Morphology and phylogenetic position of two novel marine gregarines (Apicomplexa, Eugregarinorida) from the intestines of North-eastern Pacific ascidians

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Eugregarine apicomplexans parasitize marine, freshwater and terrestrial invertebrates, and have lifecycles involving trophozoites (feeding stages) with complex morphologies and behaviour. The genus *Lankesteria* refers to marine aseptate eugregarines that parasitize ascidians. We described the surface ultrastructure of two new gregarine species, *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. that inhabit the intestines of *Chelyosoma columbianum* and *Cystodytes lobatus*, respectively, collected from the North-eastern Pacific Ocean. Apart from inhabiting different hosts and major differences in the cell size of *L. chelyosomae* sp. n. (mean length 182 μm) and *L. cystodytae* sp. n. (mean length 70 μm), the morphology of both gregarine species was quite similar. The trophozoites ranged from elliptoid to obdeltoid in shape and were brownish in colour. The nucleus was situated at the anterior end of the cell just behind a pointed mucron. A dense array of epicytic knobs was present over the entire surface of trophozoites in both species, and longitudinal epicytical folds were only weakly developed. We also sequenced the small subunit rDNA from the gregarines collected from both hosts, which supported the establishment of two new *Lankesteria* species. Phylogenetic analyses of the new DNA sequences and those derived from other alveolates, demonstrated that both new species clustered in a strongly supported clade consisting of other *Lankesteria* species, *Lecudina* species, and some environmental sequences. These morphological and molecular phylogenetic data suggested that improved knowledge of gregarine diversity could lead to the recognition of more than one distinct clade (genus) of gregarines within ascidian hosts.

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Introduction

Gregarine apicomplexans inhabit the intestines, coeloms and reproductive vesicles of aquatic and terrestrial invertebrates and comprise about 1400 known species. They have monoxenous (single host) life cycles with extracellular trophozoite stages that are very diverse in morphology and behaviour (Perkins *et al*. 2002; Leander 2008). Traditionally, gregarines have been thought to represent the most ancestral lineages within the Apicomplexa (Levine 1977). However, molecular phylogenetic and comparative ultrastructural data demonstrate that most gregarines are much more evolutionary derived than is usually assumed (Leander 2008). There are three major, albeit poorly defined, groups of gregarines: archigregarines, eugregarines and neogregarines (Grassé 1953). Archigregarines occur exclusively in marine habitats, and the trophozoites (feeding stages) resemble the morphology of the infective sporozoites. Eugregarines can be found in marine, freshwater and terrestrial habitats, and the morphology and behaviour of the trophozoites is significantly different from that of the sporozoites. Neogregarines infect terrestrial hosts, have reduced trophozoite stages, and are found in and on the host tissues rather than within the intestines *per se*.

Lankesteria Mingazzini, 1891 belongs to the aseptate eugregarine family Lecudinidae. The composition of this genus was at one time problematic, because *Lankesteria* consisted of gregarines from four major invertebrate phyla: Plathyhelminthes (turbellarians), Chordata (ascidians), Chaethognatha and Arthropoda (mosquitoes) (Vávra 1969; Levine 1981). Gregarines, however, tend to show high host specificity, and previous members of *Lankesteria* have been subsequently separated as follows: (i) *Lankesteria sensu stricto* Mingazzini, 1891 — parasites of ascidians, (ii) *Monocystella* Valkanov, 1935 and *Pseudolankesteria* Ormières, 1965 — parasites of turbellarians, (iii) *Tricystis* Hamon, 1951 — parasites of chaetognaths, and (iv) *Ascocystis* Grassé, 1953 — parasites of mosquitoes (Ormières 1965). We follow this scheme and refer only to parasites of ascidians when discussing *Lankesteria*. Levine (1977) reported 27 known *Lankesteria* species from ascidians, and most of them were described from European waters. Thirteen species are known to occur in ascidians collected from the Pacific Ocean (Levine 1981).

Like most eugregarines, the trophozoites of *Lankesteria* species appear brown because the cytoplasm is filled with amylopectin granules (carbohydrate storage products). The anterior end of the trophozoites or 'mucron' is devoid of granules and appears clear under the light microscope. The cell shape varies considerably, even within the same species, from ovoid to obpyriform (i.e. broader anterior part and narrower, tail-like posterior part; terms for plane cell shapes are in accordance with the nomenclatural system established by Clopton 2004). The nucleus is located in the anterior half of the cell, and in most cases just behind the rounded mucron. Very little is known about the surface morphology and ultrastructure of *Lankesteria* species. Currently, only *L. ascidiae* and *L. parascidiae* have been studied with transmission electron microscopy (TEM) (Ormières 1972; Ciancio *et al*. 2001) and *L. abbotti* has been examined with scanning electron microscopy (SEM) (Leander *et al*. 2006); moreover, both SEM and TEM were used to describe *L. cyrtocephala* and *L. levinei* (Simdyanov 1995). Nonetheless, the identification of *Lankesteria* species is very difficult, as most of the original descriptions are based only on line drawings (e.g. see Ormières 1965; Levine 1981).

