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ORIGINAL ARTICLE

Molecular phylogeny of the Lecudinoidea (Apicomplexa): A major group of marine gregarines with diverse shapes, movements and hosts

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Abstract

Gregarine apicomplexans are ubiquitous endosymbionts of invertebrate hosts. Despite their ecological and evolutionary importance, inferences about the phylogenetic relationships of major gregarine groups, such as the Lecudinidae and Urosporidae, have been hindered by vague taxonomic definitions and limited molecular and morphological data. In this study, we investigated five gregarine species collected from four families of polychaete hosts (Nereididae, Oenonidae, Hesionidae, and Phyllodocidae) using light microscopy (LM) and scanning electron microscopy (SEM). We also generated small subunit ribosomal DNA sequences from these species and conducted molecular phylogenetic analyses to elucidate the evolutionary relationships within the Lecudinoidea. Our results include new molecular and morphological data for two previously described species (*Lecudina* cf. *platynereidis* and *Lecudina* cf. *arabellae*), the discovery of a new species of *Lecudina* (*L. oxydromus* n. sp.), and the discovery of two novel species, namely *Amplectina cordis* n. gen. et. n. sp. and *Sphinctocystis inclina* n. sp. These two species exhibited unique shapes and movements, resembling those of urosporids but with a phylogenetic affinity to lecudinids, blurring the border between lecudinids and urosporids. Our study emphasizes the need for further investigations into this highly diverse group, which has achieved great success across multiple animal phyla with diverse shapes and movements.

KEYWORDS

Gregarinasina, *Lecudina*, molecular phylogeny, scanning electron microscopy, Urosporidae

INTRODUCTION

APICOMPLEXANS include important pathogens of animals (e.g. humans, livestock, and birds), causing diseases such as malaria, toxoplasmosis, and cryptosporidiosis (Current & Garcia, 1991; Sato, 2021; Weiss & Dubey, 2009). "Gregarines" form a major group of apicomplexans that infect the gut, coeloms and other extracellular spaces within diverse invertebrate hosts (Desportes & Schrével, 2013; Leander, 2008; Levine, 1977a). Discovery and documentation of gregarine diversity began in the early 1800s due to their relatively abundant and conspicuous feeding stage, called the "trophozoite". As a result, more than 1600 species in over 240 genera of gregarines have been described so far (Desportes & Schrével, 2013; Perkins et al., 2000). However, gregarine systematics in general remains largely unresolved due to several factors. First, most species were described with only line drawings of usually trophozoites, because documenting different, inconspicuous life stages requires intensive

sampling and effort (Schrével, 1969, 1970). Second, reliable characters for classification at higher and lower taxonomic levels are not readily available in gregarines (Clopton, 2009; Kamm, 1922; Levine, 1977a; Perkins et al., 2000). In this context, molecular phylogenetic data combined with comparative ultrastructural data has become a highly useful tool to elucidate important characteristics and resolve phylogenetic relationships within the group (Landers & Leander, 2005; Leander, 2008; Leander et al., 2006; Paskerova et al., 2021; Rueckert & Leander, 2008; Simdyanov et al., 2017; Wakeman & Leander, 2013).

Traditionally, three subgroups have been recognized within the Gregarinasina: archigregarines, eugregarines, and neogregarines (Grassé, 1953; Perkins et al., 2000). Eugregarines (Eugregarinorida Léger, 1900) are further classified based on the presence or absence of a septum dividing a cell into a protomerite and a deutomerite, resulting in two groups: septate and aseptate gregarines (Chakravarty, 1960; Clopton, 2009; Levine, 1971). Most marine aseptate eugregarines are placed within two families: Lecudinidae Kamm, 1922 and Urosporidae Léger 1892 (Levine, 1977a, 1977b). The Lecudinidae was established to contain *Lecudina* with a description stating "non-septate gregarines inhabiting digestive tract of polychaetes. Epimerite a simple knob" (Kamm, 1922). Later, this group was redefined as "gamonts elongate, with gliding rather than pendulum-like or coiling motion; first stage of development generally intracellular; syzygy present; oocysts ellipsoidal or ovoid with wall slightly thickened at one end; intestinal parasites of annelids, sipunculids, echiurids or arthropods" (Levine, 1977a; Perkins et al., 2000). Over several decades, many gregarine genera from annelids (polychaetes, echiurans, and sipunculids), as well as genera from arthropods, were classified within the Lecudinidae (24 genera are currently registered in the World Register of Marine Species; WoRMS), mostly because the trophozoites had a relatively simple morphology and the definition of the group was broad (Grassé, 1953; Levine, 1977a). Consequently, this family became a taxonomic mixture containing most marine eugregarines, as mentioned by several authors (Levine, 1977a; Perkins et al., 2000; Rueckert et al., 2015; Simdyanov, 2004). Although insect-infecting genera such as *Ascogregarina* and *Kofoidina* were moved out of the Lecudinidae, this group still represents a 'hodge-podge' of taxa with unclear phylogenetic affinities (Ormières, 1965; Rueckert et al., 2015; Simdyanov, 2004).

The taxonomy of the Urosporidae Léger 1892 is also unclear (Levine, 1977b). This group includes gregarines isolated from annelids (polychaetes, sipunculids, echiurans), echinoderms (sea cucumbers and sea urchins), nemerteans, and gastropods. It is defined as "mucron more or less marked; syzygy frontal or lateral; oocysts with dissimilar ends, with an anterior neck and a more or less marked posterior prolongation; oocyst with a well-differentiated wall" (Levine, 1977b). With a stricter definition than the Lecudinidae, the Urosporidae includes six genera: *Urospora* Schneider, 1875, *Gonospora* Schneider, 1875, *Lithocystis* Giard, 1876, *Ceratospora* Léger, 1892, *Pterospora* Labbé and Racovitza, 1897, and *Paragonospora* Lang, 1954. Among these, molecular data are available for three genera: *Urospora*, *Lithocystis*, and *Pterospora* (Diakin et al., 2016; Leander et al., 2006). Additionally, *Difficilina*, which is highly similar to *Lankesteria* based on morphology (Simdyanov, 2009), is grouped with species of *Urospora* (Diakin et al., 2016; Rueckert et al., 2010; Valigurová et al., 2023).

The phylogenetic affinity between Lecudinidae and Urosporidae has been shown in several previous molecular phylogenetic analyses, supporting the Lecudinoidea (Simdyanov et al., 2017). However, within the Lecudinoidea, the interrelationships among the major lineages have not been clearly resolved. For instance, two major groups of Lecudinidae, *Lecudina* and *Lankesteria*, have not been resolved as monophyletic (Iritani et al., 2021). Despite *Lecudina* alone comprising over 40 described species, molecular data are available for only 6 species (Iritani, Wakeman, & Leander, 2018; Odle et al., 2024; Rueckert et al., 2010, 2011; Schrével et al., 2016). *Lankesteria* is also relatively well known with more than 40 described species, among which 14 species have molecular data. In an endeavor to expand our understanding of the phylogenetic and morphological diversity of marine gregarines, we investigated polychaete hosts for gregarines across benthic and intertidal habitats. Here, we present new molecular phylogenetic and comparative morphological data on five species of Lecudinoidea, including three novel species and two previously described species that lacked molecular data. Among these five species, *Amplectina cordis* n. gen. et n. sp. and *Sphinctocystis inclina* n. sp. demonstrated features not typical of *Lecudina*, particularly in their movement. With the increased amount of morphological and molecular data, we address the phylogenetic relationships and the known overall diversity within the Lecudinoidea.

MATERIALS AND METHODS

Sample collection

The polychaete hosts examined in this study were collected from two different locations in British Columbia, Canada: Harriot Bay on Quadra Island and Grappler Inlet in Bamfield. Host specimens were collected by SCUBA diving in Harriot Bay and during low tide in Grappler Inlet. In Harriot Bay, *Lecudina* cf. *platynereidis*, *Lecudina oxydromus* n. sp., and *Amplectina cordis* n. gen. et n. sp. were collected from *Platynereis* sp., *Oxydromus pugettensis*, and Phyllodocidae sp., respectively. In Grappler Inlet, *Lecudina* cf. *arabellae* and *Sphinctocystis inclina* n. sp. were collected from Oenonidae sp. and

Phyllodoce medipapillata, respectively. The collected animals were transported to a laboratory at the University of British Columbia (UBC), Canada. The polychaete specimens were dissected with precise forceps to extract gregarine cells from either the digestive tract or body cavity of the host. The gregarine cells were transferred to a glass slide for examination under a differential interference contrast (DIC) microscope. A Zeiss Axioplan 2 microscope or an Axiovert 200 inverted scope with a Zeiss Axiocam 503 color camera were used for micrographs. Individual gregarine cells or syzygies were isolated using a hand-drawn glass pipette and subsequently washed multiple times in filtered seawater and then kept in PCR tubes for single-cell PCR. The host tissue was stored in 95% ethanol for DNA extraction for host identification.

