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Molecular systematics of marine gregarine apicomplexans from Pacific tunicates, with descriptions of five novel species of *Lankesteria*

Sonja Rueckert,[1,2] Kevin C. Wakeman,[3,4] Holger Jenke-Kodama[4] and Brian S. Leander[3]

Correspondence
Sonja Rueckert
s.rueckert@napier.ac.uk

[1]School of Life, Sport and Social Sciences, Edinburgh Napier University, Sighthill Campus, Sighthill Court, Edinburgh EH11 4BN, UK

[2]Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, 415-0025 Shizuoka, Japan

[3]Departments of Zoology and Botany, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada

[4]Microbiology and Biochemistry of Secondary Metabolites Unit, Okinawa Institute of Science and Technology, 1919-1 Tancha, Kunigami, 904-0412 Okinawa, Japan

The eugregarines are a group of apicomplexan parasites that mostly infect the intestines of invertebrates. The high level of morphological variation found within and among species of eugregarines makes it difficult to find consistent and reliable traits that unite even closely related lineages. Based mostly on traits observed with light microscopy, the majority of described eugregarines from marine invertebrates has been classified into a single group, the Lecudinidae. Our understanding of the overall diversity and phylogenetic relationships of lecudinids is very poor, mainly because only a modest amount of exploratory research has been done on the group and very few species of lecudinids have been characterized at the molecular phylogenetic level. In an attempt to understand the diversity of marine gregarines better, we surveyed lecudinids that infect the intestines of Pacific ascidians (i.e. sea squirts) using ultrastructural and molecular phylogenetic approaches; currently, these species fall within one genus, *Lankesteria*.
We collected lecudinid gregarines from six ascidian host species, and our data demonstrated that each host was infected by a different species of *Lankesteria*: (i) *Lankesteria hesperidiiformis* sp. nov., isolated from *Distaplia occidentalis*, (ii) *Lankesteria metandrocarpae* sp. nov., isolated from *Metandrocarpa taylori*, (iii) *Lankesteria halocynthiae* sp. nov., isolated from *Halocynthia aurantium*, (iv) *Lankesteria herdmaniae* sp. nov., isolated from *Herdmania momus*, (v) *Lankesteria* cf. *ritterellae*, isolated from *Ritterella rubra*, and (vi) *Lankesteria didemni* sp. nov., isolated from *Didemnum vexillum*. Visualization of the trophozoites with scanning electron microscopy showed that four of these species were covered with epicytic folds, whereas two of the species were covered with a dense pattern of epicytic knobs. The molecular phylogenetic data suggested that species of *Lankesteria* with surface knobs form a clade that is nested within a paraphyletic assemblage species of *Lankesteria* with epicytic folds.

Abbreviations: AIC, Akaike information criterion; DIC, differential interference contrast; GTR, general-time reversible; ML, maximum-likelihood; SEM, scanning electron microscopy; SSU, small-subunit.

The GenBank/EMBL/DDBJ accession numbers for the SSU rRNA gene sequences determined in this study are KR024700–KR024702 (*Lankesteria hesperidiiformis* sp. nov. isolate 1 and isolate 2 clones 1 and 2), KR024698 and KR024699 (*Lankesteria metandrocarpae* sp. nov. isolate 1 clones 1 and 2), KR024693 and KR024694 (*Lankesteria halocynthiae* sp. nov. isolate 1 clones 1 and 2), KR024697 (*Lankesteria herdmaniae* sp. nov.), KR024695 and KR024696 (*Lankesteria* cf. *ritterellae* isolates 1 and 2) and KR024689–KR024692 (*Lankesteria didemni* sp. nov. isolate 1 clones 1 and 2 and isolate 2 clones 1 and 2).

INTRODUCTION

The Apicomplexa encompasses a diverse group of unicellular parasites, including well-studied pathogens of humans and livestock (e.g. *Plasmodium*, the causative agent of malaria, and *Cryptosporidium*) and poorly studied parasites, called gregarines, that infect the intestines, coeloms and reproductive vesicles of terrestrial, freshwater and marine invertebrates. Grassé (1953) suggested that gregarines be separated into three major groups: (i) archigregarines, which occur exclusively in marine habitats and have intestinal extracellular trophozoite (feeding) stages that

resemble the general morphology of infective sporozoite stages; (ii) eugregarines, which occur in marine, freshwater and terrestrial habitats and have extracellular trophozoites that are significantly larger and morphologically more complex than the sporozoites; and (iii) neogregarines, which exclusively infect insects and have reduced trophozoite stages on host tissues other than the intestines. Although these groupings are commonly used to discuss gregarine diversity, molecular phylogenetic data suggest that they are almost certainly oversimplifications (Leander, 2008). Current data are still too scarce to revise gregarine taxonomy confidently as a whole, despite the fact that one author has recently attempted to do so (Cavalier-Smith, 2014).

Most species of marine eugregarines (25 genera) are classified within the Lecudinidae Kamm, 1922 (Levine, 1976, 1988). Generally speaking, lecudinids tend to be host specific, so differences in host taxa have been used in combination with other traits to discriminate different species of marine lecudinids from one another (Levine, 1979; Perkins *et al.*, 2000; Rueckert & Leander, 2008). Trophozoites are the most conspicuous stage in the life cycle of lecudinds, so the diversity of traits associated with this stage, such as surface ultrastructure, provides additional evidence for determining the boundaries between different lineages of lecudinids. For example, the trophozoites of intestinal lecudinids typically have numerous epicytic folds (up to 400) that run along the longitudinal axis of the cell (Leander, 2008; Desportes & Schrével, 2013). Marine gregarines that inhabit the hosts' coelom, by contrast, often lack longitudinal folds and possess either hair-like projections (Dyakin & Simdyanov, 2005) or organized arrays of surface crenulations (Landers & Leander, 2005). Species boundaries are reinforced when host specificity and traits associated with surface ultrastructure are consistent with molecular phylogenetic data.

In an attempt to understand the diversity of marine gregarines better, we surveyed lecudinids that infect the intestines of Pacific ascidians (i.e. sea squirts) using ultrastructural and molecular phylogenetic approaches; currently, these species fall within one genus, *Lankesteria* Mingazzini, 1891. The trophozoites different species of *Lankesteria* have surfaces covered in either dense arrays of surface knobs or epicytic folds (Ormières, 1972; Ciancio *et al.*, 2001; Rueckert & Leander, 2008). Currently, there are small-subunit (SSU) rRNA gene sequences available for only four species of *Lankesteria*, which limits inferences about whether or not certain trophozoite traits (surface knobs) reflect phylogenetic relationships. In this study, we characterized six different species of *Lankesteria* from six different ascidian hosts collected from both coasts of the Pacific Ocean (Canada and Japan). Five of the six species of *Lankesteria* we characterized are novel. Molecular phylogenetic analyses of the more comprehensive dataset from *Lankesteria* enabled us to establish boundaries between the different species and to evaluate the reliability of surface ultrastructural traits in predicting phylogenetic relationships.

METHODS

Collection and isolation of organisms. The tunicates *Metandrocarpa taylori* Huntsman, 1912, *Halocynthia aurantium* (Pallas, 1787), *Ritterella rubra* Abbott & Trason, 1968 and *Didemnum vexillum* Kott, 2002 were collected by SCUBA diving (5–10 m depth) in August 2013 from wooden dock pilings at Bamfield Marine Science Centre (48° 50′ 03″ N 125° 08′ 13″ W), British Columbia, Canada. During this time, *Distaplia occidentalis* Bancroft, 1899 was collected from a dredge haul conducted at Wizard Islet (48° 51′ 06″ N 125° 09′ 04″ W), at a depth of 20 m, during a trip on the research vessel M/V *Alta* from the Bamfield Marine Science Centre. *Herdmania momus* (Savigny, 1816) was collected from a drained tank (34° 40′ 02″ N 138° 56′ 08″ E) in 2011 at the Shimoda Marine Research Centre, Shimoda, Japan. All tunicates were stored temporarily in flowing seawater tables prior to dissection. Some hosts were later transported in cooled seawater to Vancouver, British Columbia, Canada, for further study.

Gregarine trophozoites were released from the tunicate intestines by micro-dissection using fine-tipped forceps under a low-magnification stereomicroscope (Leica MZ6). Gut contents were examined with an inverted compound microscope (Leica DM IL or Zeiss Axiovert 200). Hand-drawn glass pipettes were used to collect individual trophozoites by micromanipulation from well slides. Trophozoites were washed in filtered seawater before being prepared for microscopy and DNA extraction.

