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ORIGINAL ARTICLE

# Molecular Phylogenetic Positions of Two New Marine Gregarines (Apicomplexa)—*Paralecudina anankea* n. sp. and *Lecudina caspera* n. sp.—from the Intestine of *Lumbrineris inflata* (Polychaeta) Show Patterns of Co-evolution

Davis Iritani[a], Kevin C. Wakeman[b,c] & Brian S. Leander[a]

a Department of Botany and Zoology, University of British Columbia, #3529 - 6270 University Blvd., Vancouver, BC V6T 1Z4, Canada
b Office of International Affairs, Hokkaido University, North 10, West 8, Sapporo 060-0810, Japan
c Faculty of Science, Hokkaido University, North 10, West 8, Sapporo 060-0810, Japan

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**Correspondence**
N.D. Iritani, Department of Zoology, University of British Columbia, #3529 - 6270 University Blvd., Vancouver, BC V6T 1Z4, Canada
Telephone number: +1-604-822-2474;
FAX number: +1-604-822-6089;
e-mail: davisiritani@gmail.com

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## ABSTRACT

Gregarine apicomplexans are unicellular parasites commonly found in the intestines and coeloms of invertebrate hosts. Traits associated with the conspicuous feeding stage of gregarines, known as the trophozoite, have been used in combination with molecular phylogenetic data for species delimitation and the reconstruction of evolutionary history. Trophozoite morphology alone is often inadequate for inferring phylogenetic relationships and delimiting species due to frequent cases of high intraspecific variation combined with relatively low interspecific variation. The current study combined morphological data with small subunit (SSU) rDNA sequences to describe and establish two novel marine gregarine species isolated from the intestine of a polychaete host *Lumbrineris inflata* collected in British Columbia (Canada): *Paralecudina anankea* n. sp. and *Lecudina caspera* n. sp. The sister species to the host is *Lumbrineris japonica*, which can be found on the opposite side of the Pacific Ocean (Japan) and contains two different species of gregarine parasites: *Paralecudina polymorpha* and *Lecudina longissima*. Molecular phylogenetic analyses placed *P. anankea* n. sp. as the sister species to *P. polymorpha* and *L. caspera* n. sp. as the sister species to *L. longissima*. This phylogenetic pattern demonstrates a co-evolutionary history whereby speciation of the host (*Lumbrineris*) corresponds with simultaneous speciation of the two different lineages of intestinal gregarines (*Paralecudina* and *Lecudina*).

GREGARINES are an understudied group of apicomplexan parasites that offer an opportunity to elucidate the earliest stages of apicomplexan evolution. Marine gregarines are of particular interest for apicomplexan systematics because they are uniquely positioned at the base of the apicomplexan tree and have retained key plesiomorphic characters since their divergence from an ancient biflagellate ancestor (Leander 2008a). For example, most marine gregarines are monoxenous and many parasitize host cells via myzocytosis. Molecular phylogenetic analyses of environmental DNA sequences and individually isolated species have shown that gregarines have a high prevalence across marine habitats and that most species remain undiscovered (Leander 2008b; Rueckert et al. 2011; Sitnikova and Shirokaya 2013; Wakeman and Leander 2013a). Where formal taxonomic descriptions do exist, the original sources are often scattered across obscure journals in various languages; these earlier approaches to species delimitation of gregarines relied on morphology observed with light microscopy and line drawings. Gregarine morphology, however, is often difficult to interpret due to the challenge of accurately identifying the high degree of intraspecific morphological variation within the context of relatively low interspecific variation.

Gregarines are characterized by numerous traits including their particularly large extracellular feeding stages (trophozoites), monoxenous life cycles, and infection of invertebrate hosts (Leander 2008b). Host species, location

within the host body, and cell surface morphology vary among gregarine species. In most cases gregarine infections have been documented from marine annelids and terrestrial insects (Clopton et al. 1992; Leander 2008b; Rueckert and Leander 2009; Schilder and Marden 2006; Wakeman and Leander 2013b; Zuk 1987). The host compartment targeted by gregarines is generally confined to the intestinal lumen, but some infect coelomic spaces (e.g. urosporidians; Leander et al. 2006) and reproductive organs (e.g. *Monocystis agilis*; Field and Michiels 2005). These morphological and life history traits were traditionally employed to divide the gregarines into three major categories: archigregarines, neogregarines, and eugregarines (Adl et al. 2012; Grassé 1953; Leander 2008b).

Eugregarines (Eugregarinorida Léger 1900) are further divided into two groups based on a transverse groove (i.e. the septum) that separates the trophozoite stage into two cellular compartments: the aseptate and septate gregarines. Whether the septum is a reliable character for taxonomic division of gregarines is currently still under question. The Lecudinidae Kamm 1922 is a poorly defined group of aseptate eugregarines to which most aseptate marine eugregarines belong (Levine 1977). There are 25 genera of lecudinids recognized within the Lecudinidae representing roughly 90 named species. About 40 of these named species belong to the single genus *Lecudina*. Although improved resolution of lecudinid phylogeny has been achieved through the integration of molecular data with some morphological data, the deepest relationships within the group are still unresolved. The discovery of additional taxa, however, has continually revealed subclades within the Lecudinidae and indicates progress toward taxonomic clarity (Rueckert et al. 2010, 2013).