In this study, we characterize the general form and surface morphology of two new gregarine species, namely *Lankesteria chelyosomae* sp. n. and *L. cystodytae* sp. n. that inhabit the intestines of ascidians collected from the North-eastern Pacific Ocean. We also sequenced the small subunit rDNA from these two new species and analysed their phylogenetic position within the context of sequences derived from other gregarines, apicomplexans, dinoflagellates and ciliates. These DNA 'barcodes' are extremely useful in delimiting different gregarine species that show considerably intraspecific morphological variation associated with the development of trophozoites from sporozoite stages.

Materials and methods

Collection and isolation of organisms

Trophozoites of *L. chelyosomae* sp. n. were isolated from the intestines of *Chelyosoma columbianum* Huntsman, 1912, the transparent disc top tunicate; trophozoites of *L. cystodytae* sp. n. were isolated from *Cystodytes lobatus* (Ritter, 1900), the lobed compound tunicate. Hosts were collected during a trip on the research vessel MV/Alta from the Bamfield Marine Science Centre. A dredge haul was conducted at Wizard Islet (48°51.6′N, 125°09.4′W) near Bamfield Marine Station, Vancouver Island, Canada in July 2007 at a depth of 20 m. The hosts were dissected within 3 days after collection.

Gregarine trophozoites were isolated in seawater by teasing apart the intestine of *C. columbianum* and *C. lobatus* under a dissecting microscope (Leica MZ6). The gut material was examined under an inverted microscope (Zeiss Axiovert 200) and parasites were removed by micromanipulation and washed three times in seawater.

Light and Scanning Electron Microscopy

Differential interference contrast light micrographs were produced by securing parasites under a cover slip with Vaseline and viewing them with an imaging microscope (Zeiss Axioplan 2) connected to a colour digital camera (Leica DC500).

Six trophozoites of *L. chelyosomae* sp. n. and 30 trophozoites of *L. cystodytae* sp. n. were prepared for SEM. Individuals were deposited directly into the threaded hole of a Swinnex filter holder, containing a 5-μm polycarbonate membrane filter (Coring Separations Div., Acton, MA), that was submerged in 10 mL of 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. A 10-mL syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with 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, San Jose, CA).

DNA isolation, PCR, cloning, and sequencing

One hundred trophozoites of *L. chelyosomae* sp. n. were isolated from the intestines of *C. columbianum*; 146 trophozoites of *L. cystodytae* sp. n. were isolated from the intestines of *C. lobatus*.

The trophozoites of both species were washed three times in filtered seawater, and deposited into a 1.5-mL Eppendorf tube. DNA was extracted by using the total nucleic acid purification protocol specified by EPICENTRE (Madison, WI). Small-subunit (SSU) rDNA was PCR amplified using puReTaq Ready-to-go PCR beads (GE Healthcare).

SSU rDNA sequences from *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. were amplified in one fragment using universal eukaryotic PCR primers F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander *et al.* 2003) and internal primers designed to match existing eukaryotic SSU sequences F2 5′-TGCGCTACCTGGTT GATCC-3′, F3 5′-AAGTCTGGTGCCAGCAGCC-3′ and R2 5′-GCCTYGCGACCATACTCC-3′. All amplifications consisted of an initial denaturing period (95 °C for 2 min), 35 cycles of denaturing (92 °C for 45 s), annealing (45 °C for 45 s) and extension (72 °C for 1.5 min) and a final extension period (72 °C for 5 min) (Leander *et al.* 2003). 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, Frederick, MD). Eight cloned plasmids were digested with EcoR1 and screened for size. Three clones from each species (six in total) were sequenced with ABI bigdye reaction mix using vector primers and internal primers orientated in both directions. The SSU rDNA sequences were initially identified by BLAST analysis prior to phylogenetic analysis (GenBank Accession numbers EU670240–EU670241).

Molecular phylogenetic analysis

Representative sequences from *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. were aligned with 49 alveolate SSU rDNA sequences using MacClade 4 (Maddison & Maddison 2000) and visual fine-tuning. A 20-taxon alignment was also created and consisted of ciliates, all marine eugregarines and the three clones from each of the two new species. Maximum likelihood (ML) and Bayesian methods under different DNA substitution models were performed on the 51-taxon alignment (1042 unambiguous sites) and on the 20-taxon alignment (1102 unambiguously aligned sites). All gaps were excluded from the alignments before phylogenetic analysis. The α-shape parameter was estimated from the data using the general-time-reversible (GTR) model for base substitutions (Posada & Crandall 1998) and a γ-distribution with invariable sites (51-taxon alignment: eight rate categories, $\alpha = 0.429$, fraction of invariable sites = 0.052; 20-taxon alignment: eight rate categories, $\alpha = 0.372$, fraction of invariable sites = 0.007). ML bootstrap analyses were performed using PhyML* (Guindon & Gascuel 2003; Guindon *et al.* 2005) on 100 resampled data under an HKY model using an α-shape parameter and transition/transversion ratio (Ti/Tv) estimated from each data set.

We also examined the 51-taxon data set with Bayesian analysis using the program MrBayes 3.0 (Huelsenbeck & Ronquist 2001). The program was set to operate with GTR, a γ-distribution, and four Monte Carlo Markov chains (MCMC; default temperature = 0.2). A total of 2 000 000 generations were calculated with trees sampled every 100 generations and with a prior burn-in of 200 000 generations (2000 sampled trees were discarded). A majority-rule consensus tree was constructed from 18 000 postburn-in trees with PAUP* 4.0 (Swofford 1999). Posterior probabilities correspond to the frequency at which a given node is found in the postburn-in trees.

