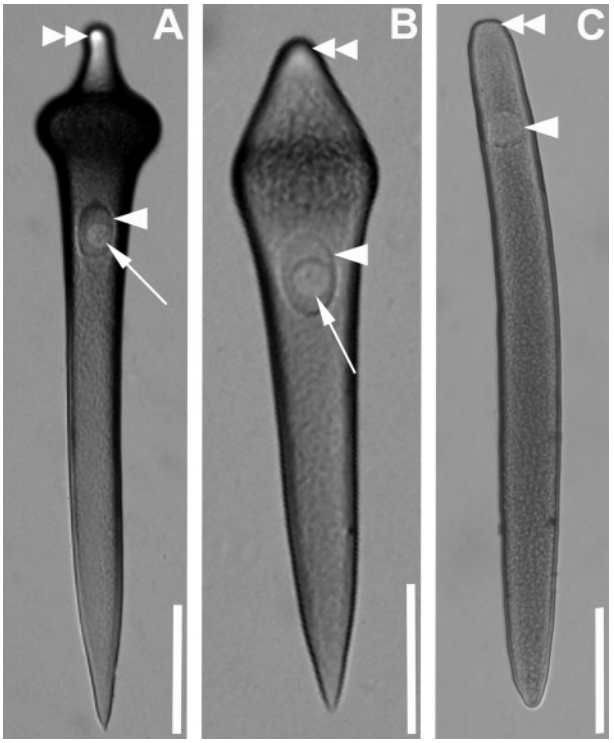

Fig. 2. LM, collected with our portable field-based microscopy system, showing the general trophozoite morphology of *Lecudina polymorpha* (a, b) and *Lecudina longissima* (c). Small subunit rDNA sequences were obtained from trophozoites with these morphologies. (a, b) Trophozoites of *Lecudina polymorpha* (morphotype 2) with a very narrowly obdeltoid cell shape, terminating in a pointed posterior tip. The anterior mucron (double arrowhead) was rounded and free of amylopectin. The ovoid nucleus (single arrowhead) was situated in the anterior half of the cell and contained a large spherical nucleolus (arrow). The trophozoites possessed a prominent bulge between the mucron and the nucleus. Bars, 115 μm (a); 70 μm (b). (c) Trophozoite of *Lecudina longissima* with a linearly elliptoid cell shape. The cell terminated with a rounded mucron (double arrowhead) and possessed a spherical to ellipsoidal nucleus (single arrowhead) that was situated in the anterior half of the cell. Bar, 100 μm.

sequences; the deepest relationships within this *Lecudina*/*Lankesteria* clade were mostly unresolved. Nonetheless, the new sequences from *Lecudina phyllochaetopteri* sp. nov. and *Lecudina longissima* clustered within this *Lecudina*/*Lankesteria* clade (excluding *Lecudina polymorpha*). The new sequence from *Lecudina polymorpha* morphotype 2 (collected in 2006) clustered strongly with the two sequences previously published for *Lecudina polymorpha* (collected in 2002); these three sequences were extraordinarily divergent and formed a moderately supported sister group to the clade consisting of the *Lecudina*/*Lankesteria* clade, the *Difficilina* clade and urosporids (i.e. coelomic gregarines) (Fig. 4).

A total of 1760 nt was compared between *D. tubulani* sp. nov. and *D. paranemertis* sp. nov., resulting in 221 nt differences and 28 indels. A pairwise distance calculation based on the Kimura two-parameter model (Kimura, 1980) of 1732 nt (excluding the indels) resulted in a 14.1 % sequence divergence between the two species of nemertean parasites. A total of 1704 nt was compared between the three sequences of *Lecudina polymorpha*. There were 12 nt differences between morphotypes 1 and 2 (collected in 2002); 10 differences occurred between morphotype 1 (collected in 2002) and morphotype 2 (collected in 2006); and 13 nt differences occurred between morphotype 2 collected in 2002 and morphotype 2 collected in 2006. Pairwise distance calculations based on the Kimura two-parameter model resulted in a 0.6–0.8 % sequence divergence between the three sequences of *Lecudina polymorpha*.