*Paralecudina*, for instance, was initially a monotypic genus formed by moving *L. polymorpha* out of *Lecudina* after molecular phylogenetic analyses of SSU rDNA sequences revealed that this species did not fall within the *Lecudina* clade (Rueckert et al. 2013). This was corroborated each time new lecudinid taxa such as *Difficilina* spp. grouped cleanly into subclades within the Lecudinidae (Rueckert et al. 2010), and *L. polymorpha* always associated with unidentified environmental sequences. With the discovery of the *Trichotokara* clade, the sister relationship between *Trichotokara* and *L. polymorpha* became even clearer so *L. polymorpha* was moved to *Paralecudina*. The taxonomic resolution of eugregarines is currently poor and most of its backbone is composed of unsupported branches. The discovery of additional taxa contributes to a more comprehensive catalogue of eugregarines and is expected to improve inferences about the phylogenetic relationships of gregarines.

In the current study, two new species of aseptate marine eugregarines are described from a polychaete host collected in British Columbia. One is a new *Paralecudina* species, representing the only other species described since the genus was established. The other is a new *Lecudina* species. The establishment of the two new species was based on host affiliation, geographical location, comparative trophozoite morphology, and SSU rDNA sequences. The molecular phylogenetic positions of the two new species provide evidence for co-evolution between gregarine parasites and polychaete hosts. The discovery of these two species also helps illustrate some of the challenges associated with interpretations of gregarine morphology and the indispensability of molecular phylogenetic data for understanding gregarine biodiversity.

## MATERIALS AND METHODS

### Collection of host material and isolation of gregarine trophozoites

The annelid host *Lumbrineris inflata* Moore 1911 was collected at low tides during fall of 2015 and summer of 2016 from Clover Point (48°24′14.18″N 123°21′00.91″W), British Columbia, Canada. The geography of the beach consists of a sheltered, rocky topography interspersed with patches of eelgrass beds. The hosts are infaunal and often burrow in the sediment between the roots of eelgrass, thus could be collected by pulling tufts of eelgrass by the roots. The hosts were isolated from the sediment by vigorously rinsing the eelgrass in a bucket of seawater. The collected hosts were placed in plastic bags, stored on ice, and transported to a holding tank at the University of British Columbia. All hosts were dissected within the first week of collection.

Individual trophozoites were collected from the hosts via dissection. A single host worm was placed in a Petri dish filled with filtered seawater and split longitudinally with fine forceps to spill their gut contents. Gregarine trophozoites were located among food particles and digestive debris using a Leica (Wetzlar, Germany) DM IL inverted microscope. Individual trophozoites were isolated using hand-drawn glass pipettes. Every trophozoite was washed three times with filtered seawater in a well slide and set aside to be prepared for light microscopy, scanning electron microscopy, and DNA extraction.

### Light microscopy

Trophozoites were imaged using differential interference contrast (DIC) with a Zeiss Axioplan 2 microscope (Carl-Zeiss, Göttingen, Germany) paired to a Zeiss Axiocam 503 color camera (Carl-Zeiss, Göttingen, Germany). Light micrographs were edited on Adobe Photoshop 11.

### Scanning electron microscopy

Trophozoites were isolated from *L. inflata* and fixed for SEM using 24-well tissue culture plates and plastic capsules to hold and move the trophozoites between fixation steps. The bottoms of TEM beem capsules (size: no.00) were cut off, creating a hollow cylinder, and a 50 μm mesh was added to cover one of the open ends. The mesh was held in place by pinching it between the capsule and an appropriately sized TEM embedding capsule cap. The customized capsules were submerged in the

wells of the tissue culture plates filled with filtered seawater plus a drop of 2.5% glutaraldehyde. Trophozoites were transferred to these capsules using hand-drawn glass pipettes and the remaining open end was closed with more mesh and a cap. The trophozoites were left to fix in the 2.5% glutaraldehyde for 30 min on ice. Each capsule holding trophozoites was then moved to an adjacent well and was rinsed with 0.1 M sodium cacodylate ($(CH_3)_2AsO_2Na$) and left to soak for several min. The capsules were moved to the next well filled with filtered seawater plus three drops of 1% $OsO_4$ and left to soak for 30 min on ice. Each capsule was rinsed and soaked again with 0.1 M sodium cacodylate. The trophozoites were then dehydrated with serial ethanol baths by submerging the capsules for 3 min at 30%, 50%, 75%, 85%, 95%, and 100% dilutions. Following the ethanol baths, the capsules were placed in a Tousimis Autosamdri® 815B critical point dryer. Individual trophozoites were transferred from the 50 μm mesh onto SEM stubs using an eyelash glued to a glass pipette and then sputter coated with 6 nm of gold/palladium alloy. SEM images were taken on a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Ltd., Pleasanton, CA) and edited on Adobe Photoshop 11.