Results

General morphology and surface ultrastructure

The descriptors used here for plane cell shapes are in accordance with the nomenclatural system established by Clopton (2004). The trophozoites of *L. chelyosomae* sp. n. were spatulate to narrowly obpyriform, usually with a broader head-like anterior region and a distinctively narrower tail-like posterior region (Figs 1A–D, 2A and 3A). The trophozoites of *L. cystodytae* sp. n. were elliptoid to obdeltoid, usually with a broad anterior end that narrowed to a blunt posterior end (Figs 1E–H, 2B and 4A–B). Under the light microscope, the trophozoites of both species were brown, suggesting an accumulation of amylopectin granules within the cytoplasm. For both species the nucleus was located at the anterior end of the cell, just behind a pointed mucron (Fig. 1A–H). Some trophozoites had a more rounded mucron (Fig. 1B). In most trophozoites of both species, the area of the mucron lacked amylopectin granules and was clear. The trophozoites of both species varied considerably in size, but the range of size variation between the two species was different (Fig. 2). The trophozoites of *L. chelyosomae* sp. n. were relatively long with a mean length of 182 μm (78–287 μm, $n = 6$), whereas in *L. cystodytae* sp. n. they were relatively short with a mean length of 70 μm (15–141 μm, $n = 11$). The nucleus of *L. chelyosomae* sp. n. was spherical (25–40 μm in diameter); the nucleus of *L. cystodytae* sp. n. was smaller and more oval shaped (10×15 μm). The trophozoites of both species were generally rigid and were capable of gliding locomotion without associated changes in cell shape. However, some trophozoites could undergo slight bending movements when stationary.

Without exception, trophozoites possessed dense arrays of epicytic knobs across the entire surface of the cell (Figs 3 and 4), including the mucron (Fig. 3B). In *L. chelyosomae* sp. n., the knobs were about 70–80 nm in diameter; in *L. cystodytae* sp. n. they were 58–70 nm in diameter (Figs 3E and 4E). The density of epicytic knobs on the surface of *L. chelyosomae* sp. n. was between 18 and 25 knobs per 0.5 μm^2 (Fig. 3E) and between 30 and 32 knobs per 0.5 μm^2 on the surface *L. cystodytae* sp. n. (Fig. 4E). Epicytic folds were shallow for both species (Figs 3C,D and 4D), however, the longitudinal folds were not continuous across the length of the cell in either species. More epicytic folds were present on the posterior