Single-cell PCR

Ten microliter of distilled water was added into the PCR tubes containing isolated gregarine cells (two gamonts associated in syzygy for *Amplectina cordis*). The tubes were then incubated at 56°C for 3 min to disrupt the cell and release DNA. Two rounds of PCR were conducted for nested PCR. For the first round of PCR, 22 μL of distilled water, 0.5 μL each of forward and reverse primers (GF2; 5′-TGCGCTACCTGGTTGATCC-3′ and SSUR4; 5′-GATCCTTCTGCAGGTTCACCTAC-3′), and 2 μL of DNA template were added in a 0.2 mL tube containing a PCR bead (Cytiva PuReTaq Ready-To-Go™ PCR Beads; Cytiva, Global Life Sciences Solutions, Marlborough, MA, USA). PCR reactions were conducted with the following conditions: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 7 min. The second round of PCR was conducted using 23 μL of distilled water, 0.5 μL each of forward and reverse primers (GF5; 5′-CCTGGTTGATCCTGCCAG-3′ and LL_R1; 5′-ACAGCTGCCGTGTTATGAC-3′), 1 μL of PCR product from the first round of PCR, and a PCR bead with the following conditions: an initial denaturation at 95°C for 3 min, 26 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 7 min. 5 μL of PCR product from each PCR reaction was run on a 1.5% agarose gel. PCR products were purified using ExoSap (Applied Biosystems™), and then the purified PCR products were sequenced by Sanger sequencing by Sequencing and Bioinformatics Consortium at the University of British Columbia, Canada, using the same primers used for PCR amplification.

Host identification

COI sequences were obtained for host identification. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). For PCR, 12.3 μL of distilled water, 4 μL of reaction buffer, 0.8 μL of LCO1490 and HCO2198 primers (Folmer et al., 1994), 0.1 μL of MyTaq (Bioline), and 2 μL of DNA were used. PCR conditions were following: 94°C initial denaturation for 5 min, 35 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 40 s, and final extension for 10 min at 72°C. For species for which COI could not be obtained, 18S sequences were instead obtained using GF2 and SSUR4 primers under the conditions described above. PCR product purification and sequencing were carried out as described above.

Scanning electron microscopy

Live gregarine cells were picked up using a hand-drawn glass pipette and then transferred to a Swinnex filter holder containing an isopore membrane (pore size = 10 μm), which was filled with filtered seawater. The cells were fixed with osmium vapor for 10 min, followed by the addition of 6–7 drops of 4% osmium tetroxide directly into the Swinnex filter holder for additional fixation for 10 min. The osmium-fixed cells were rinsed twice with filtered seawater. Ethanol dehydration was performed in a series, starting with 30% to 50%, 70%, 80%, 85%, 90%, 95%, and finally 100% ethanol. The membrane was transferred to a metal basket submerged in 100% ethanol for critical point drying using the Tousimis autosamdri 815B Critical Point Dryer. Once dried, the membrane with specimens was placed onto a stub and a 2 nm layer of platinum gold was sputter coated using either the Cressington 208HR high-resolution sputter coater or the Leica EM ACE600 Coater. Images were captured using Zeiss Crossbeam XB350. SEM processing and imaging were conducted at the UBC Bioimaging Facility.

Phylogenetic analyses

Two datasets were prepared for apicomplexans and lecudinoideans. The apicomplexan tree comprised 88 SSU sequences, including five species reported in this study, as well as representatives of major groups of gregarines, other apicomplexans, and outgroups (ciliates, dinoflagellates, and stramenopiles). The second dataset was made for Lecudinoidea, consisting of 53 SSU sequences, including the five species reported in this study. Additionally, it included at least one sequence representing each known species of Lecudinoidea (*Lecudina*, *Lankesteria*, *Pterospora*, *Lithocystis*, *Difficilina*, *Urospora*, *Veloxidium*), as well as several environmental sequences. DNA sequences were aligned using the MAFFT algorithm (Katoh and Standley, 2013) within Geneious Prime. Ambiguous sites were subsequently removed from the SSU rDNA alignments using Gblocks (Castresana, 2000), resulting in an alignment of 1239 bp for the apicomplexan tree and 1377 bp for

the lecudinoidean tree. All phylogenetic analyses were conducted on CIPRES Science Gateway v3.3 (Miller et al., 2010). The best-fitting models of nucleotide evolution by using jModelTest v2.1.6 (Darriba et al., 2012). Based on the corrected Akaike information criterion (AICc), GTR+I+G was chosen as the best model for both datasets. Maximum likelihood (ML) trees were inferred using IQ-tree 2.2.2.7 (Nguyen et al., 2015). Rapid bootstrap analysis was conducted with 1000 replicates. MrBayes 3.2.7 was used for Bayesian inference (Ronquist et al., 2012). Two independent runs, consisting of four chains each, were simultaneously conducted for 5 million (for the apicomplexan tree) and 2 million (for the lecudinoidean tree) generations with a sampling frequency of 1000. The initial 25% of samples were discarded. The resulting trees were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Intraspecific genetic divergence among representative lecudinoidean species based on SSU rDNA sequences was obtained using Kimura 2 parameter (K2P) distance model in MEGA11 (Tamura et al., 2021).

RESULTS

Morphology

Lecudina cf. *platynereidis* Schrével, 1970

Trophozoites appeared bottle-like, with a rounded or dome-shaped anterior area (Figure 1A). The cells measured 100 μm long (95–110 μm, $n=4$) and 25 μm wide (23–27 μm, $n=4$). An oval-shaped nucleus was situated just posterior to the center of the cell and measured 21 μm long (20–22 μm, $n=3$) and 16.5 μm wide (15–18 μm, $n=3$) (Figure 1A). An apical papilla (an extruded structure at the tip of the epimerite) and longitudinal epicytic folds (2.5 folds/μm) were observed with SEM (Figure 1B). The longitudinal folds stopped near the posterior end, demonstrating a slight spiral pattern (Figure 1C). Gliding motility was observed, with the anterior part being capable of bending.

Lecudina cf. *arabellae* Hoshide, 1958

Trophozoites were easily recognized even under a stereoscope due to their large size, measuring 540 μm long (450–600 μm, $n=5$) and 55 μm wide (50–60 μm, $n=5$). The anterior end of the cell was blunt, while the posterior region tapered to a terminal point. A round or oval-shaped nucleus was situated near the anterior end of the cell (Figure 2A). The nucleus was 29 μm long (20–35 μm, $n=4$) and 24 μm wide (15–30 μm, $n=4$), with some cells exhibiting a double nucleolus (Figure 2B). SEM images of the trophozoites revealed an apical papilla at the anterior end, a tapered posterior end (Figure 2C), and a dense array of longitudinal epicytic folds with 3–4 folds/μm (Figure 2D,E). Gliding motility was observed, with the anterior part being capable of bending.

Lecudina oxydromus n. sp.