## DNA extraction, amplification, and sequencing

For each of the two different species, 6–10 trophozoites were pooled and washed three times with autoclaved seawater and placed in 1.5 ml Eppendorf tubes with 4 μl of molecular grade distilled water. Genomic DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Manufacturer protocols were followed except for the final step in which the DNA was eluted in 8 μl of EB buffer, to concentrate the DNA, instead of the suggested 40 μl.

SSU rDNA sequences were initially amplified by a polymerase chain reaction (PCR) using either universal eukaryote primers PF1 5′-CGCTACCTGGTTGATCCTGCC-3′ and SSUR4 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander et al. 2003a) or gregarine-specific primers 65FDeGr 5′-YDAARCTGCGRAKRGCTCAT-3′ and 1958RAp 5′-TGTGTACAAAGGGCAGGGAC-3′. Template DNA and primer pairs were added to distilled water and a PCR bead according to the manufacturer's instructions (Illustra, PuReTaq Ready-To-Go PCR beads, GE Healthcare, Quebec City, Canada). The following thermal cycle was used: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 2:00 min, and a final extension at 72 °C for 5 min. For both species, the product from this initial amplification was used as the template for a second round of nested PCRs using internal primers LecuF 5′-GTDAATCGGCGTGTTCYACG-3′ and LecuR 5′-GAATGCCCTCARCCGTTC-3′ (Rueckert et al. 2015). The PCR products were screened on a 1% agarose gel to confirm that the reactions yielded expected sequence lengths. Bands were cut from the gels using a sterile razor blade and cleaned using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Nested PCR products for *Paralecudina anankea* n. sp. were cloned and with a TOPO TA cloning kit (Invitrogen, Frederick, MD) according to manufacturer protocols. Eight clone colonies were isolated and amplified with TOPO vector primers in a 25 μl reaction with EconoTaq 17″ Master Mix (Lucigen Corp. Middleton, WI). The clones were then fingerprinted using an HAE III digestion reaction, and clone variants were sequenced using the cloning vector primers in an ABI BigDye® reaction mix. Uncloned PCR products from the *Lecudina caspera* n. sp. amplifications were sequenced with the following internal primers: 648F: 5′-CGCGGTAATTCCAGCTTCA-3′; 648R: 5′-TGGAGCTGGAATTACCGCG-3′; 1300F: 5′-ATGGTTGCAAGACTGAAACT-3′; 1300R: 5′-AGTTTCAGTCTTGCAACCAT-3′; 1321F: 5′-AAAGGAATTGACGGAAGGGCA-3′; and 1321R: 5′-TGCCCTTCCGTCAATTCCTTT-3′. Sequences were assembled and edited using Sequencher™ (version 4.5, Gene Codes Corporation, Ann Arbor, MI). Acquired sequences were initially identified by Basic Local Alignment and Search Tool (BLAST) analysis.

## Molecular phylogenetic analyses

The phylogenetic positions of the two new species were determined using a 74-taxon alignment of SSU rDNA sequences, including three dinoflagellate sequences (outgroup) and representatives from the major clades of apicomplexans. The SSU rDNA sequences were aligned using the MAFFT algorithm (Katoh et al. 2002) on Geneious version 10.0.2 (Kearse et al. 2012). The MAFFT algorithm was chosen over others for its ability to account for the secondary structure of ribosomal subunits. Ambiguously aligned regions and gaps were cut from the final alignment using Aliscore version 2.0 (Kück et al. 2010; Misof and Misof 2009) and Alicut version 2.3. The resulting alignment included 1,497 unambiguously aligned sites.

The GTR + I + Γ model (proportion of invariable sites = 0.1970, gamma shape = 0.7250) was selected by jModelTest version 2.1.10 (Darriba et al. 2012; Guindon and Gascuel 2003) for maximum likelihood and Bayesian analyses under Akaike Information Criterion (AIC). The maximum likelihood (ML) tree and ML bootstrap values were inferred using RAxML version 8.2.9 (Stamatakis 2014) through the Cipres Science Gateway version 3.3 (Miller et al. 2010). Bayesian posterior probabilities were calculated using Mr. Bayes version 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012) using the GTR substitution model with invariable sites over a gamma distribution (lset nst = 6, rates = invgamma) and Monte Carlo Markov Chains (MCMC) run with the following parameters: 10,000,000 generations (ngen = 10,000,000), 2 runs (nruns = 2), 4 chains (nchains = 4), temperature parameter at 0.2 (temp = 0.200), sample frequency of 100, prior burn-in of 0.25 of sampled trees, and a stop rule of 0.01 to terminate the program when the split deviation fell below 0.01.