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

Individual trophozoites of the six gregarine species were deposited either directly into the threaded hole of separate Swinnex filter holders, containing a 3 μm polycarbonate membrane filter (Millipore), that was submerged in 25 ml seawater, contained within a shortened Falcon tube, or in hand-made baskets (top end of a 1000 μl pipette tip fixed with silicon to the same 3 μm filters) placed in 24-well culture plates. A piece of Whatman filter paper was mounted on the inside of the lids of both sample holders. The Whatman filter paper was saturated with 4 % OsO_4 and the lids closed. The trophozoites were fixed by OsO_4 vapour for 40 min. Following the initial fixation, a single drop of 4 % OsO_4 was added to the hand-made baskets, and four drops were added to the Swinnex filter holders; the parasites were fixed for an additional 30 min on ice. The samples from Canada were washed with distilled water and then dehydrated with a graded ethanol series for 10–15 min at each step (50, 70, 85, 90, 95 and 100 %) and critical-point dried with CO_2. Filters were mounted on stubs, sputter coated with 5 nm gold and viewed under a scanning electron microscope (Hitachi S4700). The sample from Japan was freeze-dried using tert-butanol as a solvent. Solvents were exchanged in a graded series of ethanol and tert-butanol, for 5 min in each concentration, finishing with four exchanges of 100 % tert-butanol. The sample was put in the freezer for 5 min, before being transferred to a JEOL JFD-320 dry-freezer. Filters were also mounted on stubs, sputter coated with 5 nm gold and viewed under a scanning electron microscope (JEOL NeoScope JCM 5000). Samples that were already collected in the field were stored in 70 % ethanol for transportation. All samples were critical-point dried within 7 days of collection. Some scanning electron microscopy (SEM) data were presented on a black background using Adobe Photoshop CS5 (Adobe Systems).

DNA isolation, PCR amplification, cloning and sequencing. Single trophozoites were isolated using hand-drawn glass pipettes. Trophozoites were washed in filtered, autoclaved seawater until clean and

deposited into a 1.5 ml microfuge tube. Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification kit (EPICENTRE). SSU rRNA gene sequences were amplified by PCR using puReTaq Ready-to-go PCR beads (GE Healthcare) and the eukaryotic PCR primers F1 (5′-GCGCTACCTGGTTGATCCTGCC-3′) and R1 (5′-GATCCTTCTGCAGGTTCACCTAC-3′) (Leander *et al.*, 2003). The thermocycler was set to an initial denaturation at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min and a final elongation step at 72 °C for 9 min. For *Lankesteria herdmaniae* sp. nov., PCR products corresponding to the expected size (~1800 bp) were gel-isolated and cloned into the pSC-A-amp/kan vector, using the StrataClone PCR cloning kit (Agilent Technologies). For all other species, 1 µl PCR product was diluted 100-fold in distilled water and 1 µl of this dilution was used in subsequent amplifications, pairing internal primers specific for lecudinid marine gregarines, F2 (5′-GTDAATCGGCGTGTTCYACG-3′) and R2 (5′-GAATGCCCTCARCCGTTC-3′), with R1 and F1, respectively, under the following conditions: initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s and a final elongation step at 72 °C for 9 min.

PCR products corresponding to the expected size (~1000–1200 bp) were gel-isolated and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen). Eight cloned plasmids for each PCR product were digested with *Eco*RI, and inserts were screened for size using gel electrophoresis. Two clones were sequenced with ABI BigDye reaction mixture using vector primers orientated in both directions. The SSU rRNA gene sequences were identified by BLAST analysis and molecular phylogenetic analyses.

Molecular phylogenetic analyses. Six representative SSU rRNA gene sequences [of 14 sequences in total: two isolates of *Lankesteria* cf. *ritterellae*, four clones from two isolates of *Lankesteria didemni* sp. nov., three isolates (two cysts) from *Lankesteria hesperidiiformis* sp. nov., two clones each from isolates of *Lankesteria halocynthiae* sp. nov. and *Lankesteria metandrocarpae* sp. nov., and an isolate of *Lankesteria herdmaniae* sp. nov.] from six different species of *Lankesteria* were aligned with 74 additional alveolate sequences (to cover the diversity of apicomplexans) using MUSCLE 3.8.31 (Edgar, 2004). The 80-sequence alignment was subsequently edited and fine-tuned using MacClade 4.08 (Maddison & Maddison, 2005). The program Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) was used to analyse the 80-sequence alignment (1170 unambiguously aligned positions; gaps excluded) with maximum-likelihood (ML). Jmodeltest 0.1.1 selected a general-time reversible (GTR) model of nucleotide substitution (Posada & Crandall, 1998) that incorporated invariable sites and a discrete gamma distribution (eight categories) (GTR+I+Γ model: α=0.6430 and fraction of invariable sites=0.2160) under the Akaike information criterion (AIC) and AIC with correction (AICc). ML bootstrap analyses were performed on 500 pseudoreplicates, with one heuristic search per pseudoreplicate (Zwickl, 2006), using the same program set to the GTR model+I+Γ. Bayesian analysis of the 80-sequence dataset was performed using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The program was set to operate with GTR, a gamma distribution and four Monte Carlo Markov chains (MCMC; default temperature=0.2). A total of 10 000 000 generations were calculated with trees sampled every 100 generations and with a prior burn-in of 1 000 000 generations (10 000 sampled trees were discarded; burn-in was checked manually). When the average split rate fell below 0.01, the program would terminate. All other parameters were left at the default. A majority-rule consensus tree was reconstructed from 90 000 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees.

A second, 21-sequence alignment, containing all 14 *Lankesteria* sequences generated in this study, was constructed in order to increase the resolution along the backbone of the clade containing *Lankesteria* and close relatives. This dataset was constructed and analysed in the same manner as the larger 80-taxon dataset, but included 1347 unambiguously aligned sites. Jmodeltest selected a GTR model of nucleotide substitutions (GTR+I+Γ model: α=0.6220 and fraction of invariable sites=0.6020) under AIC, and AICc. MrBayes 3.1.2 was set to operate with GTR, a gamma distribution and four MCMC chains (default temperature=0.2). A total of 6 500 000 generations were calculated with trees sampled every 100 generations and with a prior burn-in of 650 000 generations (6500 sampled trees were discarded; burn-in was checked manually). When the average split rate fell below 0.01, the program would terminate. All other parameters were left at the default. A majority-rule consensus tree was reconstructed from 58 500 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees. ML bootstrap values of less than 50 % and Bayesian posterior probabilities <0.90 are considered low support, while values of 90 % and >0.95 are considered high support.

A pairwise distance calculation based on Kimura's two-parameter model (Kimura, 1980) of 1550 nt (excluding gaps and missing data) was performed using MEGA 5.0 (Tamura *et al.* 2011) on the 14 new *Lankesteria* sequences plus four previously published *Lankesteria* sequences.

RESULTS

Trophozoite morphology

***Lankesteria hesperidiiformis* sp. nov.** The trophozoites were isolated from the tunicate *Distaplia occidentalis* and were lemon-shaped [length 54 (35–80) µm, width 33 (25–42) µm, n=28] (Fig. 1a–d, h). The anterior end was often more pointed than the posterior end (Fig. 1a–d). Both ends were free of amylopectin granules (Fig. 1a–d). The mostly spherical nucleus [14 (10–22) × 15 (11–20) µm, n=25] was positioned in the middle of the cell, or shifted slightly towards the anterior end. A round nucleolus [8 (6–10) µm diameter, n=14] was visible in some of the nuclei. Gamonts in syzygy were spherical (Fig. 1e); therefore, it cannot be determined in which orientation the two gamonts were attached to each other. The spherical gametocysts [89 (50–150) µm, n=15] contained oval oocysts [10 (9–10) × 6 (6–7) µm, n=11] (Fig. 1f, g). SEM demonstrated that the trophozoites were covered by narrow and flat epicytic folds (sometimes more reminiscent of ridges) separated by broad grooves. [2 (2–3) folds per µm, n=4] (Fig. 1h–k). The trophozoites did not show any evidence of gliding motility, but showed some cellular deformations in the mucron area.