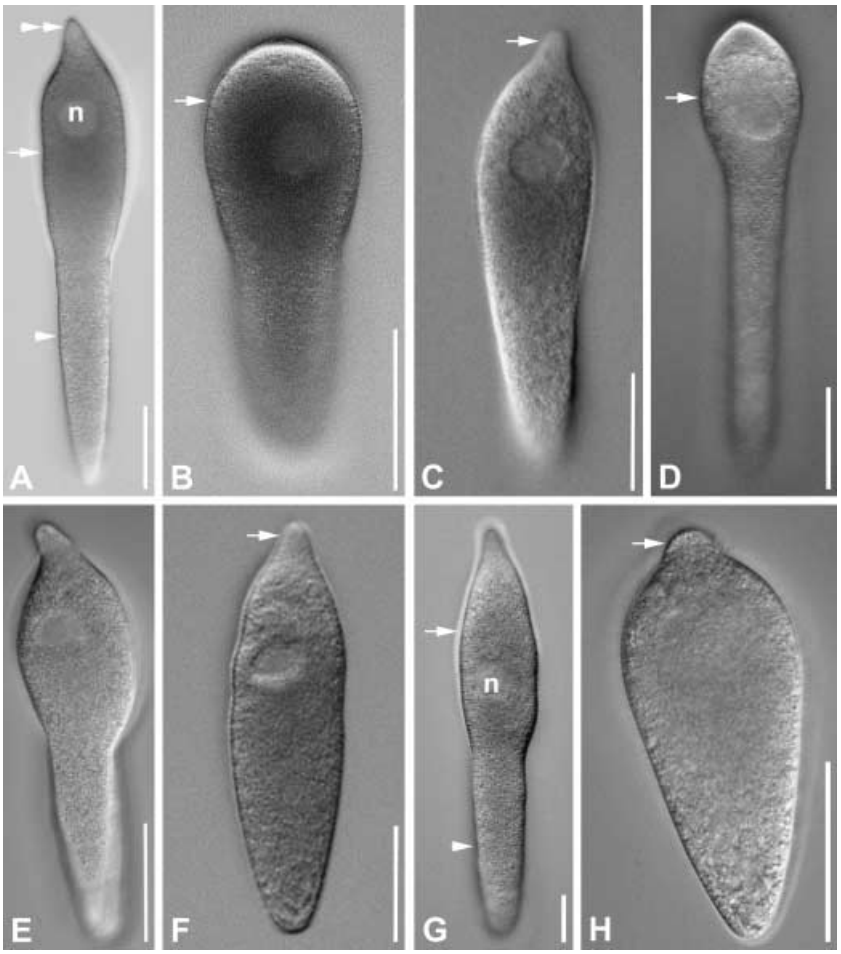


Fig. 1 A–H. Differential interference contrast (DIC) light micrographs showing the general morphology of *Lankesteria chelyosomae* sp. n. (A–D) and *Lankesteria cystodytae* sp. n. (E–H). —A. Trophozoite with a distinct head-like (arrow) and tail-like (arrowhead) region. The mucron is relatively pointed (double arrowhead). The nucleus (n) is positioned in the anterior part of the cell. (Bar = 50 μm). —B. Trophozoite with a swollen head-like region (arrow) (Bar = 65 μm). —C. Trophozoite with a spatulate cell shape, the anterior part terminates with a pointed mucron (arrow) (Bar = 35 μm). —D. Trophozoite with a bulbous head-like (arrow) and a long tail-like region (Bar = 30 μm). —E. Trophozoite with a bulbous head-like region, terminating with a pointed mucron. The tail-like region is relatively short (Bar = 40 μm). —F. Trophozoite with elliptoid cell shape. The anterior part terminates in a relatively pointy mucron (arrow) (Bar = 30 μm). —G. Trophozoite with head-like (arrow) and a tail-like (arrowhead) region. The nucleus (n) is situated in the posterior part of the head-like region (Bar = 15 μm). —H. Trophozoite with an obdeltoid cell shape and a rounded mucron (arrow) (Bar = 40 μm).

tail-like region of the trophozoites in *L. chelyosomae* sp. n., whereas in *L. cystodytae* sp. n., they were present over the entire cell surface.

Molecular phylogeny of Lankesteria *as inferred from SSU rDNA*

The 51-taxon data set recovered a strongly supported clade of ciliates, a weakly supported clade consisting of dinoflagellates and *Perkinsus*, a well-supported clade of *Colpodella* species, and an apicomplexan clade with a poorly resolved backbone (Fig. 5). Within the apicomplexans, a moderately supported clade of piroplasmids and coccidians formed the sister group to a weakly supported clade consisting of rhytidocystids, cryptosporidians, neogregarines, monocystid eugregarines, archigregarines and eugregarines. The new sequences from *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. clustered within a weakly supported clade of mainly marine gregarines including archigregarines, such as *Selenidium terebellae*, and eugregarines, such as *Lecudina tuzetae*. The genera *Lecudina* and *Lankesteria* (excluding *Lecudina polymorpha*) and several environmental sequences formed a highly supported clade within the marine eugregarines (Fig. 5). This *Lankesteria-Lecudina* clade consisted of three main subclades: (i) *Lankesteria abbotti* and *Lankesteria* sp. (from *Clavelina picta*), (ii) *Lecudina tuzetae* and an environmental sequence (AY179977), and (iii) *L. chelyosomae* sp. n. and *L. cystodytae*. The subclade consisting of the two new *Lankesteria* sequences clustered most closely with an environmental sequence (AB191437), albeit with weak support.

Moreover, a total of 1746 base pairs were compared between *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. resulting in 52 different bases and 13 indels. A pair-wise distance calculation based on the Kimura 2-parameter model (Kimura 1980) of 1728 base pairs (excluding the indels) resulted in a 2.1–3.1% sequence divergence between the two species. Variation between the three clones of *L. chelyosomae* sp. n. was 0.3–0.5%; variation between the three clones of *L. cystodytae* sp. n. was 1.3–1.5%. Phylogenetic analyses of the 20-taxon data set containing all six clones, demonstrated that the three clones of *L. chelyosomae* sp. n. clustered together as a sister group to the three clones of *L. cystodytae* sp. n. with very strong support (data not shown).

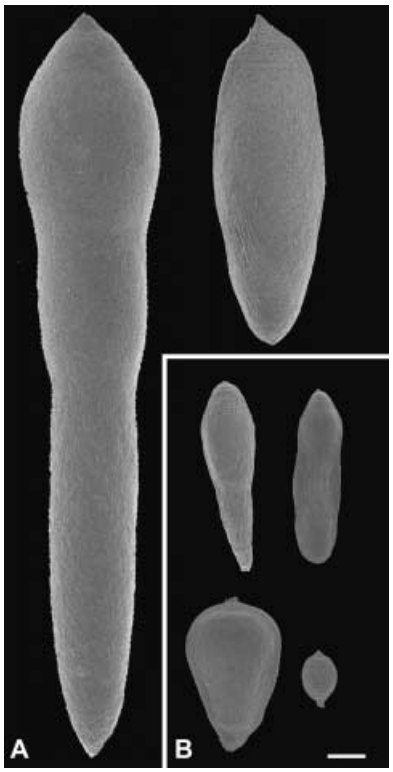


Fig. 2 A, B. Scanning electron micrographs (SEM) of trophozoites showing general morphology and size differences in between *Lankesteria chelyosomae* sp. n. —A and *Lankesteria cystodytae* sp. n. —B (scale bar 10 μm).