DISCUSSION

Within the eugregarines, the suborder Aseptatina Chakravarty, 1960 contains ten families, of which the largest is the family Lecudinidae (Levine, 1976). The literature is widely scattered and, in some cases, it is exceedingly difficult to obtain original descriptions. Most of the original descriptions are based only on line drawings and quite a few species and genera are poorly described and circumscribed (Levine, 1977). Accordingly, the aim of this study was to report on the utility of coupling SSU rDNA sequences with LM and SEM of trophozoites for the delimitation of marine gregarine lineages. For instance, DNA sequences are not vulnerable to changes in gregarine morphology due to developmental plasticity or to an interruption of the normal feeding behaviour of the host. The molecular sequence data also provide insights into the phylogenetic relationships of gregarines that are simply unattainable through comprehensive analyses of morphometric data. In our view, DNA sequences coupled with high-resolution micrographs provide a much more efficient and precise approach for distinguishing gregarine lineages from one another and represent a much-needed step forward in our understanding of gregarine biodiversity.

Molecular phylogeny and systematics of the genus *Lecudina*

Except for the genus *Ascogregarina* Ward *et al.*, 1982, host organisms for gregarines within the family Lecudinidae are exclusively marine invertebrates. The genus *Lankesteria*, for example, refers to gregarines that only infect urochordates (Ormières, 1965; Vávra, 1969; Levine, 1977; Perkins *et al.*, 2000; Leander, 2008; Rueckert & Leander, 2008). In contrast, species within the genus *Lecudina* have been described from chaetognaths, echiurans, nemerteans, oligochaetes and polychaetes (see Levine, 1974). Most species of the genus *Lecudina*, however, have been described from polychaetes. In this study, we describe one novel species of the genus *Lecudina* from a new polychaete host, namely the parchment tubeworm *Phyllochaeopterus prolifica* (Polychaeta). Although many of the known species of the genus *Lecudina* are