Trophozoites were slightly bent showing a boomerang-like shape (Figure 3A,B). They appeared brown due

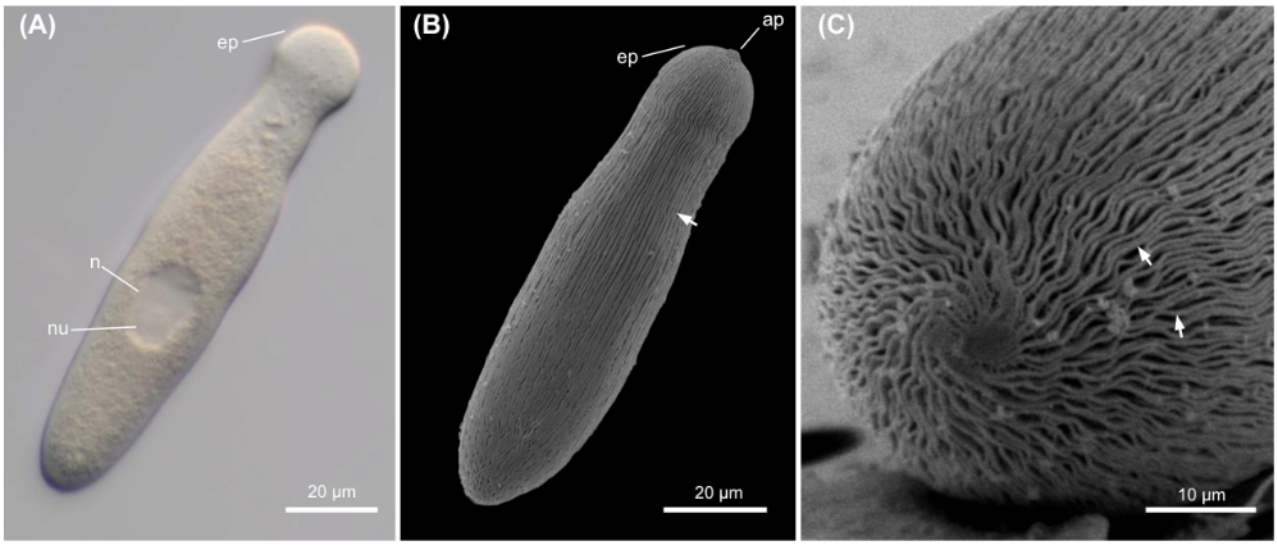

FIGURE 1 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of a trophozoite of *Lecudina* cf. *platynereidis*. (A) LM showing an elongated, bottle-like shaped trophozoite. The anterior end exhibits a ball- or dome-like shape. The oval nucleus is located near the middle of the cell slightly toward the posterior end (scale bar: 20 μm). (B) SEM showing dense longitudinal folds on the trophozoite. An apical papilla is visible at the tip of the epimerite (scale bar: 20 μm). (C) The posterior end of a trophozoite displaying dense epicytic folds with undulations, shows slight spiral patterns that stop near the end (scale bar: 10 μm). ap, Apical papilla; ep, Epimerite; n, Nucleus; nu, Nucleolous.

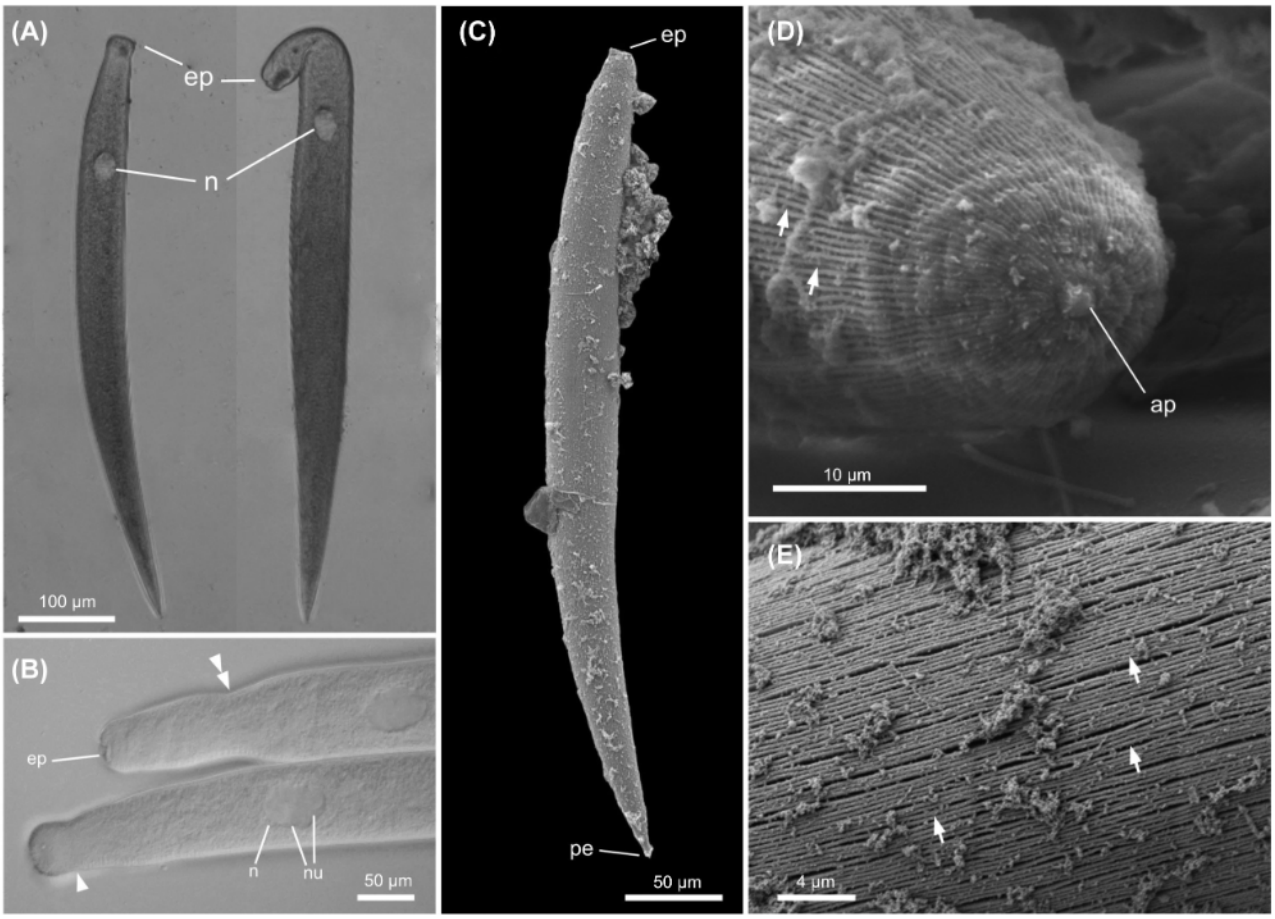

FIGURE 2 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of trophozoites of *Lecudina* cf. *arabellae*. (A) LM images of two different trophozoites observed on an inverted microscope showing slightly different shapes. Both cells exhibit a blunt anterior end and a sharp posterior end. A rounded nucleus is located between the middle and anterior end of the cell. The cell on the left shows a more crescent shape, while the one on the right shows a straight shape and bending movement at the anterior part, which was commonly observed in many cells (scale bar: 100 µm). (B) LM images of the anterior parts of two trophozoites. A slight indentation is observed after an epimerite (indicated with an arrowhead) or where the bending movement occurs (indicated with a double arrowhead). Some cells harbored two nucleoli. Some transverse striations were visible (scale bar: 50 µm). (C) SEM of a trophozoite showing a crescent shape, blunt end, and a sharp posterior end (scale bar: 50 µm) (D) SEM showing the anterior part of a cell showing an apical papilla (scale bar: 10 µm). (E) High magnification SEM showing longitudinal epicytic folds with a density of 3–4 folds/µm (scale bar: 4 µm). ap, Apical papilla; ep, Epimerite; n, Nucleus; nu, Nucleolus; pe, Posterior end.

to accumulated amylopectin granules in the cytoplasm, while the anterior part was devoid of granules (Figure 3A). Trophozoites measured 93 µm long (70–120 µm, $n=4$) and 29 µm wide (25–33 µm, $n=4$). A round nucleus was situated near the anterior end of the cell, measuring 13 µm long (10–18 µm, $n=3$) and 15 µm wide (12–18 µm, $n=3$) (Figure 3A). The longitudinal epicytic folds had a density of 4–5 folds/µm (Figure 3C). Gliding motility was observed.

Amplectina cordis n. gen. et n. sp.