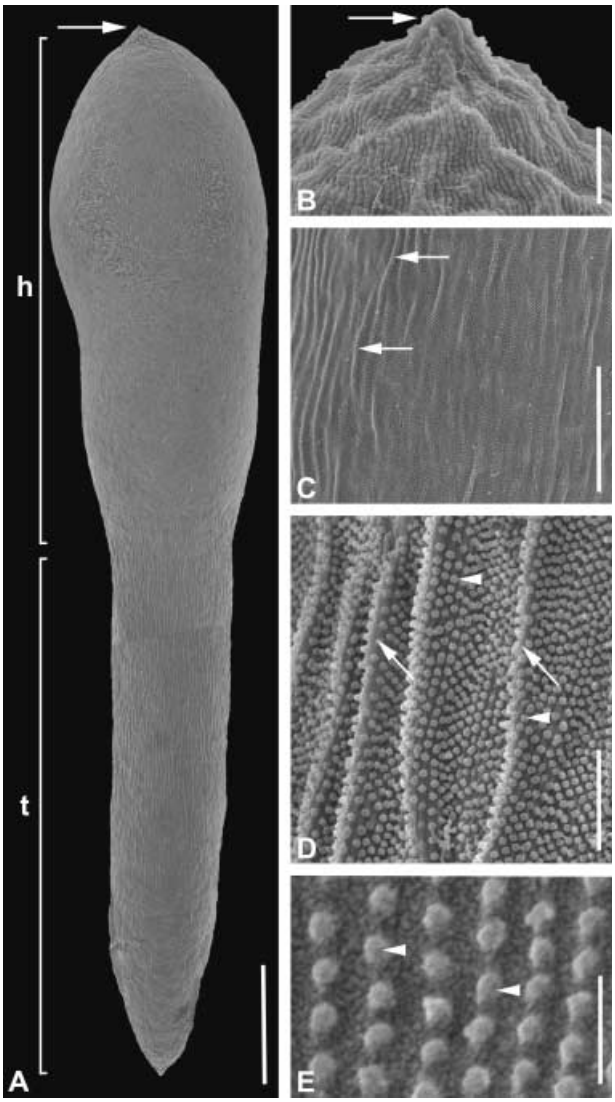


Fig. 3 A–E. Scanning electron micrographs (SEM) of *Lankesteria chelyosomae* sp. n. showing surface ultrastructure. —A. A trophozoite with a head-like (h) anterior region and a tail-like (t) posterior region. The mucron (arrow) is pointed (Bar = 20 μm). —B. Higher magnification view of the mucron (arrow) (Bar = 1 μm). —C. Higher magnification view of the tail-like region. The arrows indicate weakly developed epicytical folds (Bar = 5 μm). —D. High magnification view of epicytical folds (arrows) and epicytic knobs (arrowheads) (Bar = 1 μm). —E. High magnification view of the epicytic knobs (Bar = 250 nm).

Species descriptions

Family Lecudinidae Kamm, 1922

Genus *Lankesteria* Mingazzini, 1891

Lankesteria chelyosomae sp. n. (Figs 1A–D, 2A and 3)

Hapantotype. Wizard Islet (48°51.6′N, 125°09.4′W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (Figs 1A–D and 3).

Etymology. The name of this species refers to the genus of the ascidian type host, *Chelyosoma columbianum* Huntsman, 1912.

Type host. *Chelyosoma columbianum* Huntsman, 1912 (Metazoa, Chordata, Ascidiacea, Corellidae).

Location in host. Intestinal lumen.

Diagnosis. The overall appearance of this species resembles that of other *Lankesteria* species, but the length of the cell reaches 287 μm. The small subunit rDNA sequence EU670240 and host distinguishes *L. chelyosomae* from all other *Lankesteria* species.

Description. Body spatulate to narrowly obpyriform (Figs 1A–D, 2A and 3A), mean length 182 μm (78–287 μm), brownish in colour. Spherical nucleus (25–40 μm diameter) at the anterior end of the cell. Anterior tip terminates in a pointed