## RESULTS

### General ultrastructure of the trophozoites

The trophozoites of *P. anankea* n. sp. were spindle-shaped with a permanent, superficial indentation that ran transversely to divide the cell into a posterior region, including the nucleus, and an elongated anterior region (Fig. 1a, b). Trophozoites were light brown overall, but translucent at the edges all around. The oval, centrally located nucleus was entirely translucent and range 18–27 μm ($\bar{X}$ = 24 μm; $n$ = 4) along the major axis and 14–18 μm ($\bar{X}$ = 19 μm, $n$ = 4) along the minor axis. The elongated anterior end was 36–109 μm long ($\bar{X}$ = 71 μm, $n$ = 6). The cells were 203–383 μm ($\bar{X}$ = 272 μm, $n$ = 6) in length and 23–56 μm ($\bar{X}$ = 37 μm, $n$ = 6) in width at the broadest point. The anterior region tapered to an inconspicuous mucron. The trophozoites displayed gliding motility in a single direction and could bend along its minor axis. Epicytic folds ran longitudinally down the entire length of the trophozoite at a density of six folds per micron. The depth of each epicytic fold appeared uniform throughout the cell. Some trophozoites were covered in host sperm and other organic material.

*Lecudina caspera* n. sp. trophozoites were acorn-shaped measuring 226–420 μm ($\bar{X}$ = 308 μm, $n$ = 9) in length and 61–153 μm ($\bar{X}$ = 106 μm, $n$ = 6) at the narrowest point (Fig. 2a, b). A circular nucleus with a 22–55 μm ($\bar{X}$ = 36 μm, $n$ = 8) diameter was situated at a transverse constriction on the gregarine. The cells were rigid and capable of gliding forward; they were never observed to contract, elongate, or fold. A permanent constriction was visible around the nucleus that demarcated the posterior region from a round, stub-nosed anterior. The anteriormost portion of the cell ended with a nipple-like mucron (Fig. 2c) that was the only translucent structure on the trophozoite. Epicytic folds lined the entire length of the trophozoite (Fig. 2d) at a density of four folds per micron. The depth of each epicytic fold was uniform across the cell.

### Molecular phylogenetic analyses of SSU rDNA sequences

The 74-taxon alignment of SSU rDNA sequences yielded a strongly supported outgroup of dinoflagellates (84 Maximum likelihood bootstrap [MLB], 1.00 Bayesian posterior probability [BPP]) and an ingroup of apicomplexans with a poorly resolved backbone (Fig. 3). Both maximum likelihood and Bayesian analyses recovered identical tree topologies. The apicomplexan backbone gave rise to piroplasmid, coccidian, rhytidocystid, cryptosporidian, and gregarine clades. The so-called archigregarines were paraphyletic with *Platyproteum vivax* and *Filipodium phascolosomae* forming the most basal apicomplexan branch. Two terrestrial gregarine clades were also recovered. Terrestrial gregarine clade I was well-supported (92 MLB, 1.00 BPP), but included genera comprised of species with extremely short branches. The clade also included environmental sequences (AF372779 and AY179988) acquired from marine environmental PCR surveys. The support for terrestrial gregarine clade II was robust (100 MLB, 1.00 BPP) and included only gregarines described from terrestrial hosts. Marine gregarines formed clades composed of members that infect similar hosts (e.g. capitellid gregarines and *Lankesteria* collected from tunicates).

*Lecudina caspera* n. sp. branched as the sister lineage to *L. longissima* with strong support; this subclade was part of a more inclusive and strongly supported lecudinid clade (Fig. 3). A paralecudinid clade was highly supported (100 MLB, 1.00 BPP) within which there were two species, *P. anankea* n. sp. and *P. polymorpha*, and an unidentified environmental sequence (AB252765). *Paralecudina anankea* n. sp. and *P. polymorpha* were recovered as sister species (72 MLB, 0.99 BPP).

## DISCUSSION

Molecular phylogenetic analyses of SSU rDNA sequences demonstrated a clade composed of environmental sequences, *Trichotokara*, and *Paralecudina*. *Paralecudina*