Numerous syzygies (two gamonts associated laterally), as well as some single trophozoites, were found in one heavily infected host specimen (Figure 4). Gamonts associated with syzygy measured 135 µm long (130–140 µm, $n=4$) and 35 µm wide (30–40 µm, $n=4$). The nuclei of gamonts were 21 µm long (20–22 µm, $n=2$) and 21 µm wide ($n=2$). Single trophozoites were smaller, measuring 58 µm long (55–60 µm, $n=3$) and 17 µm wide (15–20 µm, $n=3$). The nuclei of single trophozoites were 13.5 µm long (12–15 µm, $n=2$) and 15 µm wide (12–15 µm, $n=2$). Both gamonts in syzygies (Figure 4A,B) and single trophozoites (Figure 4C,D) exhibited dynamic metabolic (or peristaltic) movement. Specifically, cytoplasmic contents consistently moved from one end to the other, forming a bulge that traversed the longitudinal axis. The position of the nucleus was not fixed but moved along with the cytoplasm.

Sphinctocystis inclina n. sp.

Trophozoites showed distinct annular constrictions under the light microscope (Figure 5A,B), were 333 µm

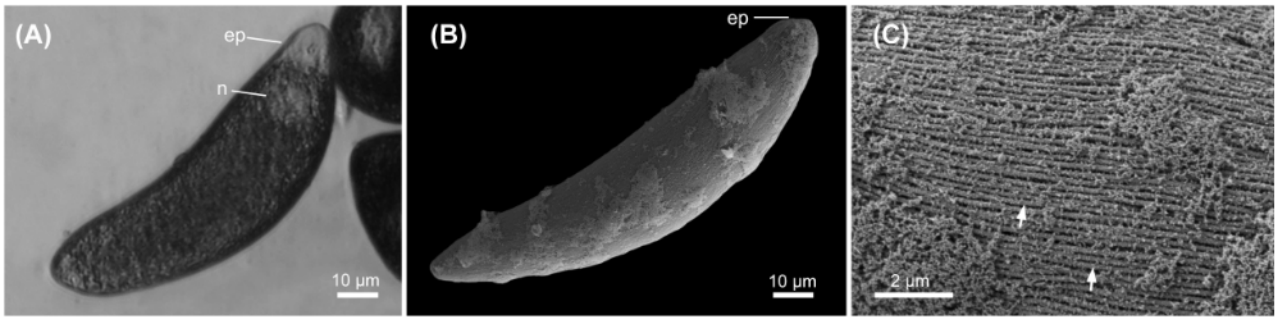

FIGURE 3 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of trophozoites of *Lecudina oxydromus*. (A) The trophozoite exhibits a crescent shape and brown color due to dense amylopectin granules. The anterior end lacks granules. An oval nucleus is situated near the anterior end (scale bar: 10 µm). (B) SEM image showing a crescent-shaped trophozoite (scale bar: 10 µm). (C) High magnification SEM showing dense longitudinal folds with a density of 4–5 folds per µm (scale bar: 2 µm).

long (295–500 µm, $n=5$) and were 52 µm wide (40–70 µm, $n=5$). An oval nucleus was located in the widest portion of the cell, which was just anterior to the center of the cell (Figure 5A,B). The nucleus was 30 µm wide ($n=3$) and 24 µm long (22–25 µm, $n=3$). The cell became narrower toward the rounded posterior end. Some trophozoites showed a rounded and smooth epimerite (Figure 5A,C). A noticeable apical papilla was not observed (Figure 5D). Dense epicytic folds with both straight and undulated patterns were observed with a density of 3 folds/µm (Figure 5C–E). Trophozoites were not rigid but rather flexible showing dynamic movements of gliding and bending. The anterior part of the cell was especially active with continuous bending movements (Figure 5A).

Molecular phylogenetic analyses

BLAST results showed that all five species we report in this study are closely related to *Lecudina*; they were distinguished from other known *Lecudina* species at the molecular level based on the genetic distance (Table S1). Specifically, *Lecudina oxydromus*, *Lecudina* cf. *arabellae*, and *Amplectina cordis* showed similarities to *Lecudina phyllochaetopteri* (94.12%, 90.07%, and 93.7%, respectively); this was followed by other *Lecudina* and *Lankesteria* species. *Lecudina* cf. *platynereidis* and *Sphinctocystis inclina* n. sp. were similar to environmental sequences (95.29% to AY179977 and 88.50% to AB191437, respectively). These two taxa also showed similarities to *L. phyllochaetopteri* at 89.26% and 87.95%, respectively. In both ML and Bayesian trees of apicomplexans, the five species were grouped with other lecudinid and urosporid species within the Lecudinoidea with maximal support (PP=1, BS=100). However, these trees were unable to resolve relationships among the major subgroups of gregarines (Figure 6). Additionally, the tree shows that many *Lecudina*-like gregarines (i.e. genera previously classified under Lecudinidae, highlighted in Figure 6) represented several different lineages of gregarines in the tree inferred from SSU rDNA sequences.

Both ML and Bayesian phylogenetic trees of the Lecudinoidea showed two groups corresponding to "Lecudinidae" and "Urosporidae" (Figure 7). All *Lankesteria* and *Lecudina* species were grouped together, although their monophyly was only strongly supported in the Bayesian tree (PP=1, BS=62). The clade containing *Lankesteria*, *Lecudina*, and *Amplectina cordis* n. gen. et n. sp. (= "Lecudinidae") was strongly supported (PP=1, BS=100). *Sphinctocystis inclina* n. sp. was sister to the clade of Lecudinidae; however, their sister relationship was only strongly supported in the Bayesian tree (PP=0.96, BS=77). All of the genera considered as Urosporidae (i.e. *Pterospora*, *Lithocystis*, *Urospora*) as well as *Difficilina* were grouped together, although their monophyly was only strongly supported in the Bayesian tree (PP=0.91, BS=70). The clade combining "Lecudinidae", "Urosporidae", and *Sphinctocystis inclina* n. sp. was strongly supported on the Bayesian tree (PP=0.98, BS=87), which was sister to a clade containing the recently described species *Undularius glycerae* and a few environmental sequences. *Veloxidium* was shown as a sister lineage to all other members of the Lecudinoidea (Figure 7).

Comparative morphology

Figure 7 also shows important trophozoite characters and host affinities for each species included in the analyses (host phylum, host family, cell surface feature, infection sites, movement, and syzygy). All *Lankesteria* species are associated with tunicate hosts, whereas all *Lecudina* species known to data with molecular data are associated with annelid hosts (i.e. polychaetes). The Urosporidae encompasses diverse host groups, such as sea urchins, nemerteans, and annelids. Longitudinal epicytic folds are widespread among lecudinoideans; however, other surface ornamentations like knobs, superfolds, transverse striations, and the absence of folds have also been observed. While most lecudinoideans are intestinal, some urosporids (*Pterospora*, *Lithocystis*, and *Urospora*) have been found in the