***Lankesteria metandrocarpae* sp. nov.** The trophozoites were collected from the intestines of *Metandrocarpa taylori*. The cell shape was reminiscent of a flattened peapod (i.e. the pod of a sugar pea or mangetout pea) (Fig. 2a–c). Trophozoites were 115 (93–150) µm long and 37 (27–50) µm wide (n=6) at the widest point (Fig. 2a–c). The anterior mucron was rounded, but with a pointed tip, shifted laterally and free of amylopectin; the posterior end of the cell tapered also into a pointed tip (Fig. 2a–c). The

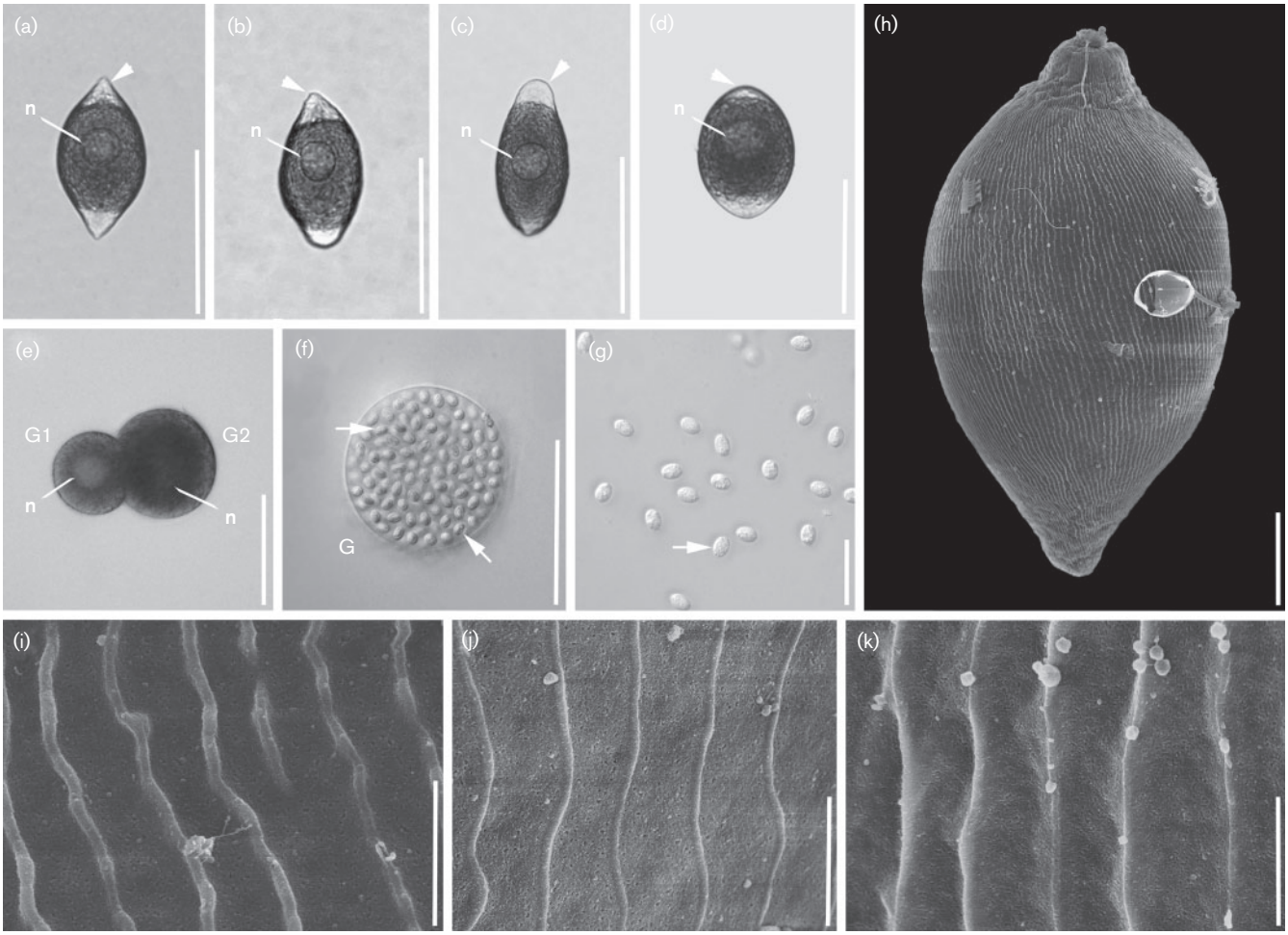

Fig. 1. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria hesperidiiformis* sp. nov. (a–d) Trophozoites showing different cell shapes from lemon-shaped to oval. The spherical nucleus (n) was mostly situated in the middle of the cell. The mucron was pointed to rounded (arrowhead). The anterior and posterior ends were free of amylopectin. Bars, 70 μm (a), 65 μm (b), 80 μm (c), 52 μm (d). (e) Two gamonts (G1, G2) in syzygy. Cells are completely rounded with a spherical nucleus (n) visible in the centre of each gamont. Bar, 50 μm. (f) Gamontocyst filled with oocysts (arrows). Bar, 100 μm. (g) Oocysts (arrow) freed from gametocyst. Bar, 30 μm. (h) SEM of a trophozoite with a nipple-like mucron. Folds or ridges are visible on the surface. Bar, 10 μm. (i–k) High-magnification views of the cell surface showing the different types of longitudinal epicytic folds. Bars, 1 μm.

ellipsoidal nucleus [15 (12–17) × 21 (20–22) μm, n=3] was situated in the anterior third of the trophozoite, at the widest point of the cell (Fig. 2a, b). SEM confirmed longitudinal epicytic folds similar to those of *Lankesteria abbotti* Levine, 1981 (Figs 2c, d). The density of folds was 4 folds per μm (Fig. 2d). The cells were rigid except for the mucron area and were capable of gliding motility.

***Lankesteria halocynthiae* sp. nov.** The trophozoites were isolated from the intestines of the tunicate *Halocynthia aurantium*. They were similar in shape to those of *Lankesteria metandrocarpae* sp. nov., but longer [length 277 (78–360) μm, width 53 (25–70) μm, n=17] (Fig. 3a–c). In some cells, the posterior end was more rounded than the anterior end (Fig. 3b). The pointed-cone-shaped mucron was free of amylopectin and situated at the anterior tip of the cell, bent to one side (Fig. 3a–c). A half-moon-shaped nucleus was situated in the anterior part of the cell [18 (13–20) × 26 (20–33) μm, n=14] (Fig. 3a, b). A spherical nucleolus was visible in some of the nuclei [5 (4–6) μm diameter, n=9]. SEM revealed longitudinal rows of epicytic folds with a density of 4 (3–4) folds per μm (Fig. 3c, d). Trophozoites were relatively rigid except for the mucron area and capable of gliding motility.

***Lankesteria herdmaniae* sp. nov.** The trophozoites were collected from the intestines of the tunicate *Herdmania momus*. The vermiform cell shape differs from all the other species of *Lankesteria* described here, as it was long and

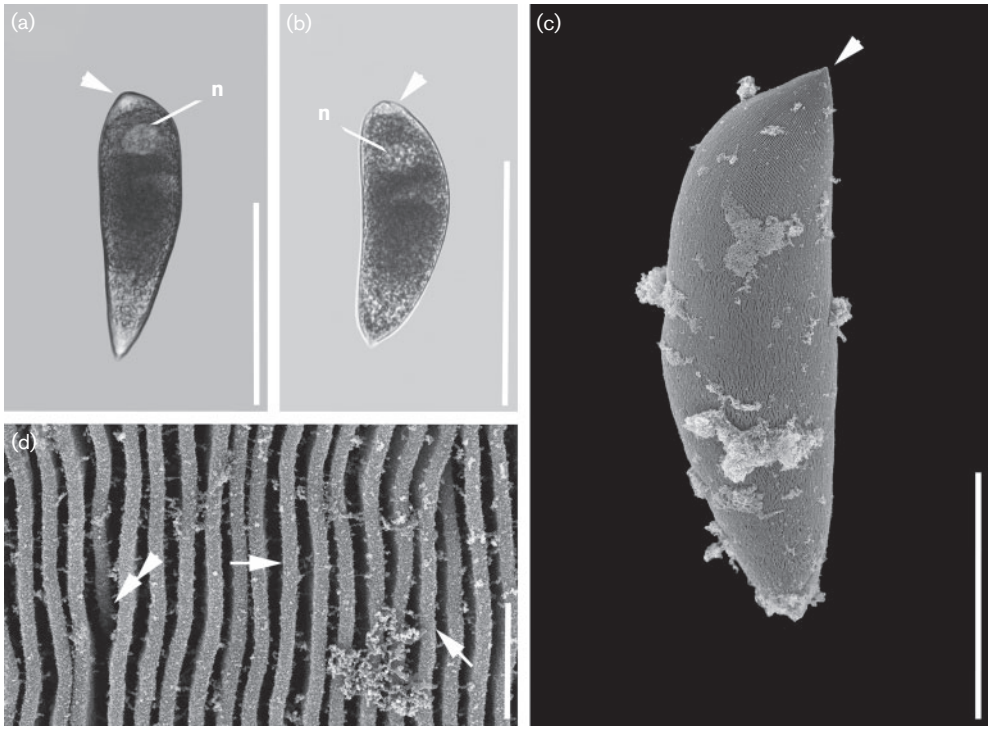

Fig. 2. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria metandrocarpae* sp. nov. (a, b) Trophozoites showing the general flattened peapod-like cell shape. The anterior mucron (arrowhead) was free of amylopectin, bent laterally with a pointed tip. The ovoid nucleus (n) was situated in the anterior half of the cell. Bars, 100 μm (a), 135 μm (b). (c) SEM of a trophozoite showing the epicytic folds and some host debris on the cell surface. Bar, 40 μm. (d) Higher-magnification SEM of the epicytic folds (arrows). Some of the epicytic folds terminate (double arrowhead) before reaching the anterior or posterior ends. Bar, 1 μm.