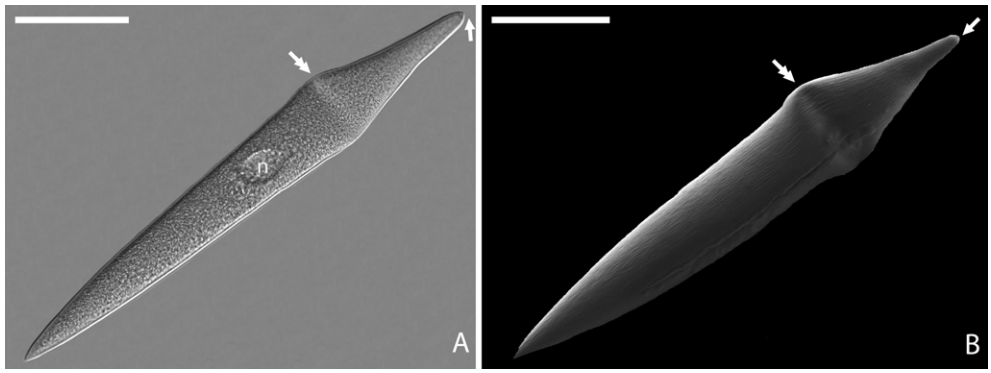


**Figure 1** Light micrograph (LM) and scanning electron micrographs (SEM) of *Paralecudina anankea* n. sp. trophozoite morphology and ultrastructure. (**A**) LM of spindle-shaped trophozoite taken in differential interference contrast (DIC). An oval nucleus (n) is visible located centrally within the cell. A superficial fold (double arrow) running transversally separates the posterior region including the nucleus with the anterior region. (**B**) SEM of the trophozoite showing the transverse fold. Epicytic folds run down the entire length of the cell longitudinally. Scale bars: a, b = 50 μm.

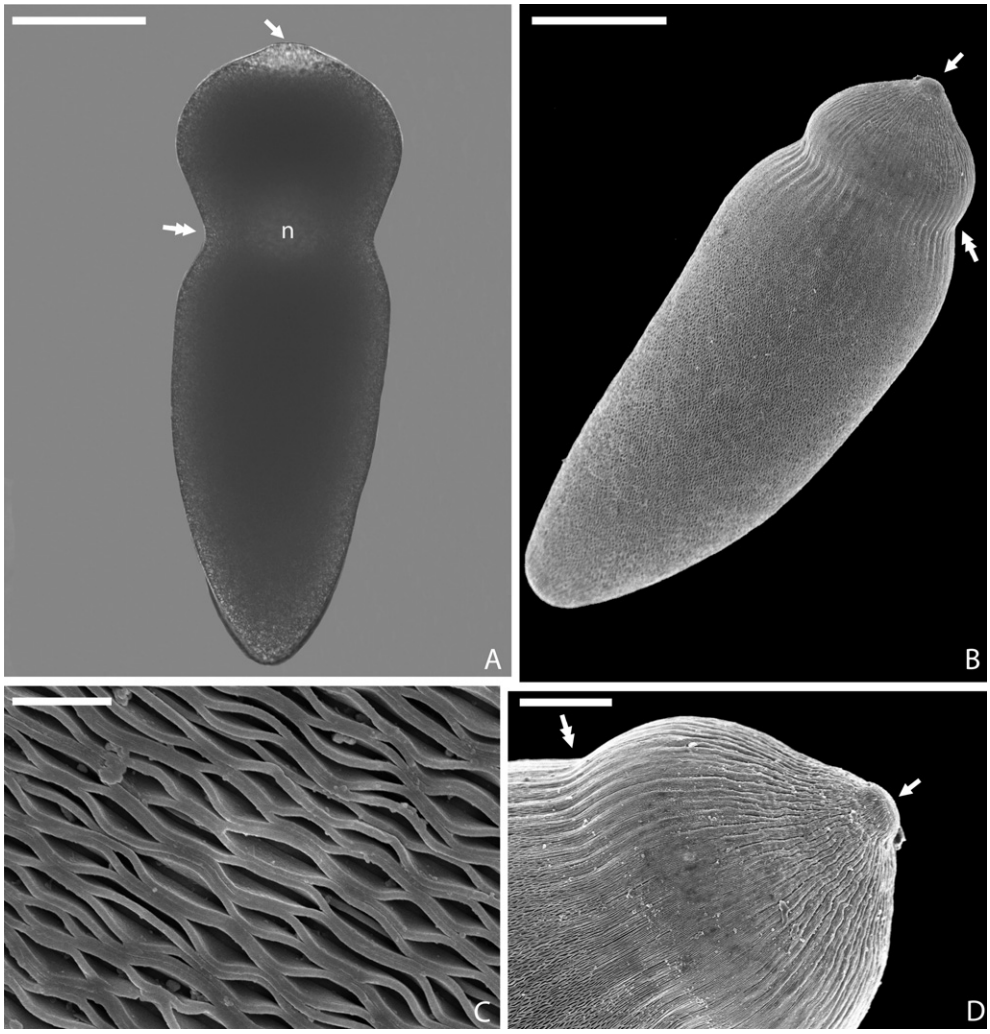


**Figure 2** Light micrograph (LM) and scanning electron micrographs (SEM) of *Lecudina caspera* n. sp. trophozoite morphology and ultrastructure. (**A**) LM of the acorn-shaped trophozoite in differential interference contrast (DIC). A circular nucleus (n) is visible, located at a transverse constriction (double arrow) of the cell. The constriction divides the cell into two regions. The mucron is translucent and forms a nipple-like protrusion (single arrow). (**B**) SEM of the trophozoite showing the constriction and mucron. Epicytic folds are also seen running along the entire length of the trophozoite. (**C**) SEM of epicytic folds close up. (**D**) SEM of the anterior end (arrow) of a trophozoite. Scale bars: a, b = 50 μm; c = 2 μm; d = 30 μm.

consists of one other species with two known morphotypes: morphotype 1 and morphotype 2 (Leander et al. 2003b; Rueckert et al. 2010). Trophozoites of both morphotypes were described as rigid and capable of gliding motility. In morphotype 1, the posterior end tapers to a distinct point, the anterior end is adorned with an elongated mucron, and an oval nucleus is situated within the anterior half of the trophozoite. Trophozoites representing morphotype 2 have long, tapering bodies with a distinct bulge around the anterior third of the trophozoite. Apart from nucleus position, *P. anankea* n. sp. shares all the above morphological characters with *P. polymorpha* and is especially similar to morphotype 2, which supports the placement of *P. anankea* n. sp. within *Paralecudina*.