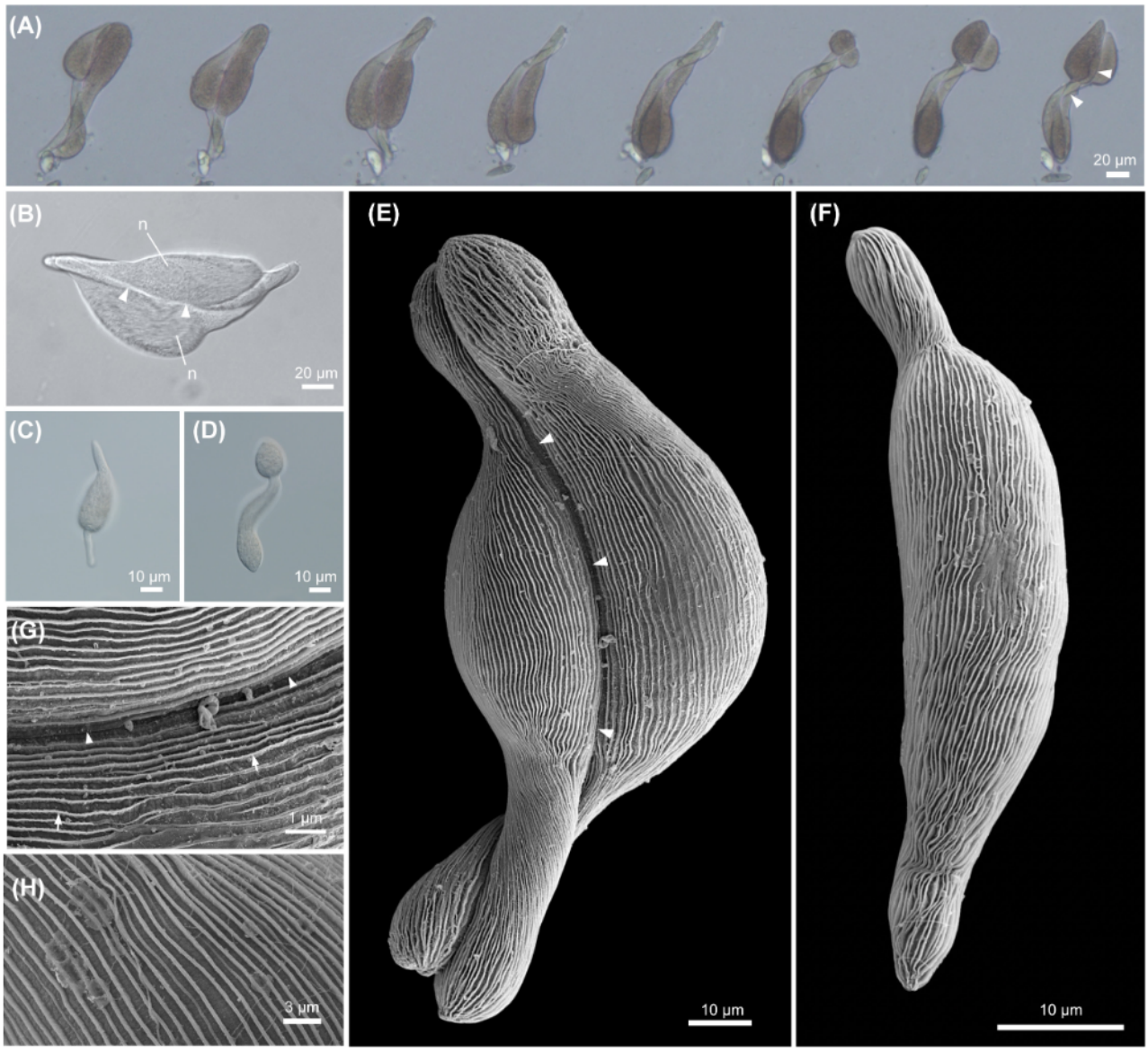

FIGURE 4 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the general morphology, movement, and surface ultrastructure of trophozoites and gamonts of *Amplectina cordis* n. gen. et n.sp. (A) sequential series of LMs of gamonts in syzygy observed with an inverted microscope, showing peristaltic movement of cells (scale bar: 20 μm). (B) LM image of gamonts in syzygy observed with a compound microscope. Nuclei in each gamont are visible (scale bar: 20 μm). (C and D) Two different single trophozoites also demonstrating peristaltic movement (scale bar: 10 μm). (E) SEM images of two gamonts in sygyzy showing longitudinal folds and the syzygy junction (indicated with arrow heads) (scale bar: 10 μm). (F) SEM image of a single trophozoite showing longitudinal folds (scale bar: 10 μm). (G) High magnification SEM showing syzygy junction (indicated with arrow heads) and longitudinal folds with a density of 2–3 folds per μm (scale bar: 1 μm). (H) High magnification SEM showing longitudinal folds 3–4-folds per μm (scale bar: 3 μm).

coelomic spaces of their hosts. Gliding mobility is common in the Lecudinidae and some *Lecudina* species have shown a slight bending movement near the anterior part (Ganapati, 1946a). However, peristaltic, metabolic, extensive bending and twisting movements have only been observed among non-lecudinid members of the Lecudinoidea. Syzygy remains largely unknown; however, lateral syzygy is the most common when observed.

DISCUSSION

All of the gregarine species reported in this study were aseptate gregarines found in polychaete hosts. Some exhibited characteristics of typical *Lecudina*, while others displayed novel shapes and movements resembling those of urosporids. We provide our rationale for species identification and the establishment of new species and genera below.

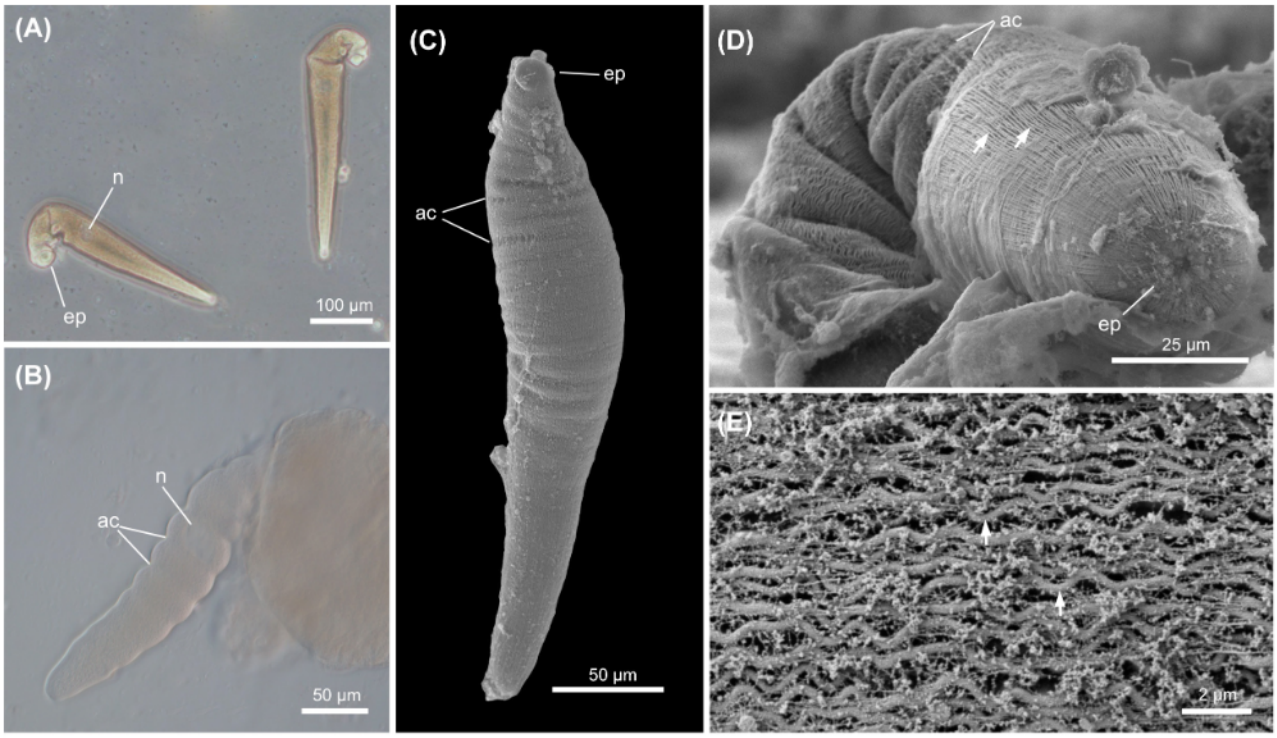

FIGURE 5 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of trophozoites and gamonts of *Sphinctocystis inclina* n. sp. (A) LM of two trophozoites observed on an inverted microscope. Both cells exhibit bending movement (scale bar: 100 µm). (B) LM of a trophozoite attached to host tissue showing annular constrictions. An oval shaped nucleus is located between the middle and the anterior end of the cell (scale bar: 50 µm). (C) SEM of a trophozoite showing annular constrictions (scale bar: 50 µm). The anterior end is smooth without noticeable apical papilla (scale bar: 50 µm). (D) SEM of a trophozoite showing its flexibility. Numerous annular constrictions are visible due to bending (scale bar: 25 µm). (E) High magnification SEM showing a dense array of undulating epicytic folds (scale bar: 2 µm).