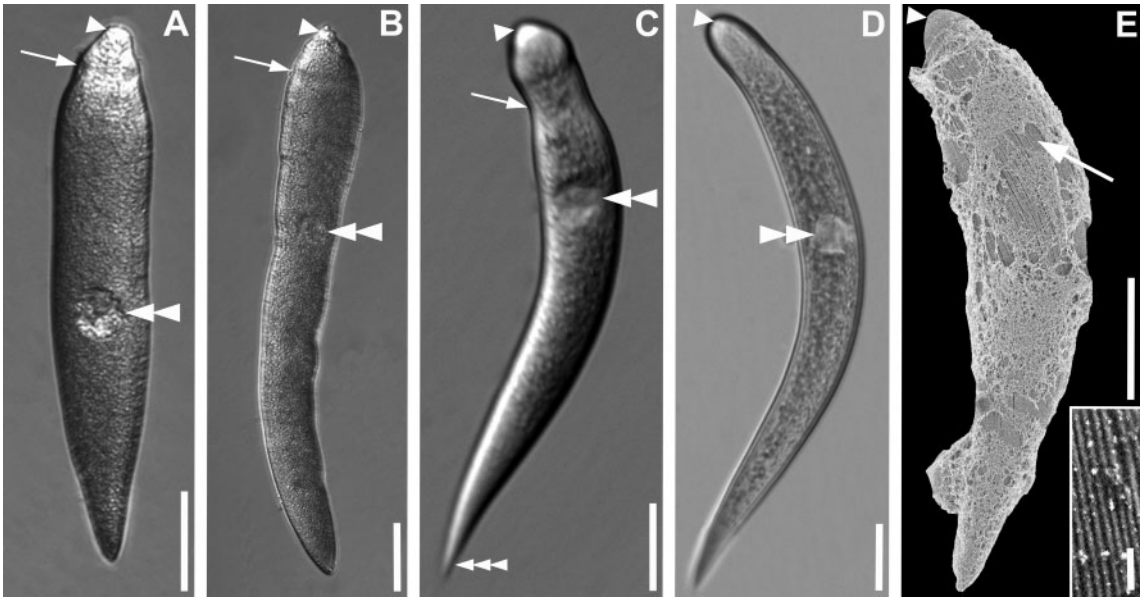

Fig. 3. LM and SEM showing the general trophozoite morphology of *Difficilina paranemertis* sp. nov. and *Difficilina tubulani* sp. nov. Small subunit rDNA sequences were obtained from trophozoites with these morphologies. (a, b) Trophozoites of *Difficilina paranemertis* sp. nov. with narrowly spatulate cell shapes. The mucron (single arrowhead) was rounded and the posterior end was more pointed. The spherical nucleus (double arrowhead) was situated in the middle of the cell. A constriction (arrow) was visible at the base of the mucron. Bars, 45 μm (a); 50 μm (b). (c, d) These images were collected with our portable field-based microscopy system. Trophozoites of *Difficilina tubulani* sp. nov. with a rounded mucron (single arrowhead) and sharply pointed posterior end (triple arrowhead). The spherical to oval nucleus (double arrowhead) was situated in the middle to anterior half of the cell. A neck-like constriction (arrow) was visible at the base of the mucron. Bar, 40 μm (c); 35 μm (d). (e) SEM of a trophozoite of *Difficilina tubulani* sp. nov. showing the rounded mucron (single arrowhead) and the longitudinally oriented epicytic folds (arrow). The sticky and viscous consistency of the nemertean (host) tissue formed a layer of mucus over the trophozoite that limited the clarity of the underlying surface folds in the SEM preparation. Bar, 15 μm. The inset shows a higher magnification view of the epicytic folds. Bar, 1 μm.

conspicuously elongated, *Lecudina phyllochaetopteri* sp. nov. was elliptoid with rounded anterior and posterior ends and was superficially reminiscent of rhytidocystid apicomplexans (Leander & Ramey, 2006). This novel species was placed within the genus *Lecudina* because the SSU rDNA sequence clustered strongly with other species of the genus, namely *Lecudina longissima* and *Lecudina tuzetae*. The genus *Lecudina*, in general, is known for rigid trophozoites with an elaborately folded cell cortex and gliding motility (Levine, 1976; Leander *et al.*, 2003b). However, a novel feature of *Lecudina phyllochaetopteri* sp. nov. was the absence of any discernible epicytic folds. Leander *et al.* (2003b) suggested that the genus *Lecudina* was paraphyletic and probably gave rise to many different lineages (e.g. genera) of marine eugregarines independently. With the available data, we have decided to place *Lecudina phyllochaetopteri* sp. nov. within the genus *Lecudina*; however, it would not be surprising if future studies demonstrated a diverse lineage of eugregarines without epicytic folds occurring within parchment tubeworms. If this were to happen, then a new genus name for a clade consisting of *Lecudina phyllochaetopteri* sp. nov. and its relatives might be warranted. A similar outcome has already occurred within the family Lecudinidae in the genus *Lankesteria*; *Lankesteria ascidiae* (Lankester, 1872) Mingazzini, 1891, *Lankesteria chelyosomae* Rueckert & Leander, 2008, *Lankesteria cystodytae* Rueckert & Leander, 2008 and *Lankesteria parascidiae* Duboscq & Harant, 1932 have all replaced epicytic folds with epicytic knobs (Ormières, 1972; Ciancio *et al.*, 2001; Rueckert & Leander, 2008).

The trophozoites of *Lecudina phyllochaetopteri* sp. nov. were observed in two different states: (1) trophozoites had complete, undeformed margins when examined less than 3 h after the collection of the hosts (Figs 1a, b) and (2) trophozoites had notched margins in both LM and SEM observations when examined two or more days after the collection of the hosts (Fig. 1c). The cells were also covered with a very regular net of mucilaginous material that appeared to be extruded from the cell surface (Figs 1c–e). Similar extruded material has been observed on the surface of coelomic gregarines such as *Pterospora floridiensis* (Landers & Leander, 2005). We infer that the trophozoites displaying the notched margins and the extruded material were in a distressed state as a result of changes in the health of the host following several days of captivity (host worms