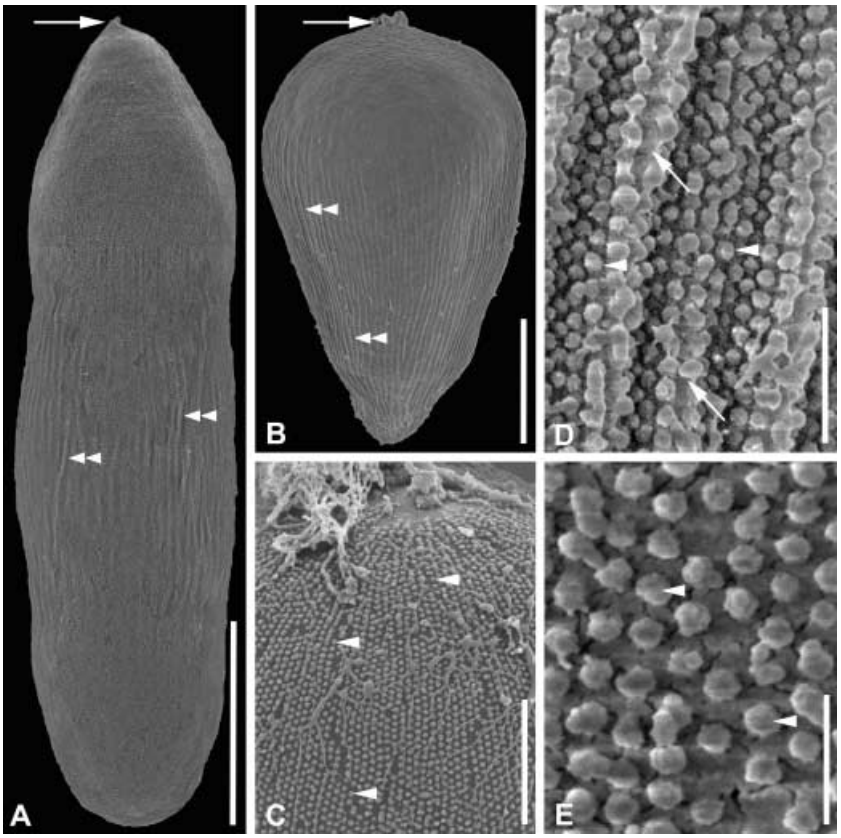


Fig. 4 A–E. Scanning electron micrographs (SEM) of *Lankesteria cystodytae* sp. n. showing surface ultrastructure. —A. A trophozoite showing an elliptoid cell shape and a pointed mucron (arrow). Double arrowheads indicate epicytical folds on the cell surface (Bar = 10 μm). —B. A trophozoite showing an obdeltoid cell shape and a more rounded mucron (arrow). Double arrowheads indicate epicytical folds on the surface (Bar = 10 μm). —C. Higher magnification view of the anterior end of a trophozoite. Epicytic knobs are clearly visible (arrowheads) (Bar = 1.5 μm). —D. High magnification of epicytical folds (arrows) and epicytic knobs (arrowheads) (Bar = 500 nm). —E. High magnification view of epicytic knobs (Bar = 250 nm).

mucron (Fig. 1A–D). Dense array of epicytic knobs over the entire surface of trophozoites (Fig. 3), 70–80 nm in diameter Shallow epicytical folds (Fig. 3C–D), mostly on posterior tail-like region of the body. Small subunit rDNA sequence EU670240.

Remarks. No gregarines have previously been reported from *Chelyosoma columbianum*. With a size of up to 287 μm and a nucleus up to 40 μm, *L. chelyosomae* is larger than any other *Lankesteria* species described from Pacific ascidians (compare Levine 1981).

Lankesteria cystodytae sp. n. (Figs 1E–H, 2B and 4)

Hapantotype. Wizard Islet (48°51.6′N, 125°09.4′W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Research Centre (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Fig. 1E–H, Fig. 4.

Etymology. The name of the species refers to the genus of the ascidian type host, *Cystodytes lobatus* (Ritter, 1900).

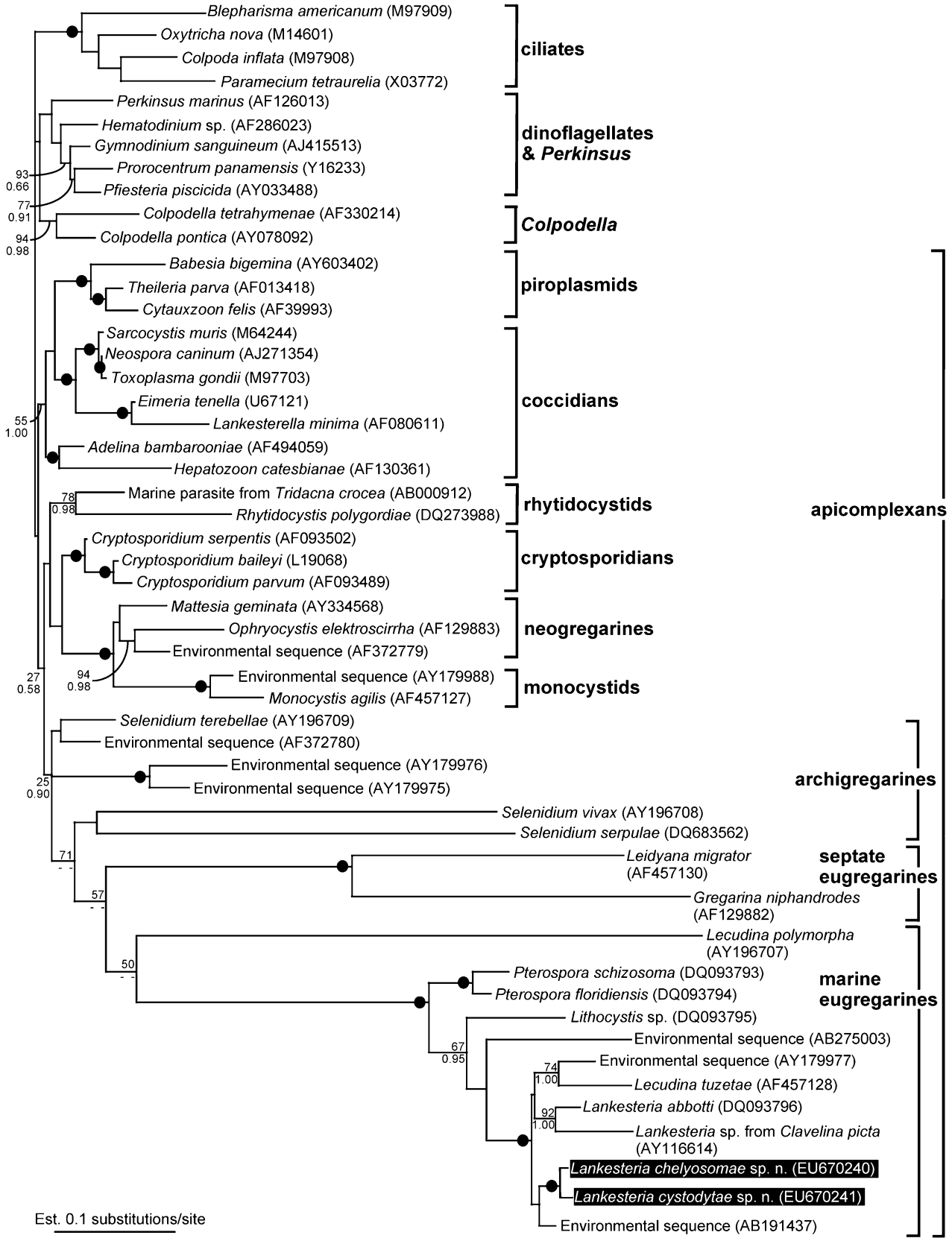
Type host. *Cystodytes lobatus* (Ritter, 1900) (Metazoa, Chordata, Ascidiacea, Clavelinidae).