Three species of *Lecudina*

Lecudina cf. *platynereidis*

Fourteen *Lecudina* species have been documented from Nereididae host species in the literature, among which two were discovered in *Platynereis*: *L. krusadiensis* Ganapati, 1946b, and *L. platynereidis* Schrével, 1969. *L. krusadiensis* was isolated from *P. abnormis* collected in India (Ganapati, 1946b; Théodoridès, 1969), while *L. platynereidis* was found in *P. massiliensis* and *P. dumerilii* in Europe (Schrével, 1969; Théodoridès, 1969). The *Lecudina* species identified in our study was isolated from *Platynereis* sp. (likely of a *Platynereis bicanaliculata* species complex) in Canada (PP815646; the COI sequence is identical to the *Platynereis* sp. collected previously in Bamfield, BC and Alaska). This gregarine species exhibited a distinctive bottle-like shape with a domed anterior end, closely resembling *L. platynereidis* described in Europe (Schrével, 1969). Without comparing molecular sequences, we cannot definitively confirm if this species is identical to *L. platynereidis*. However, some *Lecudina* species have shown to have the ability to infect multiple congeners and exhibit a wide geographic distribution (Odle et al., 2024; Rueckert et al., 2011). Therefore, based on the highly similar shape and host association (*Platynereis*), we classify this species as *Lecudina* cf. *platynereidis* (PP819648).

Lecudina cf. *arabellae*

This species is characterized by large trophozoites (540 µm × 55 µm), a blunt anterior end, and a tapering posterior end (Figure 3). This species was found in *Arabella iricolor* (18S: PP819654), and only one gregarine species (*Lecudina arabellae* Hoshide, 1958) has been found in the same host species (Levine, 1976). Although there is no molecular data available from the original description of *L. arabellae*, our isolate from the same host species has highly similar trophozoites, including the large size, blunt anterior ends, and tapering posterior ends—suggest that they are the same species. In addition, *L. arabellae* was discovered in two distant locations (Asia and Europe) indicating a wide geographic range. Although it is possible that both the hosts and the gregarines are of species complexes, we classify the species we found from *Arabella iricolor* in Canada as *Lecudina* cf. *arabellae* (PP819649).

Lecudina oxydromus n. sp.

The trophozoites of this species closely resemble those of *Lankesteria* species, exhibiting a crescent shape. This species was found in the polychaete host *Oxydromus pugettensis* (18S: PP819653) in the family Hesionidae. Only one gregarine species is known from the Hesionidae:

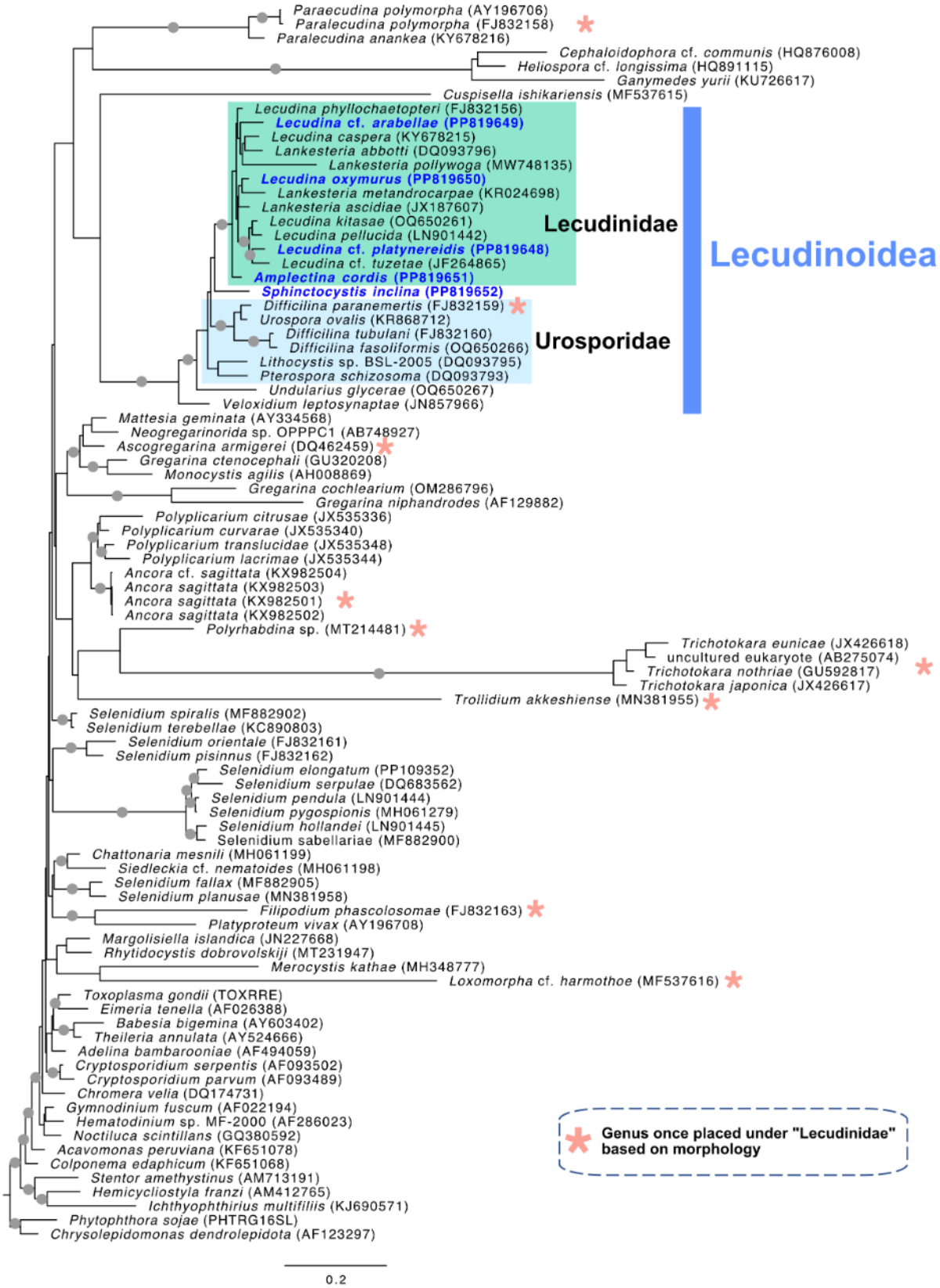

FIGURE 6 A phylogenetic tree of apicomplexans inferred from 88 SSU rDNA sequences and 1239 sites using IQ-tree. Only branches with strong support (BS>90 on IQ-tree, PP>0.95 in MrBayes) are shown with gray circles. Although some family-level and genus-level groups received strong support, the relationships among them are not clear. All the sequences reported in this study (highlighted in blue font) belong to the Lecudinoidea (highlighted with a blue bar). Lecudinidae (green box) and Urosporidae (light blue box) are shown. Genera that were once placed within the Lecudinidae are highlighted with pink asterisks.