slender [length 276 (198–478) μm, width 13 (7–17) μm, n=16] (Fig. 4a–c). The mucron was cone shaped with a rounded tip and free of amylopectin granules (Fig. 4a, b, d). Light microscopy revealed a constriction at the base of the mucron (Fig. 4d). Most cells tapered into a rounded posterior end (Fig. 4b); some cells displayed a blunt end (Fig. 4a). The nucleus was oval [21 (15–25) × 10 (8–13) μm, n=4] situated in the anterior half of the trophozoite (Fig. 4a, b, d). SEM showed longitudinal epicytic folds (Fig. 4c, e). The posterior half of the cell was rigid. Trophozoites displayed gliding movements and the ability to bend and curl up the anterior end.

***Lankesteria* cf. *ritterellae*.** Trophozoites were collected from the intestines of the tunicate *Ritterella rubra* and were club- to peanut-shaped (Fig. 5a–e). The cells were 43 (38–50) μm long and 18 (12–27) μm wide (n=15). The mucron was broadly rounded with a pointed tip and free of amylopectin, and the posterior end was tapered and rounded (Fig. 5a–e). A large, almost spherical, nucleus was situated in the middle of the cell body or shifted slightly to the anterior end of the cell [8 (5–10) × 8 (5–12) μm, n=13] (Fig. 5a, b, d). SEM showed that the trophozoites were free of folds, but covered in dense rows of epicytic knobs (Fig. 5e–g). There were 14–16 knobs per μm, and each epicytic knob had a diameter of 62 (56–67) nm (Fig. 5f, g). The cell cortex had a crinkled appearance at the anterior end (Fig. 5e). The cells were rigid and capable of gliding motility.

***Lankesteria didemni* sp. nov.** Trophozoites were isolated from the invasive colonial tunicate *Didemnum vexillum*. The cells were similar in shape to those of *Lankesteria* cf. *ritterellae*, but larger (Fig. 6a, b, e). They were broadly rounded at the anterior end, tapering into a more pointed, but rounded posterior end. Trophozoites were 59 (31–100) μm long and 31 (19–42) μm wide (n=17). The nucleus was half-moon-shaped [12 (8–15) × 15 (10–26) μm, n=13). Some trophozoites were more rounded (Fig. 6c). Gamonts were observed in syzygy (Fig. 6d). SEM revealed dense rows of epicytic knobs that were 76 (70–78) nm long and 58 (50–78) nm wide (n=10) with 11 (8–13) knobs per μm (Fig. 6f, g). The cell cortex had a crinkled appearance at the anterior end (Fig. 6f). The cells were rigid and capable of gliding motility.

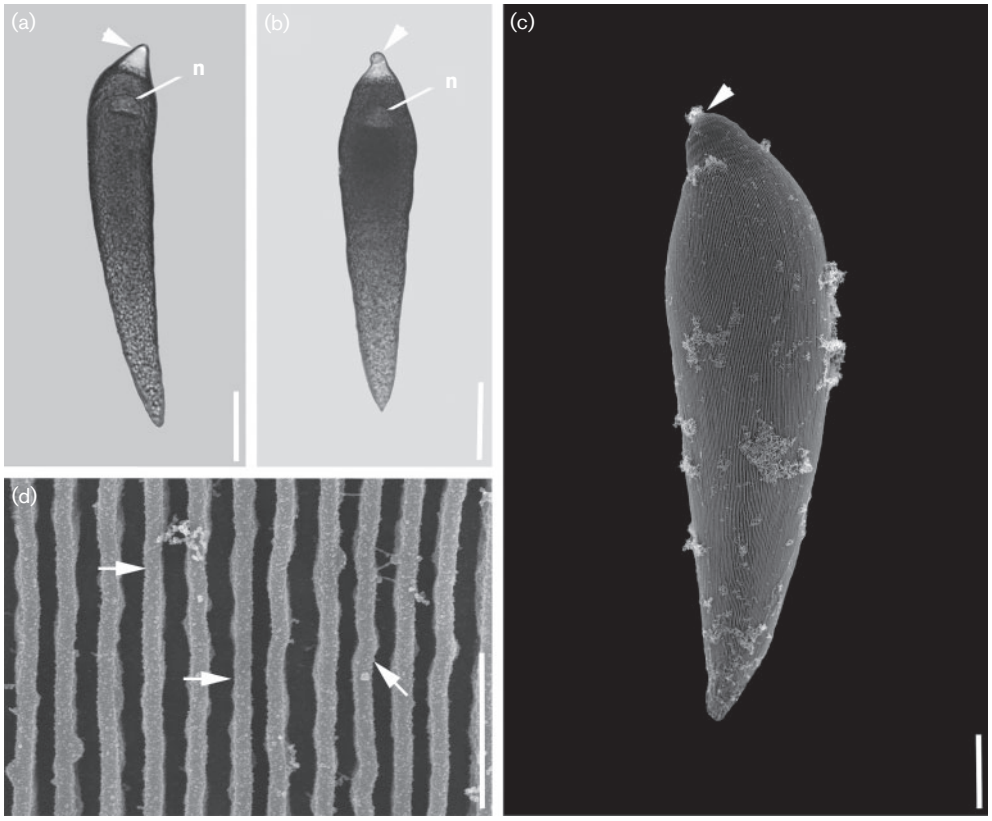

Fig. 3. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria halocynthiae* sp. nov. (a, b) Trophozoites showing the general cell shape. The cone-shaped mucron (arrowhead) was free of amylopectin. The half-moon-shaped nucleus (n) was situated in the anterior half of the cell. Bars, 55 μm (a), 70 μm (b). (c) SEM of a trophozoite showing the epicytic folds and some host debris. Bar, 20 μm. (d) High-magnification SEM of the epicytic folds (arrows). Bar, 1 μm.

Molecular phylogenetic analyses of SSU rRNA gene sequences

Molecular phylogenetic analyses of the 80-sequence dataset produced a tree topology with unresolved nodes on deeper branches. The analyses demonstrated a weakly supported clade of dinoflagellates (outgroup) and a moderately supported clade of apicomplexan sequences (ingroup) (Fig. 7). The six new *Lankesteria* sequences formed a strongly supported clade with *Lecudina* species (i.e. 'lecudinids') that was nested within a strongly supported clade consisting of marine intestinal eugregarines (i.e. lecudinids, urosporids and *Veloxidium*) (Fig. 7). Sequences from all the species of *Lankesteria* with epicytic knobs (*Lankesteria chelyosomae*, *Lankesteria ascidiae*, *Lankesteria cystodytae*, *Lankesteria* cf. *ritterellae* and *Lankesteria didemni* sp. nov.) formed a weakly supported clade. The sequences of *Lecudina longissima* and *Lecudina phyllochaetopteri* clustered together in a clade that formed the weakly supported sister group to all other *Lankesteria* and *Lecudina* sequences.

The analysis of the 21-sequence dataset included the 14 new sequences of our six species of *Lankesteria* and all other available *Lankesteria* and *Lecudina* sequences, using *Lecudina phyllochaetopteri* and *Lecudina longissima* as the outgroup. There were three main differences in the tree topology derived from the 21-sequence alignment when compared with the tree based on the 80-sequence alignment (Fig. 7). (i) The SSU rRNA gene sequences belonging to species of *Lankesteria* with surface knobs (*Lankesteria ascidiae*, *Lankesteria cystodytae*, *Lankesteria chelyosomae*, *Lankesteria* cf. *ritterellae* and *Lankesteria didemni* sp. nov.) formed a strongly supported monophyletic group (Fig. 8). (ii) All of the sequences from species of *Lankesteria* with epicytic folds, including *Lankesteria hesperidiiformis* sp. nov., but excluding *Lankesteria abbotti*, formed a weakly supported clade that was the sister group to the clade of species with surface knobs (Fig. 8). (iii) A weakly supported clade consisting of *Lankesteria abbotti* and *Lecudina tuzetae*, both with epicytic folds, formed the sister group to the clade containing all other SSU rRNA gene sequences from *Lankesteria*.