Location in host. Intestinal lumen.

Diagnosis. The overall appearance of this species resembles that of other *Lankesteria* species. The small subunit rDNA sequence EU670241 and the host distinguishes *L. cystodytae* from other *Lankesteria* species.

Description. Body shape elliptoid to obdeltoid (Figs 1E–H, 2B and 4A–B), mean length is 70 μm (15–141 μm), brownish in colour. Trophozoite terminates with a pointed mucron (Fig. 1E–H). Oval nucleus (10 × 15 μm) at the anterior end of the cell. Dense array of epicytic knobs over the entire surface of trophozoites (Fig. 4), 58–70 nm in diameter

Fig. 5 γ-corrected maximum likelihood tree (–ln L = 14401.24868, α = 0.429, eight rate categories) inferred using the GTR model of substitution on an alignment of 51 SSU rDNA sequences and 1042 unambiguously aligned sites. Numbers at the branches denote bootstrap percentage (top) and Bayesian posterior probabilities (bottom). Black dots on branches denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. The sequences of the species derived from this study are highlighted in the shaded boxes.


Blepharisma americanum (M97909)
Oxytricha nova (M14601)
Colpoda inflata (M97908)
Paramecium tetraurelia (X03772)
ciliates
Perkinsus marinus (AF126013)
Hematodinium sp. (AF286023)
Gymnodinium sanguineum (AJ415513)
Prorocentrum panamensis (Y16233)
Pfiesteria piscicida (AY033488)
dinoflagellates & Perkinsus
93
0.66
77
0.91
Colpodella tetrahymenae (AF330214)
94
0.98
Colpodella pontica (AY078092)
Colpodella
Babesia bigemina (AY603402)
Theileria parva (AF013418)
Cytauxzoon felis (AF39993)
piroplasmids
Sarcocystis muris (M64244)
Neospora caninum (AJ271354)
Toxoplasma gondii (M97703)
Eimeria tenella (U67121)
Lankesterella minima (AF080611)
coccidians
55
1.00
Adelina bambarooniae (AF494059)
Hepatozoon catesbianae (AF130361)
78
0.98
Marine parasite from Tridacna crocea (AB000912)
Rhytidocystis polygordiae (DQ273988)
rhytidocystids
Cryptosporidium serpentis (AF093502)
Cryptosporidium baileyi (L19068)
Cryptosporidium parvum (AF093489)
cryptosporidians
Mattesia geminata (AY334568)
Ophryocystis elektroscirrha (AF129883)
Environmental sequence (AF372779)
neogregarines
27
0.58
94
0.98
Environmental sequence (AY179988)
Monocystis agilis (AF457127)
monocystids
apicomplexans
Selenidium terebellae (AY196709)
Environmental sequence (AF372780)
Environmental sequence (AY179976)
25
0.90
Environmental sequence (AY179975)
archigregarines
Selenidium vivax (AY196708)
Selenidium serpulae (DQ683562)
71
- -
Leidyana migrator (AF457130)
septate eugregarines
57
- -
Gregarina niphandrodes (AF129882)
Lecudina polymorpha (AY196707)
50
- -
Pterospora schizosoma (DQ093793)
Pterospora floridiensis (DQ093794)
Lithocystis sp. (DQ093795)
Environmental sequence (AB275003)
67
0.95
Environmental sequence (AY179977)
74
1.00
Lecudina tuzetae (AF457128)
92
1.00
Lankesteria abbotti (DQ093796)
Lankesteria sp. from Clavelina picta (AY116614)
Lankesteria chelyosomae sp. n. (EU670240)
Lankesteria cystodytae sp. n. (EU670241)
Environmental sequence (AB191437)
marine eugregarines
Est. 0.1 substitutions/site

(Fig. 4E). Shallow irregular epicytical folds on the body surface. Small subunit rDNA sequence EU670241.

Remarks. No gregarines have previously been reported from *C. lobatus*. *L. cystodytae* is smaller than *L. abbotti*, but all other species described for pacific ascidians are within the size range of this newly described gregarine (compare Levine 1981). Because the species descriptions in Levine (1981) provide only line drawings and nothing is known about their ultrastructure, a detailed comparison is impossible. Nevertheless, the knobbed surface ultrastrucure, small subunit rDNA sequence and host species distinguishes this new species from *L. abbotti* and all other *Lankesteria* species described from Pacific ascidians.