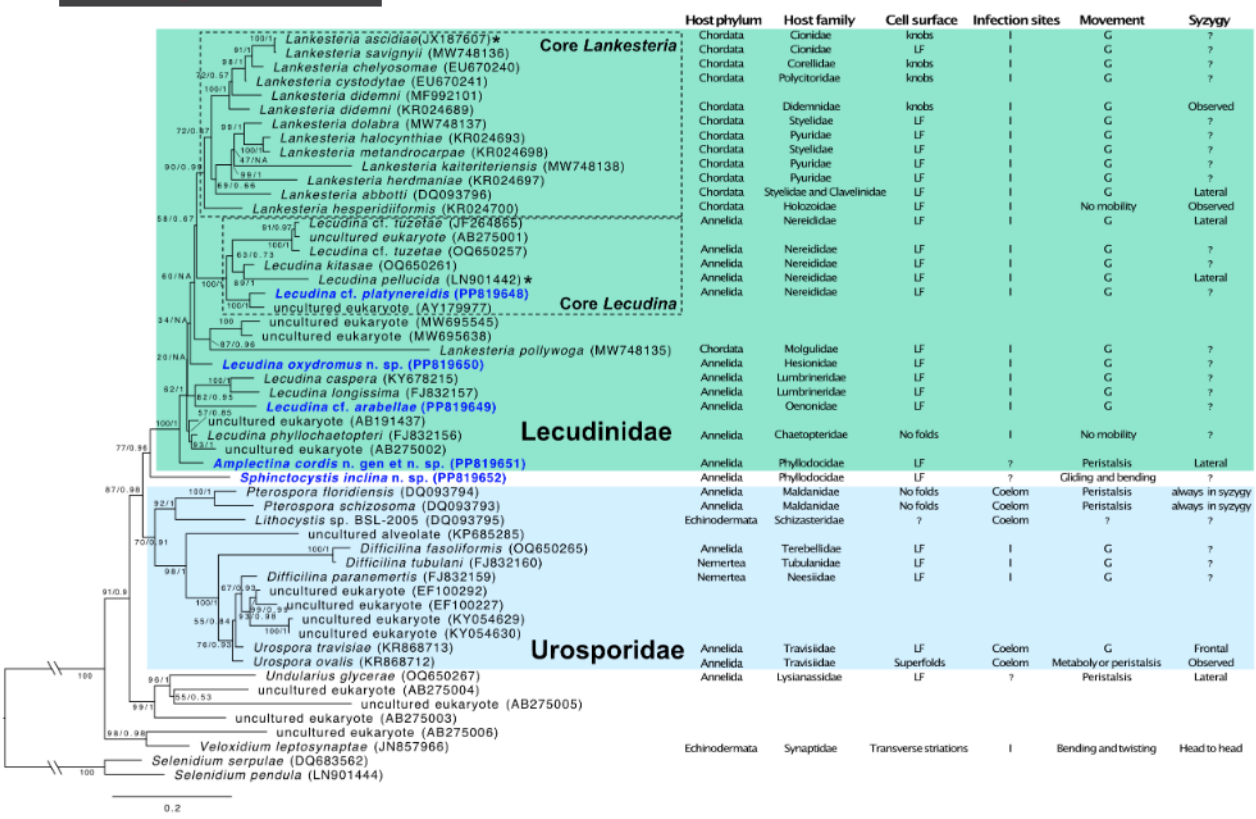

FIGURE 7 A phylogenetic tree of the Lecudinoidea inferred from 53 SSU rDNA sequences and 1377 sites using IQ-tree. Both bootstrap support (BS) from IQtree and posterior probabilities (PP) from MrBayes analyses are displayed for internal branches. Two major families of Lecudinoidea, "Lecudinidae" and "Urosporidae", are shown (highlighted with colored boxes). Well-supported clades containing the type species of *Lankesteria* and *Lecudina* are highlighted with dashed boxes. Host information, as well as some important characteristics of the trophozoites (cell surface, infection sites, movement, and syzygy), are shown next to the tree. LF, Longitudinal folds; I, intestinal; G, Gliding; ?, Unknown. 'observed' under the syzygy category indicates that syzygy was observed but the exact orientation was not determined.

Lecudina hesionis Théodoridès, 1969 isolated from *Hesione pantherina* (Théodoridès, 1969). However, *L. hesionis* showed distinct transverse striations which were not observed on our species (Figure 3). Our phylogenetic tree inferred from SSU rDNA sequences demonstrates that this species is distinct from other known *Lecudina* species at the molecular level (Table S1). Therefore, we establish this new species here.

Two novel species closely related to *Lecudina* but showing urosporid-like behavior

Amplectina cordis n. gen. et n. sp.

This species was discovered in a single heavily infected host specimen of an undescribed species of Phyllodocidae (COI: PP815648). Two gregarine species have previously been identified in Phyllodocidae species: *Lecudina phyllodocis* Théodoridès, 1969, and *Sphinctocystis phyllodoces* Simdyanov, 2004. However, the shapes and movements of this gregarine species are distinct from those of *L. phyllodocis* and *S. phyllodoces*. This gregarine species exhibited novel peristaltic movements, generating waves of bulges moving across the cell length, which closely resembles the shape and movement described in *Undularius glycerae*, a recently discovered species found in *Glycera* sp. (Glyceridae) (Odle et al., 2024). Despite their highly similar morphology, however, this species did not cluster with *U. glycerae* (Figure 7); instead, it belonged to a well-supported "Lecudinidae" clade containing all known *Lecudina* and *Lankesteria* (Figure 7). The peristaltic movement has only been observed in urosporids, not in *Lecudina* or *Lankesteria* (Figure 7). Similarly, syzygy is not as commonly observed in *Lecudina* or *Lankesteria* as it is in urosporids; most of the cells of this species were in syzygy, although some single trophozoites were also present (Figure 4C,D). Although this species is phylogenetically closely related to *Lecudina* and *Lankesteria* forming a well-supported clade (= Lecudinidae), its morphology differs significantly and resembles that of urosporids. Therefore, we propose the establishment of a new genus for this species.

Sphinctocystis inclina n. sp.

This species was discovered in *Phyllodoce medipapillata* (COI: PP815647). This species was highly similar to one of the two gregarine species reported in Phyllodocidae, namely *Sphinctocystis phyllodoces* (Simdyanov, 2004). *Sphinctocystis phyllodoces* was found in *Phyllodoce citrina*

specimens collected from the White Sea and exhibited unique annular constrictions. Due to the lack of available molecular data for *S. phyllodoces*, we cannot confirm their close phylogenetic relatedness. Although the gregarine species we found in *P. medipapillata* shares similarities in cell shape and movements with *S. phyllodoces*, it did not show the clearly defined apical papilla surrounded by a smooth platform, which is an important characteristic of *S. phyllodoces*. Therefore, we establish a new species within the genus *Sphinctocystis*.

Lecudina-like gregarines are phylogenetically divergent

More than 20 genera are placed under the 'Lecudinidae' and some have shown to be distantly related to the type genus '*Lecudina*'; however, the phylogenetic boundary of the Lecudinidae remains unclear. Molecular data of genera once placed within the Lecudinidae, such as *Ancora*, *Polyrhabdina*, *Trichotokara*, *Loxomorpha*, *Filipodium*, *Difficilina*, have revealed that annelid-infecting gregarines are highly divergent, as they are distantly related to *Lecudina* in molecular phylogenetic analyses (Rueckert & Leander, 2010; Rueckert et al., 2010, 2013; Simdyanov et al., 2017; Iritani, Horiguchi, & Wakeman, 2018; Mathur et al., 2021; Paskerova et al., 2021; see also Figure 6). For example, *Ancora* and *Polyrhabdina* are now included in the Ancoroidea (Paskerova et al., 2021; Simdyanov et al., 2017). *Filipodium*, originally thought to be a gregarine within Lecudinidae (Grassé, 1953; Levine, 1977a), later transferred to Selenidiidae (Rueckert & Leander, 2009), is actually an early branching apicomplexan within the Squirmida (Mathur et al., 2019), and *Difficilina* has been shown to be closely related to *Urospora* (Simdyanov et al., 2017), the type genus of Urosporidae. Given that *Lecudina*-like genera actually represent several divergent lineages (Figure 6), the actual diversity of *Lecudina*-like gregarines is expected to be substantial.

In this study, we propose that Lecudinidae should be restricted to a clade containing *Lecudina*, *Lankesteria*, and *Amplectina* (Figure 7). We consider *Sphinctocystis inclina* as an insertae sedis species within the Lecudinoidea until its relationship with other lecudinoideans become clearer. Unfortunately, there are few morphological characters available for classification at the microscopic level, but SSU rDNA sequences will provide useful information for species delimitation and classification. While data from electron microscopy is currently limited to a small fraction of the known diversity of gregarines, additional insights can be gained at the ultrastructural level.

Lecudinidae comprises several lineages, including core *Lecudina* and *Lankesteria*

Two main groups of the Lecudinidae, *Lecudina* and *Lankesteria*, are relatively well studied. Although 48 species have been described within each of these genera, molecular data has only been available for 6 species (12.5%) for *Lecudina* and 14 species (29.1%) for *Lankesteria*. Among the 6 *Lecudina* species with molecular data, *L. pellucida*, *Lecudina* cf. *tuzetae*, and *L. kitasae* are known from Nereididae; *L. caspera* and *L. longissimi* were obtained from Lumbrineridae; and *L. phyllochaetopteri* was obtained from hosts within the Chaetopteridae (Iritani, Horiguchi, & Wakeman, 2018; Odle et al., 2024; Rueckert et al., 2010, 2011; Schrével et al., 2016). *Lecudina* cf. *platynereidis* isolated from the Nereididae in this study also grouped with other *Lecudina* species from nereidid hosts, which we call 'core *Lecudina*' (Figure 7). The core *Lecudina* clade is a monophyletic group that includes the type species *L. pellucida*. *Lecudina oxydromus* and *Lecudina* cf. *arabellae*, which were isolated from Hesionidae and Oenonidae, respectively, formed independent lineages within the Lecudinidae, suggesting some degree of host affiliation and the possibility of more diverse lineages potentially being found in other groups of polychaete hosts. In our molecular phylogenetic analyses, all *Lankesteria* species, except *L. pollywoga* (MW748135), grouped together (BS=90, PP=0.99), which we refer to as 'core *Lankesteria*' (Figure 7). We could ultimately assign a new genus to *L. pollywoga*; however, more molecular phylogenetic data from gregarines in other tunicates should be obtained first. Although its phylogenetic relationship with *Lecudina* and *Lankesteria* is unclear, *Amplectina* n. gen. belongs to the well-supported clade containing all known *Lecudina* and *Lankesteria*. Therefore, we include these three genera within "Lecudinidae" (Figures 6 and 7).