However, several morphological differences between *P. polymorpha* and *P. anankea* n. sp. indicate that *P. anankea* n. sp. is not a third morphotype of *P. polymorpha* but a distinct species. A stark contrast in morphology lies in the distinct transverse fold seen in the anterior region of the trophozoites in *P. anankea* n. sp. The fold has not been observed in *P. polymorpha*. The trophozoites of *P. anankea* n. sp. are also flexible and capable of folding along the transverse axis. *P. anankea* n. sp. also has a more centrally positioned nucleus and epicytic folds arranged at a density of 6-folds/micron; *P. polymorpha* has either 3-folds/micron (morphotype 1) or 5-folds/micron (morphotype 2). The molecular phylogenetic data also support *P. anankea* n. sp. as a new species and gives robust support to a paralecudinid clade (100 MLB, 1.00 BPP) that also includes *P. polymorpha* and an unidentified environmental sequence (AB252765). The SSU rDNA sequences of *P. anankea* n. sp. and *P. polymorpha* were 12% divergent.

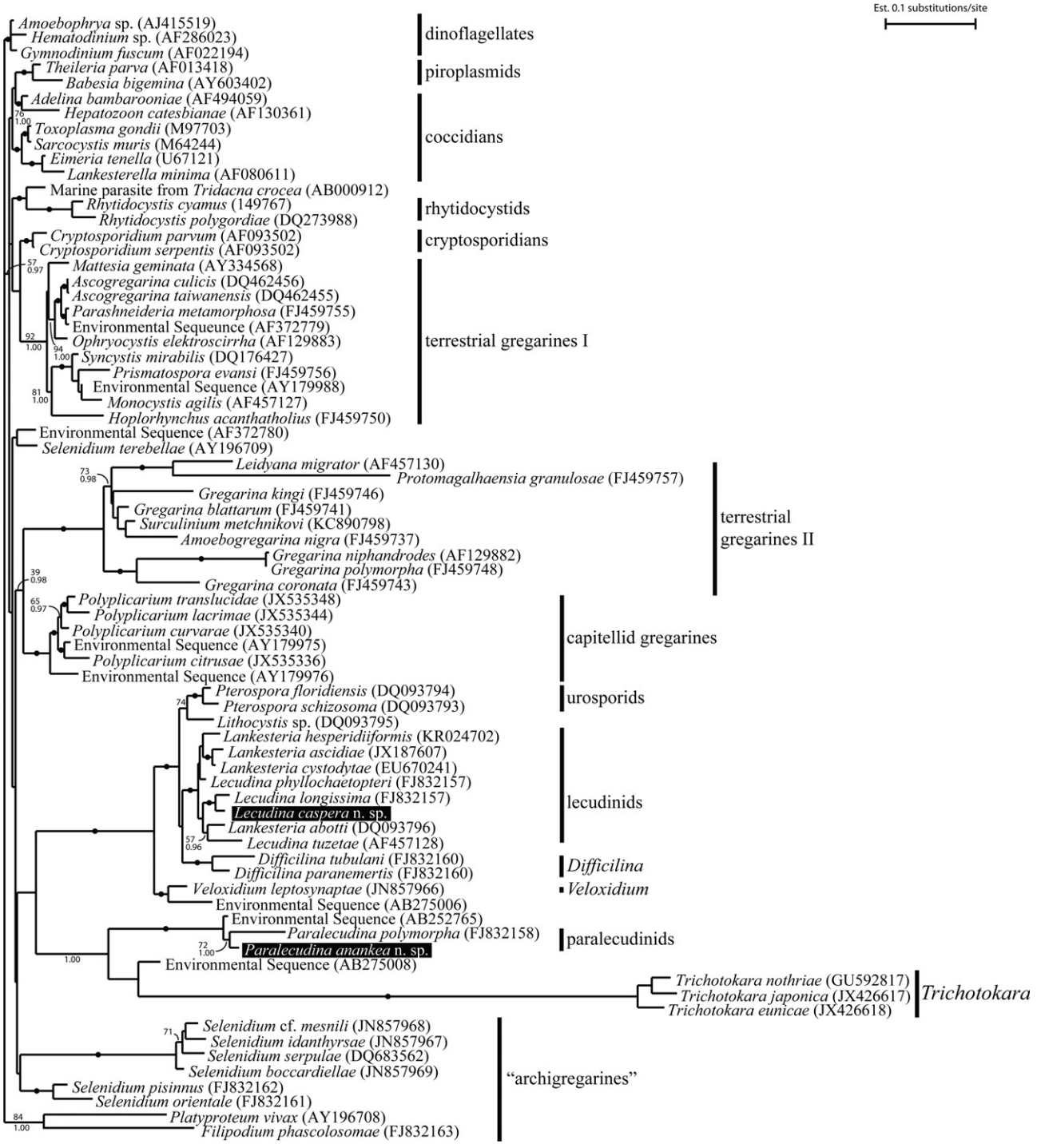


**Figure 3** Maximum likelihood tree inferred from a 74 taxa dataset of SSU rDNA sequences with 1,497 unambiguously aligned sites using the GTR + I + Γ model of substitution (gamma shape = 0.7250, proportion of invariable sites = 0.1970). Numbers denote support values with the top values indicating bootstrap support and the bottom indicating Bayesian posterior probabilities. The black dots were used on branches when both bootstrap support and Bayesian posterior probabilities were ≥ 95 and 0.99 respectively. Support values were excluded from this tree when both bootstrap support and Bayesian posterior probabilities fell below 55 and 0.95, respectively, for any given branch. The new species described in the current study is highlighted with black boxes.

*Lecudina* is a problematic genus inferred to be paraphyletic because several other genera of marine gregarines fall within it, such as *Lankesteria* and potentially *Difficilina* (Leander et al. 2003b; Rueckert et al. 2013) (Fig. 3). In general, the trophozoites of *Lecudina* are rigid, capable of gliding motility, and have a dense array of epicytic folds (Levine 1976). *Lecudina caspera* n. sp. formed the sister species to *L. longíssima* in molecular phylogenetic analyses (Fig. 3). Trophozoites of *L. longissima* are linearly ellipsoid (Rueckert et al. 2010) and are morphologically dissimilar to *L. caspera* n. sp. Both species, however, have a circular nucleus that is situated in the anterior third of the trophozoite. The SSU rDNA sequences from *L. caspera* n. sp. and *L. longissima* were 8% divergent. The divergent SSU rDNA sequences in combination with the different trophozoite morphologies in *L. caspera* n. sp, and *L. longissima* justify the establishment of *L. caspera* n. sp. as a new species.