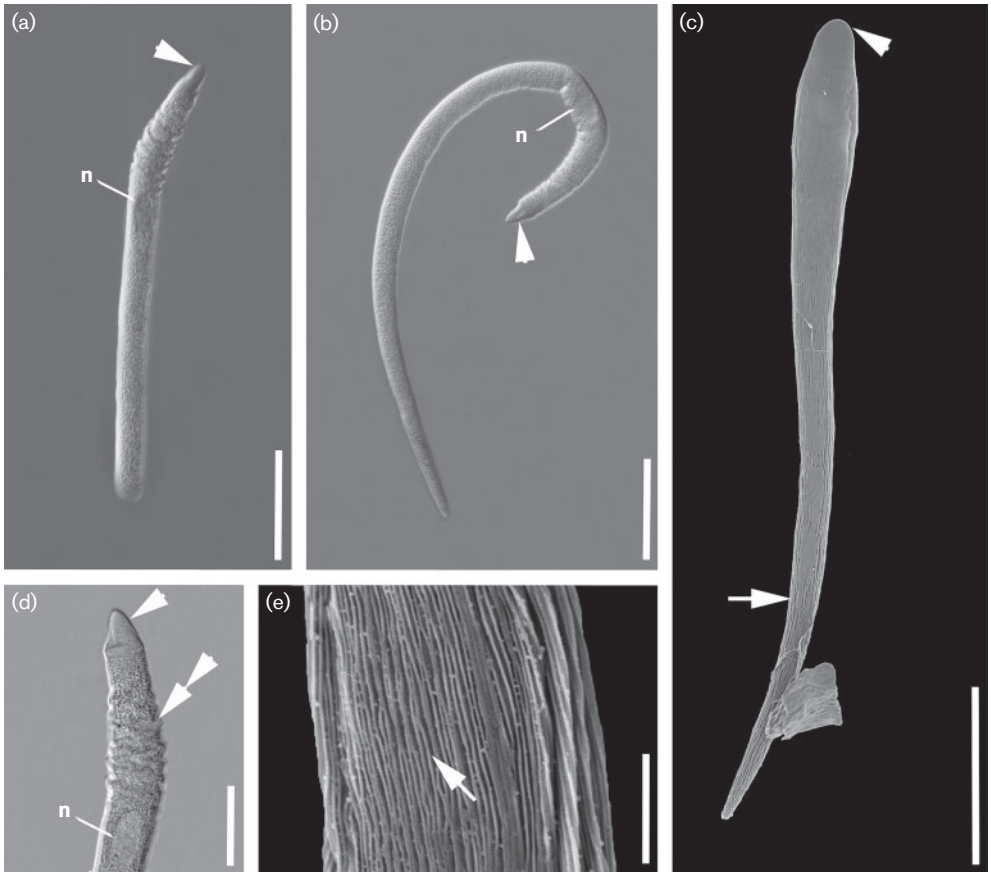

Fig. 4. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria herdmaniae* sp. nov. (a, b) Trophozoites showing long and slender cell shape. The cone-shaped mucron (arrowhead) was rounded at the tip and free of amylopectin. The oval nucleus (n) was situated in the anterior half of the cell. Bars, 50 μm. (c) SEM of a trophozoite showing the mucron (arrowhead) free of epicytic folds (arrow). Bar, 50 μm. (d) High-magnification light micrograph of mucron (arrowhead) region. The surface was crinkled (double arrowhead) in association with contraction of the anterior end. Bar, 20 μm. (e) High-magnification SEM of the epicytic folds (arrow). Bar, 5 μm.

A pairwise distance calculation based on Kimura's two-parameter model (Kimura, 1980) of 1550 nt (excluding gaps and missing data) was performed on 18 *Lankesteria* sequences (Table 1). The highest divergence of 13.8 % was found between *Lankesteria abbotti* and *Lankesteria herdmaniae* sp. nov. The range of divergence between sequences from species of *Lankesteria* displaying epicytic knobs was lower (0.1–7.6 %, mean 4.2 %) than the range of divergence between sequences from species displaying epicytic folds (0.1–13.8 %, mean 9.0 %). Intraspecific sequence divergences ranged from 0.1 to 0.8 %, while inter-specific divergences ranged from 2.2 to 7.6 % for species of *Lankesteria* with epicytic knobs and 2.1 to 13.8 % for species of *Lankesteria* with epicytic folds. Specifically, there was 0.1 % sequence divergence between the two clones of *Lankesteria* cf. *ritterellae*, 0.4–0.8 % between the four clones of *Lankesteria didemni* sp. nov., 0.1–0.4 % between the three clones of *Lankesteria hesperidiiformis* sp. nov., 0.7 % between the two clones of *Lankesteria metandrocarpae* sp. nov. and 0.4 % between the two clones of *Lankesteria halocynthiae* sp. nov.

These sequences were also checked for any clade-specific genetic signatures. Four base-pair positions (416, 719, 738 and 740) were identified according to the reference sequence of *Lankesteria abbotti* that showed the same nucleotide for all sequences from species of *Lankesteria* with epicytic knobs and a single, different nucleotide for all sequences from species of *Lankesteria* with epicytic folds (Table 2, lower section). There were three other base-pair positions (167, 614 and 1399) where the sequences of the two different groups had no common nucleotides; however, while sequences of gregarines with epicytic knobs showed only one type of nucleotide in the respective positions, the nucleotides for sequences of gregarines with epicytic folds varied.

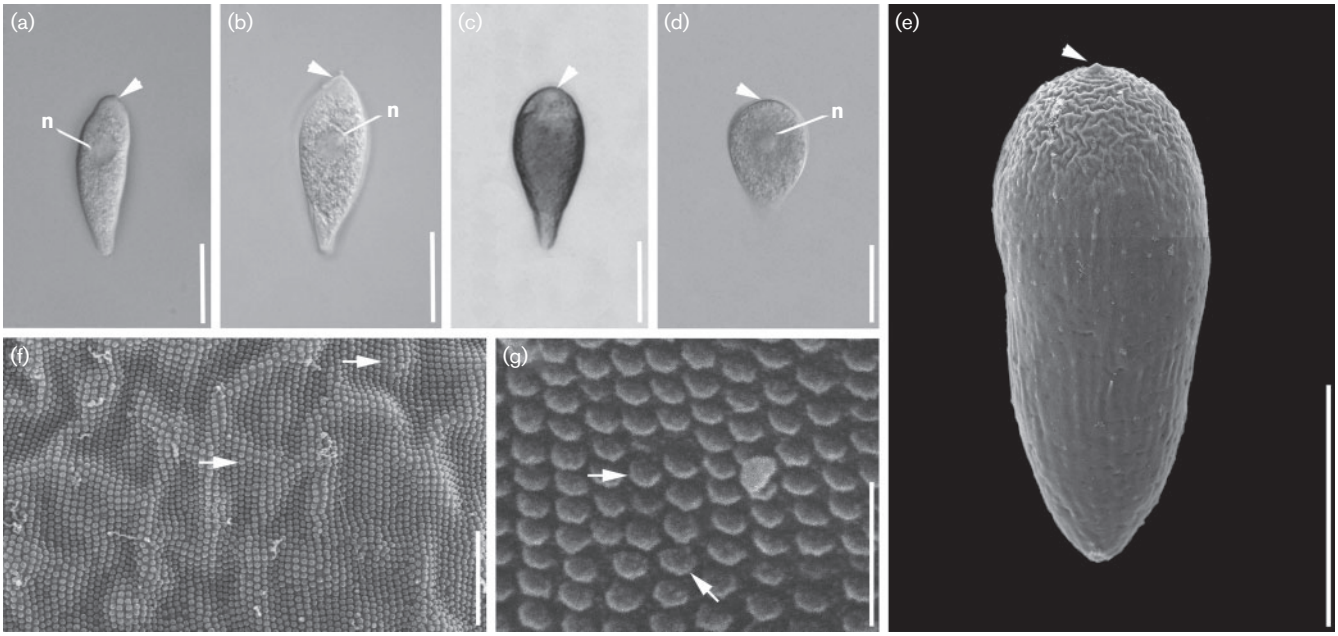

Fig. 5. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria* cf. *ritterellae*. (a–d) Trophozoites showing club-like cell shape. The mucron (arrowhead) was broadly rounded and free of amylopectin. An almost-spherical nucleus (n) was situated in the middle of the cell. Bars, 20 μm (a, b), 25 μm (c, d). (e) SEM of trophozoite showing a crinkled surface at the anterior end (arrowhead). Bar, 25 μm. (f, g) High-magnification SEMs showing the longitudinal rows of epicytic knobs (arrows). Bars, 1 μm (f), 250 nm (g).