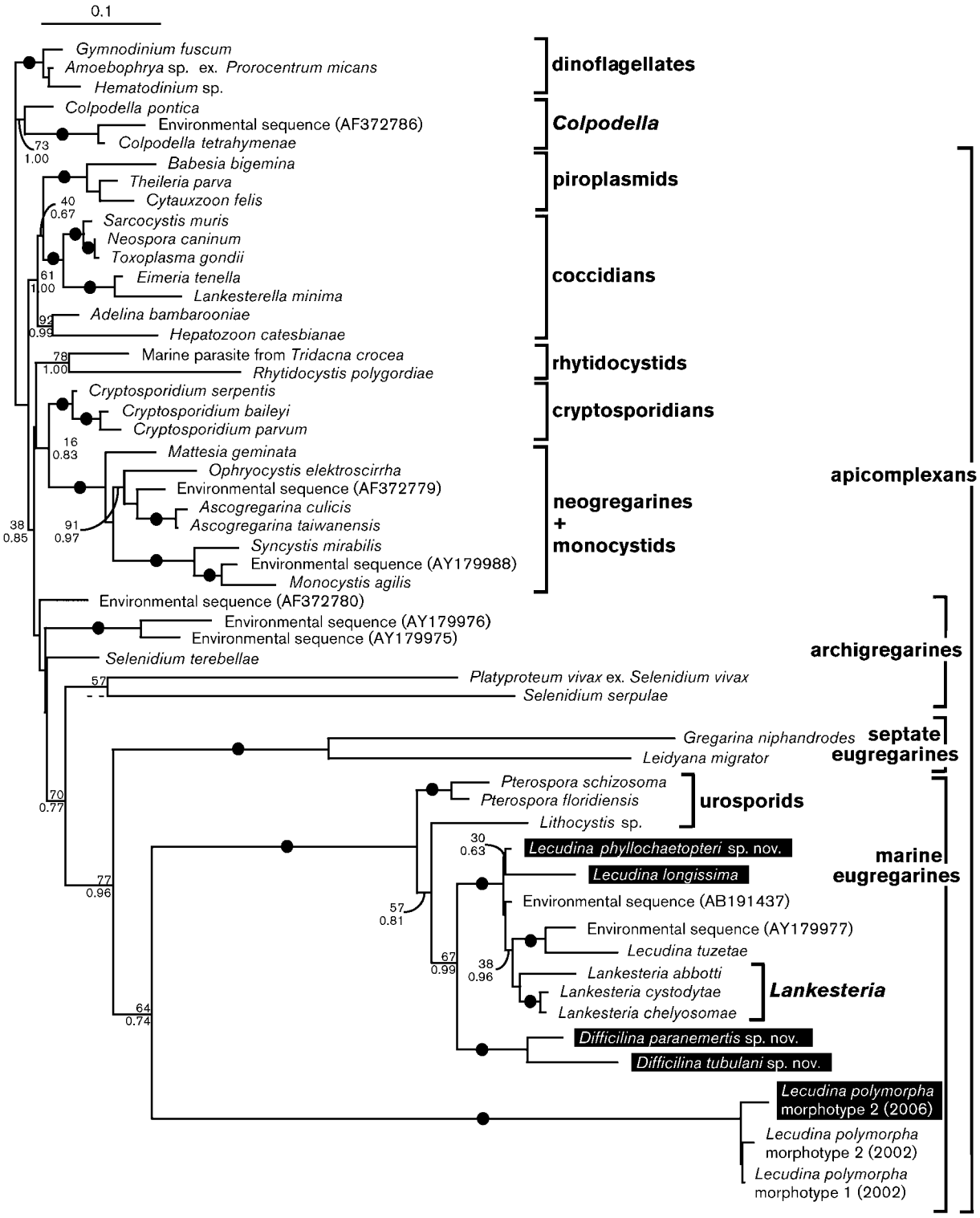

Fig. 4. ML phylogenetic tree as inferred using the GTR model of nucleotide substitutions, a gamma distribution and invariable sites on an alignment of 53 SSU rDNA sequences and 1175 unambiguously aligned sites (−ln L=15198.72287, α=0.417, fraction of invariable sites=0.051, eight rate categories). Numbers at the branches denote ML bootstrap percentages (top) and Bayesian posterior probabilities (bottom). Filled circles on branches denote Bayesian posterior probabilities $\geqslant$0.95 and ML bootstrap percentages $\geqslant$95 %. The sequences derived from this study are highlighted in the shaded boxes. The GenBank accession numbers for the sequence data used to construct the tree are available in Supplementary Table S1 in IJSEM Online. Bar, 0.1 substitutions per site.

were collected at 20 m depth). Our data also suggest that the absence of epicytic folds is irrespective of this state, since longitudinal striations were never evident in the trophozoites with complete margins when viewed at ×1000 magnification and with DIC optics (Figs 1a, b).