Most original descriptions of gregarine species are based on line drawings and are lacking molecular data. However, trophozoites often take on a great deal of intraspecific variation (e.g. the diverse morphotypes of *P. polymorpha* and *Lecudina* cf. *tuzetae*; Leander et al. 2003b; Rueckert et al. 2011), associated with motility (e.g. *Pterospora schizosoma*; Leander et al. 2006), developmental stages, and growth conditions within the host; this makes morphological traits difficult to interpret in isolation and molecular phylogenetic data are particularly important for establishing new species of gregarines. Comparative morphology and molecular phylogenetic analyses of SSU rDNA sequences indicate that both gregarines found in this study are novel species. Moreover, the host, *L. inflata* (from British Columbia), in which both *P. anankea* n. sp. and *L. caspera* n. sp. were found, has not been previously investigated for gregarines.

The different sister species to *P. anankea* n. sp. and *L. caspera* n. sp., namely *P. polymorpha* and *L. longissima*, respectively, were discovered in Bamfield, British Columbia from a closely related host: *Lumbrineris japonica* (Fig. 4; Rueckert et al. 2010). This host species was originally described from Japan, and we speculate that the host was introduced to Bamfield through the intimate historical association of fishing vessels between the two localities. Other groups of infaunal animals are thought to have moved from Japan to British Columbia through fisheries related activities (Herranz and Leander 2016). Regardless of how *L. japonica* established populations in British Columbia, the molecular phylogenetic data show that different closely related gregarines infect different closely related hosts (Rueckert et al. 2015; Wakeman and Leander 2013a,b), suggesting that gregarines and their hosts have co-evolved. In contrast with this pattern, some gregarine species have diversified within a host (Wakeman et al. 2014). For example, *Selenidium melongena* and *S. terebellae* are sister species that occupy different compartments inside the host, the coelom and intestinal lumen, respectively; in this example, niche partitioning within the host is thought to be the driver for their divergence (Wakeman et al. 2014). Neither of these phylogenetic patterns are evident from comparative morphology alone, which underscores the essential role molecular phylogenetic data play for understanding gregarine diversity and evolutionary history.