DISCUSSION

Currently, the Lecudinidae Kamm, 1922 contains about 20 to 30 genera, depending on the source (Levine, 1977; Perkins *et al.*, 2000; Desportes & Schrével, 2013). The genus *Lankesteria* did belong to the family Urosporidae (Grassé, 1953), until Théodoridès (1967) moved it to the family Lecudinidae (compare Levine, 1977). While *Lankesteria* belongs to the Lecudinidae in most classification schemes today, there is one reference that separates this genus into the Lankesteriidae Reichenow, 1953 (Reichenow, 1953). Desportes & Schrével (2013) followed this classification by Reichenow (1953) and, according to their latest checklist, 40 species of *Lankesteria* are known from ascidians. Historically, the genus *Lankesteria* itself was poorly defined (Vávra 1969), because it contained species that infect ascidians (*Lankesteria sensu stricto* Mingazzini, 1891), chaetognaths (now *Tricystis* Hamon, 1951), turbellarians (now *Monocystella* Valkanov, 1935 and *Pseudolankesteria* Ormières, 1965) and insects (especially mosquitos, now *Ascocystis* Grassé, 1953), until Ormières (1965) revised the description of the genus and removed all species that do not infect tunicates (Levine, 1977). *Ascocystis* was then renamed as *Ascogregarina* Ward, Levine & Craig, 1982, because the name *Ascocystis* was already used for a lineage of fossil echinoderms (Ward *et al.*, 1982). Votýpka *et al.* (2009) established a new genus for gregarines that infect sand flies, namely *Psychodiella* Votýpka, Lantová and Volf, 2009. Molecular phylogenetic studies have shown that species of *Lankesteria* and *Ascogregarina* (from mosquitos), as well as species of *Psychodiella* (from sand flies), are only distantly related to each other (Rueckert & Leander, 2008; Rueckert *et al.*, 2010; Votýpka *et al.*, 2009).

According to Levine (1981), species of *Lankesteria* appear to be very narrowly host specific, and he proposed that it might be possible to use the host species or genus as a valid diagnostic character for gregarines that infect ascidians. The type species *Lankesteria ascidiae* (Lankester, 1872) Mingazzini, 1891 was described in the 19th century. Most of the original descriptions provide only line drawings and measurements, which makes the delimitation of species difficult with these sources alone. Only seven species (*Lankesteria ascidiae*, *Lankesteria parascidiae*, *Lankesteria cyrtocephala*, *Lankesteria levinei*, *Lankesteria abbotti*, *Lankesteria chelyosomae* and *Lankesteria cystodytae*) have been described at the ultrastructural level using transmission and/or scanning electron microscopy (Ormières, 1972; Simdyanov, 1995; Ciancio *et al.*, 2001; Leander *et al.*, 2006; Rueckert & Leander, 2008). Our approach is, therefore, to delimit species based on a combination of comparative data derived from light microscopy, scanning electron microscopy and SSU rRNA gene sequences.

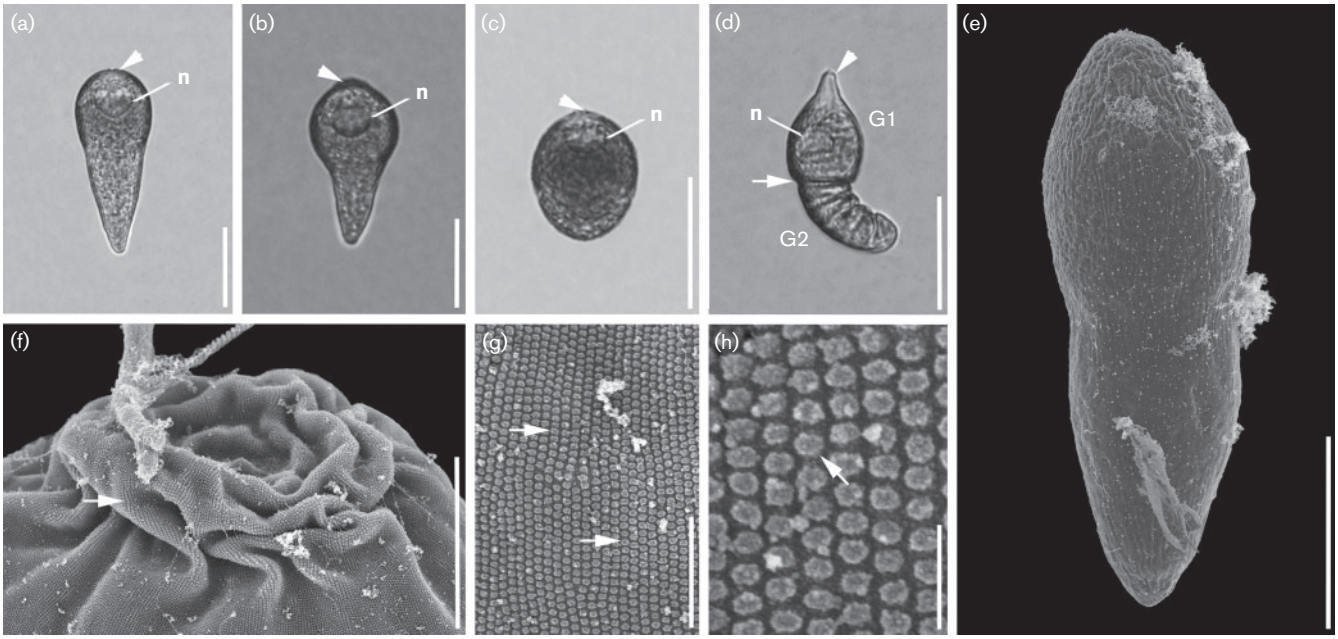

Fig. 6. DIC light micrographs and SEM showing the general trophozoite morphology and surface ultrastructure of *Lankesteria didemni* sp. nov. (a–c) Trophozoites showing the reverse teardrop-like cell shape. The mucron (arrowhead) was broadly rounded and free of amylopectin. The half-moon-shaped nucleus (n) was situated in the anterior broader part of the cell. Bars, 40 μm (a, c), 35 μm (b). (d) Two gamonts in syzygy. Gamont 1 (G1) displayed a more pointed anterior end (arrowhead) with a half-moon-shaped nucleus (n) clearly visible. No structures were visible in gamont 2 (G2). The point of attachment is marked with an arrow. Bar, 40 μm. (e) SEM of trophozoite showing a crinkled surface at the anterior end (arrow). Bar, 20 μm. (f) High-magnification SEM of the crinkled anterior end. The cell surface is covered in epicytic knobs (arrows). Bar, 5 μm. (g) High-magnification SEM of the longitudinal rows of epicytic knobs. Bar, 1 μm.

General morphology and surface ultrastructure

There are two major morphotypes of trophozoites within *Lankesteria* that appear to reflect the phylogeny of the host animals (Desportes & Schrével, 2013). Species with elongated trophozoites (e.g. *Lankesteria gigantea* and *Lankesteria butshlii*) infect ascidians in the Stolidobranchia, while the species of *Lankesteria* with more club-shaped or rounded trophozoites (e.g. *Lankesteria ascidiae* and *Lankesteria chelyosomae*) infect ascidians in the Aplousobranchia (Desportes & Schrével, 2013). *Lankesteria herdmaniae* sp. nov. is the only species of the six described here that is long and slender. It was collected from *Herdmania momus*, which belongs to the Stolidobranchia, and is consistent with previous data. Two species (*Lankesteria halocynthiae* sp. nov. and *Lankesteria metandrocarpae* sp. nov.), also collected from hosts within the Stolidobranchia, were elongated, but not distinctly long and slender. The other three species were club-shaped or rounded, and were all isolated from ascidians that belong to the Aplousobranchia, confirming the correlation stated by Desportes & Schrével (2013). The smallest gregarine found in this study, *Lankesteria* cf. *ritterellae*, was 43 μm long and 18 μm wide; the largest gregarine found in this study, *Lankesteria herdmaniae* sp. nov., was up to 478 μm long and only around 13 μm wide. One of the smallest species of *Lankesteria* described so far is *Lankesteria parascidae*, at 35 μm long; one of the longest species described so far is *Lankesteria gigantea*, at 800 μm long (Ormières 1965).