Discussion

Comparative morphology

Lankesteria belongs to the Lecudinidae (Apicomplexa, Eugregarinorida) and consists of gregarine species that infect ascidians. However, at one time, *Lankesteria* not only included parasites of ascidians, as originally described by Mingazzini (1891), but also parasites of turbellarians, chaetognaths and insects. Ormières (1965) revised the genus by transferring all species not occurring in ascidians to other genera (Levine 1977). Although one report indicates that identical morphotypes of *L. abbotti* were found in two different ascidian hosts (namely *Cnemidocarpa finmarkiensis* and *Clavelina huntsmani*; Leander *et al*. 2006), other reports indicate that species of *Lankesteria* are highly host specific (Levine 1981). For example, Levine (1981) was unable to find the same *Lankesteria* morphotypes in two different genera of ascidians, even when the hosts were side by side or attached to each other within the same environment. Although this issue needs to be substantiated with additional diversity surveys and by cross-transmission studies, previous reports are consistent with our establishment of two new species based in part on host specificity: *L. chelyosomae* sp. n. from *C. columbianum* and *L. cystodytae* sp. n. from *C. lobatus*.

Trophozoites are the most conspicuous and morphologically diverse stages in gregarine life cycles and their ultrastructural features reflect fundamental differences between species (Leander *et al*. 2006; Leander 2008). In addition to the fact that the two species of *Lankesteria* described here occurred in different hosts, there was a significant difference in the size and shape of the trophozoites in the two species (Figs 1 and 2). For instance, with a mean length of 182 μm, the trophozoites of *L. chelyosomae* sp. n. were considerably longer than the trophozoites of *L. cystodytae* sp. n. (mean length of 70 μm). However, the variation in cell size between and within the species could reflect different stages in the development of trophozoites from sporozoites. In practice, the life history of gregarines makes their identification based only on light micrographs and line drawings very challenging (e.g. Levine 1981). However, the surface ultrastructure as viewed by SEM, demonstrated that very different cell shapes had near identical surface characteristics. Moreover, in comparison to other eugregarines, such as most lecudinids and septate eugregarines, the trophozoite surface of the *Lankesteria* species described here was very distinctive.

Eugregarine trophozoites usually possess up to several hundred epicytic folds that run along the longitudinal axis of the cell; this condition is also found in *L. abbotti*, *L. cyrtocephala* and *L. levinei* (Simdyanov 1995; Leander *et al*. 2006; Leander 2007, 2008). These folds increase cell surface area and limit the degree of cellular deformations that occur during gliding motility (Leander 2008). In contrast, the two new species of *Lankesteria* described here had weakly developed epicytic folds, and instead, the surface was covered with a dense array of knoblike protrusions. These epicytic knobs (syn. 'tubercles') were detected previously with TEM on the surface of *L. parascidae* (Ormières 1972) and on the surface of *L. ascidiae* (Ciancio *et al*. 2001). Ormières (1972) mentioned another species in which epicytic knobs were observed on the trophozoite surface, namely *L. morchellii* Ormières, 1965. The epicytic knobs on the surface on all five of these *Lankesteria* species are reminiscent of the microtriches found on the surface of some cestodes (e.g. Palm 2004). The function of these protuberances in both cestodes and *Lankesteria* is presumably the same: to increase surface area, and perhaps generate microcurrents, that enhances nutrient absorption through the cell surface. Reliance on surface mediated nutrition in intestinal environments also helps to explain the loss of the apical complex (and myzocytosis) in eugregarine trophozoites and the development of a more bulbous mucron, as can be found in *Lankesteria* (Leander 2007, 2008). In addition to playing an important role in feeding, epicytic folds also facilitate gliding motility (Vávra & Small 1969). Interestingly, even though the surfaces of *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. lacked a continuous coverage of epicytic folds, they still were capable of relatively sluggish gliding motility. The mechanisms associated with this motility are expected to be modifications of the same gliding mechanisms used in marine eugregarines with well-developed epicytic folds (e.g. *Lecudina*).

SSU rDNA, phylogenetics and species 'barcodes'

The establishment of two new *Lankesteria* species, based initially on size differences and host specificity, was also supported by a 2.1–3.1% sequence divergence between the three clones from each species. Moreover, phylogenetic analyses of the 20-taxon data set demonstrated that the three clones of *L. chelyosomae* sp. n. clustered together as a sister clade to the three clones of *L. cystodytae* sp. n. with very strong statistical support (data not shown). These molecular 'barcodes' strengthened the separation of two new *Lankesteria* species.

Within the 51-taxon data set, the sequences of *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. clustered within a strongly supported group of marine gregarines consisting of lecudinids and environmental sequences (Fig. 5). Surprisingly, the clade formed by *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. did not cluster strongly with the clade formed by the known sequences from *L. abbotti* and *Lankesteria* sp. (from *Clavelina picta*). The sequences from the two new *Lankesteria* species did cluster, however, with an environmental sequence (AB191437), which indicates that the cellular identity of this sequence is also a member of *Lankesteria*. Nonetheless, there are well-developed epicytic folds (and no epicytic knobs) on the surface of *L. abbotti* (Leander *et al*. 2006), which stands in sharp contrast to the surface of epicytic knobs described here for *L. chelyosomae* sp. n. and *L. cystodytae* sp. n. As additional molecular phylogenetic and morphological data from more gregarines species of ascidians become available, it is expected that other species of *Lankesteria* with epicytic knobs (e.g. *L. ascidiae*, *L. parascidiae*) will become part of a distinct clade that also contains the two species described here.

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