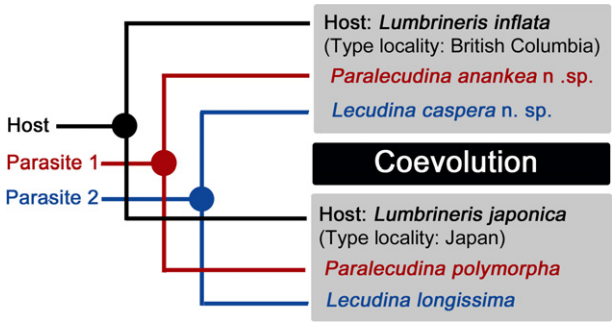


**Figure 4** Illustration showing a co-evolutionary pattern whereby two closely related polychaete hosts are infected by two different lineages (genera) of intestinal gregarines: parasite 1 and parasite 2. The sister species of *Lumbrineris* have type localities on opposite coasts of the Pacific Ocean: *L. inflata* from California and *L. japonica* from Japanese exclusive economic zone. As indicated by the shaded boxes, each host is infected by two different species of gregarine parasites. *Lumbrineris inflata* is infected by *Paralecudina anankea* n. sp. and *Lecudina caspera* n. sp.; *L. japonica* is infected by *Paralecudina polymorpha* and *Lecudina longissima*. *Paralecudina anankea* n. sp. and *L. caspera* n. sp. are the sister species of *P. polymorpha* and *L. longissima* respectively.

## TAXONOMIC SUMMARY

Phylum Apicomplexa Levine, 1970
Order Eugregarinorida Léger, 1900
Family Lecudinidae Kamm, 1922

### *Paralecudina anankea* n. sp. Iritani, Wakeman, and Leander.

**Description.** Trophozoites are spindle shaped with an elongated anterior compared to typical marine aseptate eugregarines. Cells average 272 μm (range = 203–383 μm) and 37 μm (range = 26–56 μm) in width at the broadest region. Cells are light brown except around the edges that are transparent. Both anterior and posterior ends taper to a point. Nucleus positioned centrally and is oval with a major axis of 24 μm (range = 18–27 μm) and a minor axis of 19 μm (range = 14–24 μm). Permanent superficial indentation runs transversally at anterior third of trophozoite. Gliding motility. Longitudinal epicytic folds with a density of 6/μm. Epicytic folds of even depth throughout the trophozoite.

**DNA sequence** SSU rDNA sequence (GenBank KY678216).

**Type locality.** Clover Point (48°24′14.18″N 123°21′00.91″ W), British Columbia, Canada. Host in sand between roots of seagrass; lower intertidal; 0.30 m above mean sea level.

**Type habitat.** Marine

**Type host.** *Lumbrineris inflata* Moore, 1911 (Annelida, Polychaeta, Eunicida, Lumbrineridae). The host was

barcoded with the 18S rDNA sequence and tissue has been preserved and deposited with the parasite hapantotypes in the Beaty Biodiversity Museum.
**Location in host.** Intestinal lumen
**Iconotype.** Figure 1a
**Hapantotype.** Trophozoites on SEM stubs with 6 nm of gold/palladium alloy sputter coat have been stored in the Beaty Biodiversity Museum (MI-PR145) at the University of British Columbia, Canada.
**Zoobank Registration LSID.** F063F884-197F-48E8-BB11-1F64764ACF8C
**Etymology.** The species name, *anankea*, refers to the female figure Ananke from Greek mythology who is often depicted holding a spindle which resembles the shape of the trophozoites found in this species.

### *Lecudina caspera* n. sp. Iritani, and Leander.

**Description.** Trophozoites acorn shaped with a nipple-like mucron. Cells average 308 μm in length (range = 226–420 μm) and 106 μm (range = 61–153 μm) in width at the narrowest region. Cells are brown to dark brown. Anterior compartment is bulbous and circular, whereas posterior compartment tapers to a blunt end. Circular nucleus with a diameter of 36 μm (range = 22–55 μm) positioned at the anterior third where trophozoite constricts transversally. Gliding motility. Longitudinal epicytic folds with a density of 4/μm. Epicytic folds of even depth throughout the trophozoite.
**DNA sequence.** SSU rDNA sequence (GenBank KY678215).
**Type locality.** Clover Point (48°24′14.18″N 123°21′00.91″ W), British Columbia, Canada. Host in sand between roots of seagrass; lower intertidal; 0.30 m above mean sea level.
**Type habitat.** Marine
**Type host.** *Lumbrineris inflata* Moore, 1911 (Annelida, Polychaeta, Eunicida, Lumbrineridae). The host was barcoded with the 18S rDNA sequence and tissue has been preserved and deposited with the parasite hapantotypes in the Beaty Biodiversity Museum.
**Location in host.** Intestinal lumen
**Iconotype** Figure 2a
**Hapantotype** Trophozoites on SEM stubs with 6 nm of gold/palladium alloy sputter coat have been stored in the Beaty Biodiversity Museum (MI-PR146) at the University of British Columbia, Canada.
**Zoobank Registration LSID** FAB421BB-879F-4123-B285-DDE8F843A0C7
**Etymology** The species name, *caspera*, refers to the resemblance of the trophozoite to the body shape of Casper the Friendly Ghost from the 1995 film. The suffix -a assigns a Greek, female form to the species name for consistency with *Lecudina*.

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