The Lecudinoidea contains gregarines from diverse marine invertebrate hosts

Our molecular phylogenetic analyses demonstrate that the Lecudinoidea contains two main subgroups: Lecudinidae and Urosporidae (Figure 7). However, there are several other independent lineages within the Lecudinoidea. For example, *Sphinctocystis inclina* n. sp. branches between the Lecudinidae and the Urosporidae. Although this species is more closely related to lecudinids in the molecular phylogenetic analyses, the flexibility of their trophozoites resemble those of urosporids. Also, interestingly, *Amplectina cordis* n. gen. et n. sp. is included in the Lecudinidae clade; however, it shows remarkably similar morphology and movement patterns with the recently discovered species *Undularius glycerae* (Odle et al., 2024). However, these two species do not cluster together in the molecular phylogenetic analyses; instead, *U. glycerae* forms a sister group to the clade containing all lecudinoideans except *Veloxidium* (Figure 7). The only significant differences between *Amplectina cordis* n. gen. et n. sp. and *Undularius glycerae* are an SSU rDNA sequence divergence of 82.3% and host affinity (Phyllodocidae vs. Glyceridae). When *Sphinctocystis phyllodoces* was described, it was placed under Lecudinidae because characteristics of the

trophozoites fit the definition of the group. this species could not been placed within the Urosporidae because the oocysts were not observed, which is crucial for the classification of this group (Simdyanov, 2004). We present the first molecular data of the genus *Sphinctocystis* showing its close relationship with *Lecudina* albeit forming a distinct lineage within the Lecudinoidea (Figure 7).

The trophozoites of *Difficilina* are highly similar to those in *Lankesteria* with respect to overall morphology, dense arrays of longitudinal epicytic folds, rigid cells with gliding mobility, and a crescent shape. Species of *Difficilina* are intermingled with species of urosporids in our molecular phylogenetic analyses (Figure 7). *Veloxidium* from sea cucumbers was originally described as an archigregarine because of its bending and twisting movements; however molecular phylogenetic analyses show that *Veloxidium* belongs to the well-supported Lecudinoidea clade (Figures 6 and 7; and also see Simdyanov et al., 2017). These data suggest that trophozoite rigidity and gliding motility may have evolved independently several times within the Lecudinoidea. Overeall, members of this group have diverse host affinities, including annelids, nemerteans, echinoderms, and gastropods, and trophozoites with diverse modes of behavior, such as gliding, bending, twisting, peristaltic movements, and immobility. Further exploring the diversity within the Lecudinoidea and how different traits relate to specific functions (e.g. locomotion and nutrient acquisition) within different kinds of hosts is expected to demonstrate complex evolutionary histories involving novel and reoccurring solutions to the challenges associated with the parasitic lifestyles of marine gregarines.

Taxonomic summary

Apicomplexa Levine, 1970
Eugregarinorida Léger, 1900
Lecudinidae Kamm, 1922
Lecudina Mingazzini, 1891

Lecudina oxydromus n. sp. Park and Leander

Diagnosis. A crescent-shaped trophozoites measured 93 μm long (70–120 μm, $n=4$) and 29 μm wide (25–33 μm, $n=4$). Trophozoites are brown due to dense amylopectin granules, but the anterior part is devoid of granules. A rounded nucleus measured 13 μm long (10–18 μm, $n=3$) and 15 μm wide (12–18 μm, $n=3$). Arrays of longitudinal epicytic folds with a density of 4–5 folds/μm. Trophozoites are capable of gliding.

DNA sequence. SSU rDNA sequence has been deposited in GenBank (accession ID: PP819650).

Type locality. Hyacinthe Bay, Quadra Island, British Columbia, Canada (50°6′53″N, 125°13′29″W).

Type habitat. Marine.

Type host. *Oxydromus pugettensis* (18S: PP819653).

Location in host. Intestine.

Iconotype. Figure 3.

Zoobank Registration LSID. urn:lsid:zoobank.org:act:6C9AF840-FB28-4C7A-A247-EEF0FF4B7647.

Etymology. "oxydromus" refers to the genus name of the host.

Apicomplexa Levine, 1970
Eugregarinorida Léger, 1900
Lecudinoidea Simdyanov 2013
Sphinctocystis Simdyanov, 2004

Sphinctocystis inclina n. sp. Park and Leander

Diagnosis. Trophozoites are highly flexible showing bending and gliding movements. Distinct annular constrictions are visible under a light microscope. Trophozoites were 333 μm long (295–500 μm, $n=5$) and 52 μm wide (40–70 μm, $n=5$). The cells were widest between the middle and the anterior part of the cell. The oval nucleus was 30 μm wide ($n=3$) and 24 μm length (22–25 μm, $n=3$). The anterior and posterior ends were rounded. The anterior end did not exhibit a noticeable apical papilla. Arrays of longitudinal epicytic folds had a density of 3 folds/μm.

DNA sequence. SSU rDNA sequence has been deposited in GenBank (accession ID: PP819652).

Type locality. Bamfield, British Columbia, Canada (48°50′10.8″N 125°06′52.1″W).

Type habitat. Marine.

Type host. *Phyllodoce medipapillata* (COI: PP815647).

Location in host. Intestine.

Iconotype. Figure 5.

Zoobank Registration LSID. urn:lsid:zoobank.org:act:EC6EFF84-25CD-40E2-B4F4-BE116BDBF649.

Etymology. "inclina" refers to 'bend' in Latin referring to the highly flexible bending movement of trophozoites.

Apicomplexa Levine, 1970
Lecudinoidea Simdyanov 2013
Eugregarinorida Léger, 1900

Amplectina n. gen. Park and Leander

Diagnosis. Lateral, or twisted syzygy. Peristaltic movement. Longitudinal epicytic folds with a density of 2–4 folds/μm.

Type species. *Amplectina cordis* n. sp. Park and Leander.

Etymology. 'Amplectina' means 'hug' or 'embrace' in Latin referring to the tight association of gamonts in syzygy.

Amplectina cordis n. sp. Park and Leander

Diagnosis. Lateral or twisted syzygy. Some single trophozoites are present. Gamont were 135 μm long (130–140 μm, $n=4$) and 35 μm wide (30–40 μm, $n=4$). A round nucleus of a gamont was 13.5 μm long ($n=2$) and 13.5 μm wide ($n=2$) without a fixed location in the cytoplasm. Trophozoites were 58 μm long (55–60 μm, $n=3$) and 17 μm wide (15–20 μm, $n=3$). The nucleus of a trophozoite was 21 μm long (20–22 μm, $n=2$) and 21 μm wide (20–22 μm, $n=2$). Both gamonts and trophozoites had dense arrays of longitudinal epicytic folds with dynamic peristaltic movement.

DNA sequence. SSU rDNA sequence has been deposited in GenBank (accession ID: PP819651).

Type locality. Hyacinthe Bay, Quadra Island, British Columbia, Canada (50°6′53″N, 125°13′29″W).

Type habitat. Marine.

Type host. Phyllodocidae sp. (COI: PP815648).

Location in the host. Intestine.

Iconotype. Figure 4.

Zoobank Registration LSID. urn:lsid:zoobank.org:act:07E69BA5-302A-4B7F-AC06-C267A6317C28.

Etymology. 'cordis' means 'heart' in Latin referring to the shape of some gamonts (see Figure 4A).

AUTHOR CONTRIBUTIONS

EP and BSL conceived the study. EP collected the hosts/parasites, performed the microscopy, collected the molecular data, and performed the molecular phylogenetic analyses. EP wrote the first draft of the manuscript; EP and BSL revised and approved the final version of the manuscript. BSL guided and funded the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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