Nonetheless, this study more precisely identified the Pacific host species for *Lecudina polymorpha*, namely the errant polychaete *Lumbrineris japonica*. Two morphotypes of this gregarine species have been described previously from '*Lumbrineris* sp.' collected from the same Pacific habitat (Leander *et al.*, 2003b). The trophozoites described here and those in the previous study closely match the original description of *Lecudina polymorpha* (Schrével, 1963). The different morphotypes found in *Lecudina polymorpha* reflect the high degree of cell plasticity that is typically found in gregarine populations of the same species. The SSU rDNA sequences from different morphotypes of *Lecudina polymorpha*, collected in different years, provide a preliminary foundation for interpreting trophozoite plasticity when trying to establish species boundaries. For instance, the three known sequences from *Lecudina polymorpha* are extraordinarily divergent, relative to other gregarine sequences, and are still only 0.6–0.8 % different from one another. This might be the range of SSU rDNA sequence variation we can expect within gregarine species having highly divergent DNA sequences. Moreover, because these three (divergent) sequences cluster in a strongly supported clade that forms the sister group to the clade consisting of all other marine eugregarines (e.g. *Pterospora*, *Lithocystis*, *Lankesteria*, *Difficilina* and *Lecudina*), the question arises whether *Lecudina polymorpha* should be reclassified within a new genus. Although we raise the issue here, we have chosen to leave this species within the genus *Lecudina* until additional SSU rDNA sequences from gregarines more closely related to *Lecudina polymorpha* have been determined. Nonetheless, the range of SSU rDNA variation in gregarine species with less divergent sequences (e.g. *Selenidium terebellae*) might be much narrower. Future research on molecular and morphological variation within gregarine species is expected to shed considerable light onto the utility of DNA barcoding approaches for understanding the overall diversity and systematics of this group of parasites.

Molecular phylogeny and systematics of the genus *Difficilina*

Host ranges delimit several gregarine genera within the family Lecudinidae (e.g. *Lankesteria*). The genus *Difficilina* is defined to encompass aseptate gregarine species that infect nemertean hosts (Simdyanov, 2009). In addition to the two species we describe here with molecular phylogenetic data and the type species *Difficilina cerebratuli* from *Cerebratulus barentsi* Bürger, 1895 (Simdyanov, 2009), there are three gregarine species described in the literature that also occur in nemerteans. (1) *Urospora nemertis* (Kölliker, 1845) Schneider, 1875 was described in 1845 from the intestines of the nemertean *Baseodiscus delineatus* (formerly *Nemertes delineatus*) (Kölliker, 1848), (2) *Lecudina linei* Vinckier, 1972 was described from the intestines of *Lineus viridis* (Levine, 1976; Vinckier, 1972) and (3) *Lecudina myoisophagou* Trefouël, Arnoult & Vernet, 2000, was isolated from the nemertean *Ramphogordius lacteus* (formerly *Myoisophagus lacteus*) (Trefouël *et al.*, 2000). Although only four species from different nemertean hosts have been formally described, gregarine parasites have also been reported without formal descriptions. In total, 17 nemertean species are known to act as host organisms for gregarines (ten heteronemerteans and seven hoplonemerteans) (Jennings, 1960; Roe, 1976; Kajihara *et al.*, 2001; McDermott, 2006).

It is generally thought that gregarines are host-specific at some level. Perkins *et al.* (2000) stated that most gregarines are stenoxenous, which means that they are able to mature in only one host genus or perhaps only one host species. The results of the present study and former studies indicate that the lineages within some genera are limited to certain host groups (Perkins *et al.*, 2000; Rueckert & Leander, 2008). For instance, at one time, the genus *Lankesteria* included species that infected platyhelminths, urochordates, chaetognaths and arthropods; however, Ormières (1965) erected new genera for several species within the genus *Lankesteria* in accordance with their host ranges (compare Rueckert & Leander, 2008). Available molecular phylogenetic data support this taxonomic criterion; Fig. 4 demonstrates that the genus *Ascogregarina* (which was once considered *Lankesteria*) and the genus *Lankesteria sensu stricto* (from the intestines of urochordates) are only distantly related to one another. Members of the genus *Ascogregarina* cluster strongly with terrestrial neogregarines and monocystids, and species of the genus *Lankesteria* form a clade that is nested within marine species of the genus *Lecudina*. Likewise, the genus *Difficilina* includes gregarines that infect nemerteans, and the two species described here form a distinct and strongly supported clade that forms the sister group to the clade consisting of *Lecudina–Lankesteria* sequences (excluding *Lecudina polymorpha*). These robust molecular phylogenetic data, combined with the distinctiveness of the nemertean hosts, bolster the justification for this genus of marine gregarines and it would not be surprising if future molecular phylogenetic studies demonstrate that previously described gregarines from nemerteans (e.g. *Lecudina linei*, *Lecudina myoisophagou* and *Urospora nemertis*) cluster within the *Difficilina* clade.