In general, eugregarine trophozoites are covered with up to several hundred longitudinal epicytic folds. Of the seven species of *Lankesteria* that have been studied with transmission and scanning electron microscopy so far, three (*Lankesteria abbotti*, *Lankesteria cyrtocephala* and *Lankesteria levinei*) have longitudinal epicytic folds, while four (*Lankesteria ascidiae*, *Lankesteria parascidiae*, *Lankesteria chelyosomae* and *Lankesteria cystodytae*) display dense arrays of epicytic knobs (also referred to as 'tubercles') (compare Ormières, 1972; Simdyanov, 1995; Leander *et al.*, 2006; Leander, 2008; Rueckert & Leander, 2008). Both epicytic folds and knobs increase the surface area and presumably play a role in surface-mediated nutritional uptake (Rueckert & Leander, 2008), but this has yet to be demonstrated. It has been shown, though, that epicytic folds are involved in actomyosin-based gliding motility (compare Valigurová *et al.*, 2013 and citations therein). We found longitudinal epicytic folds on four of the six newly studied gregarines in this study; the other two species in this study possessed patterns of epicytic knobs. The

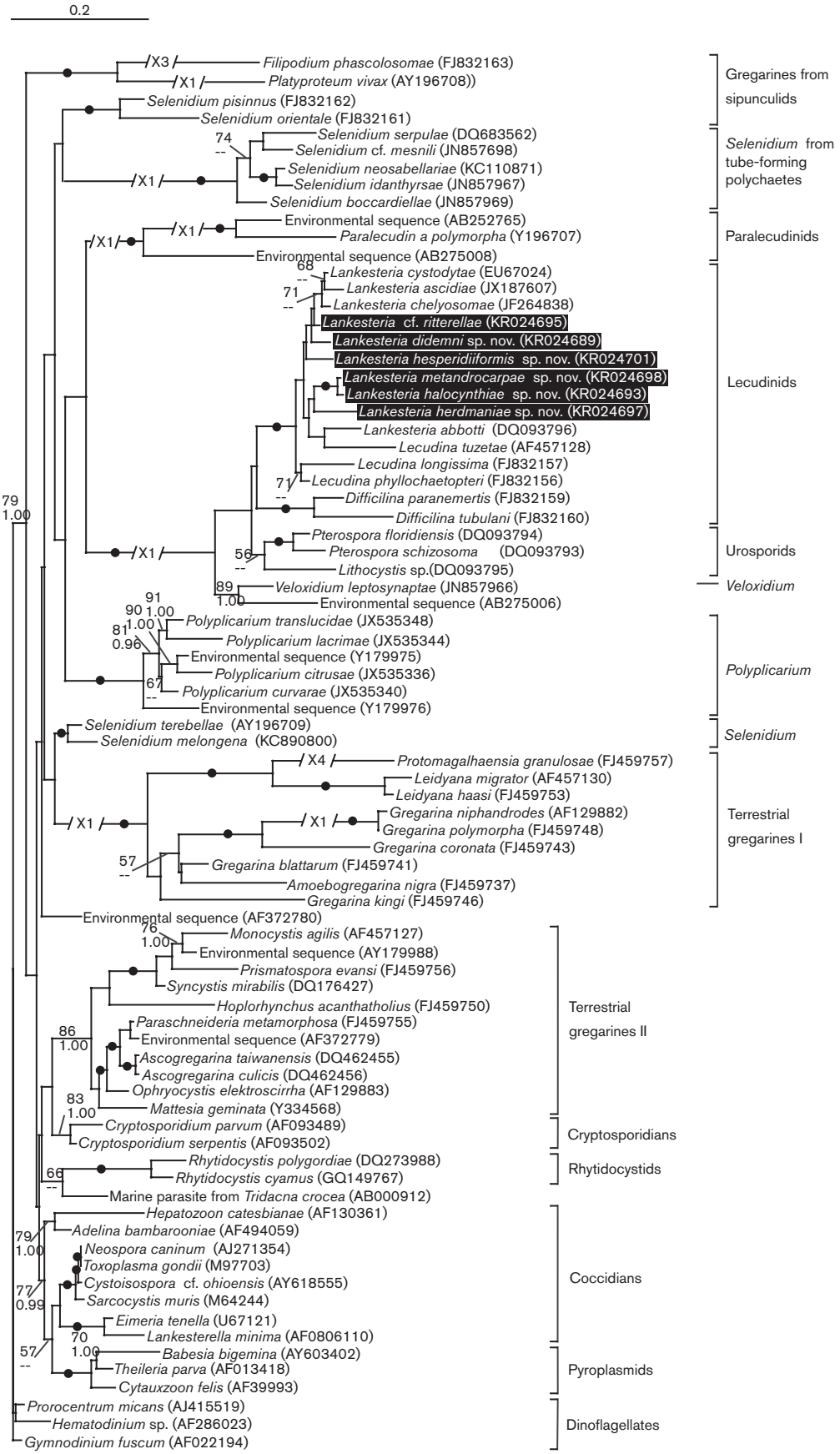

Fig. 7. ML tree as inferred using the GTR model of nucleotide substitutions, a gamma distribution and invariable sites on an alignment of 80 SSU rRNA gene sequences and 1170 unambiguously aligned sites (−ln L=15 7532.83727, α=0.6430, fraction of invariable sites=0.2160, eight rate categories). Numbers at branches denote ML bootstrap percentages (top) and Bayesian posterior probabilities (bottom). Filled circles on branches denote Bayesian posterior probabilities of 0.95 or higher and ML bootstrap percentages of 95 % or higher. Bootstrap values of less than 55 % and Bayesian posterior probabilities of less than 0.90 are not shown. Sequences derived from this study are highlighted in black boxes. Bar, 0.2 substitutions per site.

species with epicytic knobs lack the typical epicytic folds, but were still able to exhibit gliding motility. The question of how this is facilitated needs to be answered in future studies.

Molecular phylogeny of species of *Lankesteria*

Until now, SSU rRNA gene sequences were available for only four species of *Lankesteria* (*Lankesteria abbotti*, *Lankesteria ascidiae*, *Lankesteria chelyosomae* and *Lankesteria cystodytae*) (Rueckert & Leander, 2008; Rueckert *et al.*, 2010; Mita *et al.*, 2012), compared with the 40 currently described species (Desportes & Schrével, 2013). One of these four species has epicytic folds (*Lankesteria abbotti*), and the other three have surface knobs (Leander *et al.*, 2006; Rueckert & Leander, 2008; Mita *et al.*, 2012). We expected the SSU rRNA gene sequences of species with epicytic folds and species with epicytic knobs to cluster in separate clades, based on some preliminary results of previous studies (Rueckert & Leander, 2008; Rueckert *et al.*, 2010; Mita *et al.*, 2012). We now have sequence data from a total of ten species of *Lankesteria*. These sequences were