TAXONOMY

Phylum Apicomplexa Levine, 1970

Order Eugregarinorida Léger, 1900

Family Lecudinidae Kamm, 1922

Genus *Lecudina* Mingazzini, 1891

Lecudina phyllochaetopteri Rueckert & Leander sp. nov. (Figs 1a–e)

Description. Trophozoites elliptoid; mean length 29 μm (range 24–32 μm), mean width 14 μm (range 11–15 μm); brownish in colour. Rounded anterior and posterior ends; nipple-like mucron that is free of amylopectin granules; spherical nucleus (8–9 μm diameter) in posterior half of cell. Epicytic folds absent. Small subunit rDNA sequence is GenBank accession no. FJ832156.

Holotype. Figs 1(a, b).

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 (Collection number: MI-PR101).

Type locality. Wizard Islet (48° 51′ 6″ N, 125° 09′ 4″ W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada.

Habitat. Marine, rocky sediment at 20 m depth.

Etymology. Refers to the genus of the polychaete type host, *Phyllochaetopterus prolifica*.

Type host. *Phyllochaetopterus prolifica* (Metazoa, Annelida, Polychaeta, Chaetopteridae).

Location in host. Intestinal lumen.

Genus *Difficilina* Simdianov, 2009

Difficilina paranemertis Rueckert & Leander sp. nov. (Figs 3a, b)

Description. Trophozoites narrowly elliptoid to narrowly spatulate, slightly crescent-shaped, mean length=398 μm (range 240–480 μm), mean width=48 μm (range 40–55 μm), brownish in colour. Rounded mucron that is free of amylopectin. Nipple-like structure at the mucron sometimes present. Nucleus spherical to elliptoid (20–25 μm) and positioned in the middle or in the anterior half of the trophozoite. Surface covered with longitudinal epicytic folds. Trophozoites rigid and capable of gliding motility. Small subunit rDNA sequence is GenBank accession no. FJ832159.

Holotype. Figs 3(a, b).

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 (Collection number: MI-PR102).

Type locality. English Bay Beach (49° 17′ 18″ N, 123° 08′ 37″ W), Downtown Vancouver, Canada.

Habitat. Marine.

Etymology. Refers to the genus of the nemertean type host, *Paranemertes peregrina*.

Type host. *Paranemertes peregrina* (Metazoa, Bilateria, Nemertea, Emplectonematidae).

Location in host. Intestinal lumen.

Difficilina tubulani Rueckert & Leander sp. nov. (Figs 3c, d)

Description. Trophozoites very narrowly obdeltoid and distinctly crescent-shaped, mean length=309 μm (range 270–350 μm), mean width=39 μm (range 35–40 μm), brownish in colour. Rounded mucron that is free of amylopectin; posterior end sharply pointed. Spherical nucleus (20–25 μm diameter) situated in the middle or anterior half of the cell. A more or less prominent neck-like constriction between the mucron and the nucleus. Longitudinally oriented epicytic folds. Mucron area free of folds. Cells rigid, capable of gliding motility. Small subunit rDNA sequence is GenBank accession no. FJ832160.

Holotype. Figs 3(c, d).

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 (Collection number: MI-PR103).

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

Habitat. Marine.

Etymology. Refers to the genus of the nemertean type host, *Tubulanus polymorpha*.

Type host. *Tubulanus polymorpha* (Metazoa, Bilateria, Nemertea, Tubulanidae).

Location in host. Intestinal lumen.

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