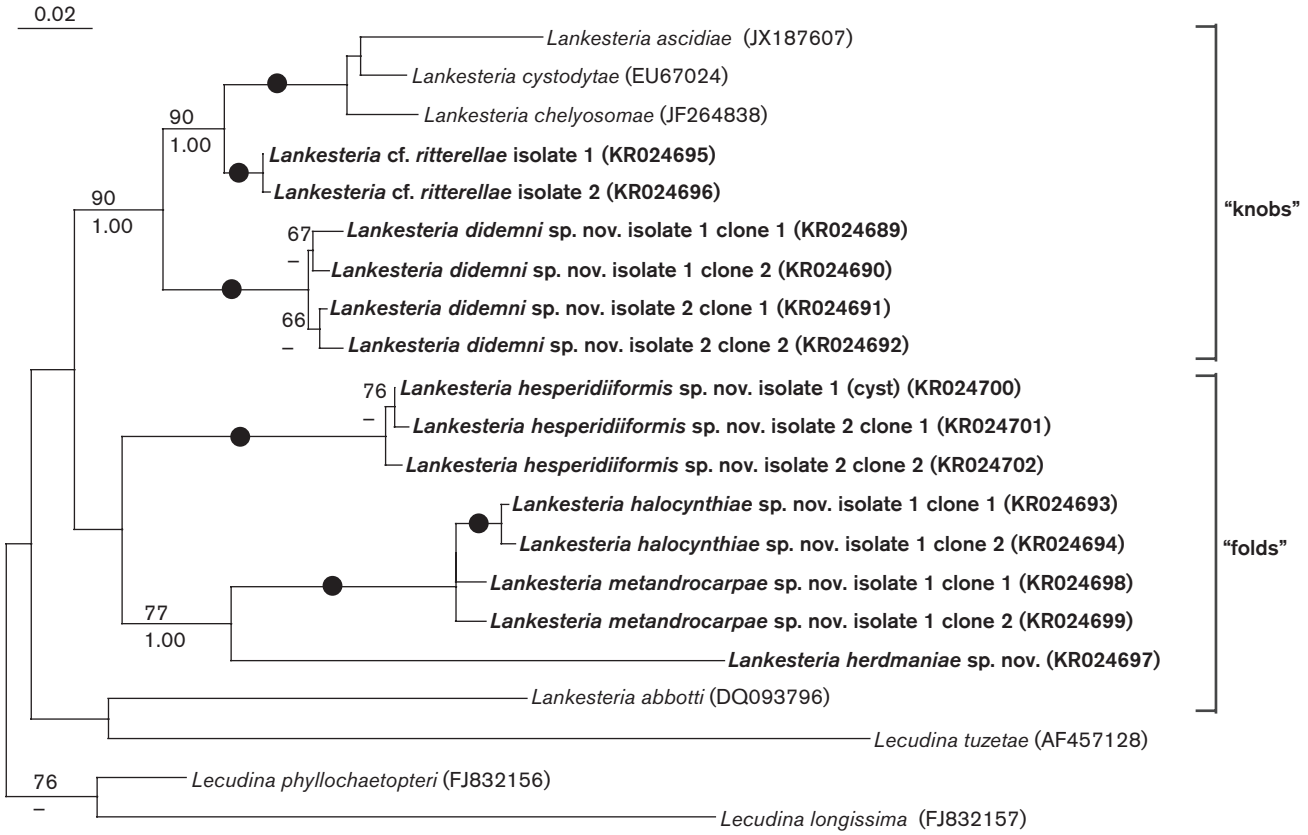

Fig. 8. More-focussed ML tree inferred from an analysis based on the GTR model of substitutions, of a gamma distribution and invariable sites on an alignment of 21 SSU rRNA gene sequences consisting of *Lankesteria* sequences plus *Lecudina tuzetae*, *Lecudina phyllochaetopteri* (outgroup) and *Lecudina longissima* (outgroup) and 1347 unambiguously aligned sites (−ln L=7398.38763, α=0.6220, fraction of invariable sites=0.6020, eight rate categories). Numbers at branches denote ML bootstrap percentages (top) and Bayesian posterior probabilities (bottom). Filled circles on branches denote Bayesian posterior probabilities of 0.95 or higher and ML bootstrap percentages of 95 % or higher. Bootstrap values of less than 55 % and Bayesian posterior probabilities of less than 0.90 are not shown. Sequences derived from this study are highlighted in bold. Bar, 0.02 substitutions per site.

Table 1. Sequence divergence of SSU rRNA gene sequences from 10 species of *Lankesteria*

Values are percentage sequence divergence. The *box* in the upper left marks intra-/inter-specific sequence divergences for species of *Lankesteria* with epicytic knobs; the **box** in the lower-right marks intra-/interspecific sequence divergences for species of *Lankesteria* with epicytic folds. Suffixes 1 and 2 indicate independent isolates; 1.1, 1.2, 2.1 and 2.2 indicate clones 1 and 2 of isolates 1 and 2.

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *L. ascidiae*	–																
2. *L. chelyosomae*	*4.0*	–															
3. *L. cystodytae*	*3.6*	*2.2*	–														
4. *L.* cf. *ritterellae* 1	*6.3*	*5.6*	*4.7*	–													
5. *L.* cf. *ritterellae* 2	*6.3*	*5.6*	*4.7*	*0.1*	–												
6. *L. didemni* 2.2	*7.5*	*6.0*	*5.0*	*3.9*	*3.9*	–											
7. *L. didemni* 2.1	*7.3*	*5.9*	*4.8*	*3.5*	*3.5*	*0.6*	–										
8. *L. didemni* 1.1	*7.6*	*5.9*	*5.0*	*3.9*	*3.9*	*0.8*	*0.6*	–									
9. *L. didemni* 1.2	*7.4*	*5.9*	*4.9*	*3.7*	*3.7*	0.7	0.5	0.4	–								
10. *L. hesperidiiformis*	10.1	9.4	8.7	*7.9*	7.8	8.6	8.4	8.5	8.3	–							
11. *L. hesperidiiformis* cyst 1	10.1	9.4	8.7	7.9	7.9	8.6	8.4	8.5	8.3	**0.4**	–						
12. *L. hesperidiiformis* cyst 2	10.0	9.2	8.6	7.8	7.8	8.4	8.2	8.4	8.1	**0.3**	**0.1**	–					
13. *L. metandrocarpae* 1.1	10.4	9.6	9.5	9.2	9.2	10.0	9.7	9.8	9.6	**10.9**	**10.9**	**10.8**	–				
14. *L. metandrocarpae* 1.2	10.6	9.7	9.5	9.3	9.3	10.1	9.8	9.8	9.7	**10.8**	**10.8**	**10.7**	**0.7**	–			
15. *L. halocynthiae* 1.1	10.7	10.0	9.9	10.0	10.0	10.4	10.1	10.2	10.0	**11.0**	**11.0**	**10.8**	**2.3**	**2.4**	–		
16. *L. halocynthiae* 1.2	10.6	9.8	9.6	9.7	9.7	10.1	9.8	9.9	9.7	**10.7**	**10.7**	**10.5**	**2.2**	**2.1**	**0.4**	–	
17. *L. herdmaniae*	13.1	11.8	11.9	11.1	11.1	11.4	11.1	11.1	10.8	**12.5**	**12.5**	**12.4**	**11.0**	**11.0**	**11.4**	**11.2**	–
18. *L. abbotti*	11.3	11.1	10.7	10.1	10.1	10.8	10.4	10.9	10.6	**11.8**	**11.8**	**11.7**	**12.3**	**12.1**	**12.7**	**12.5**	**13.8**

part of a strongly supported clade that also contained sequences from several different species of *Lecudina* (Fig. 7). Phylogenetic analyses of the 21-sequence alignment showed that *Lankesteria* is divided into two main clades: (i) a strongly supported clade of species with epicytic knobs and (ii) a weakly supported clade of species with epicytic folds (Fig. 8). When considering the phylogenetic position of a clade consisting of *Lankesteria abbotti* and *Lecudina tuzetae*, the species of *Lankesteria* with epicytic folds actually form a paraphyletic assemblage. *Lecudina phyllochaetopteri* and *Lecudina longissima*, which also have epicytic folds, formed the sister clade to the remaining *Lankesteria* and *Lecudina* sequences (Fig. 7). Even though the 21-sequence alignment contained more sites in the analyses, *Lecudina tuzetae* still clustered with *Lankesteria abbotti*, forming the sister clade to all other *Lankesteria* sequences. It is still unclear why the sequence of *Lecudina tuzetae*, a gregarine that infects the polychaete *Nereis*, clusters with species of *Lankesteria* rather than other species of *Lecudina*. Additional morphological and SSU rRNA gene sequence data from different species of *Lankesteria* and *Lecudina* are expected to improve the phylogenetic resolution of this part of the tree.

Delimitation of species of *Lankesteria*

The vast disparity in gregarine morphology and unresolved phylogenies based on molecular datasets highlight the ineffectiveness of using a single approach to study gregarines (Rueckert *et al.*, 2010, 2011a, b; Wakeman & Leander, 2013). For instance, species with low intraspecific variability at the molecular level can look deceptively dissimilar (Rueckert *et al.*, 2010), and species that appear superficially identical under a microscope may have highly divergent SSU rRNA gene sequences (Rueckert *et al.*, 2010). Therefore, a sensible approach for species delimitation should take into account: (i) host affinity, (ii) morphological characteristics that can be easily observed on the most readily encountered life stage (e.g. trophozoites) and (iii) a molecular marker that is conserved enough for alignment purposes but has enough variation that reflects phylogenetic signal at the species level (e.g. the SSU rRNA gene sequence) (Wakeman & Leander, 2013).

To the best of our knowledge, all of the host ascidians chosen in this study have never before been examined for gregarines. The trophozoites of *Lankesteria hesperidiiformis* sp. nov. were isolated from *Distaplia occidentalis*. Two other species of *Distaplia* have been described before to host one species of *Lankesteria*, namely *Lankesteria distapliae* Mingazzini, 1891. Nothing about the surface ultrastructure is mentioned in the original description (Mingazzini, 1891). As described here, the surface of *Lankesteria hesperidiiformis* sp. nov. was inscribed with flattened epicytic folds. The cell shape, even though it is diverse for most species, is more reminiscent of species of *Lankesteria* with epicytic knobs rather than those with folds. *Lankesteria distapliae* is described as similar in shape to *Lankesteria ascidiae*, which means club-shaped. It is also mentioned that syzygy is formed sideways along more or less the entire length of the gamonts

Table 2. Alignment of 18 SSU rRNA gene sequences (1549 bp) from *Lankesteria*, highlighting sequence positions that show a distinctive nucleotide for species of *Lankesteria* with either epicytic folds or epicytic knobs

Positions at which sequences from all *Lankesteria* species with epicytic knobs have one nucleotide and all sequences of species with epicytic folds have a different one are highlighted in bold. Positions where sequences of the two groups have no nucleotides in common are underlined. While sequences of gregarines with epicytic knobs have all the same nucleotide in these cases, nucleotides for sequences of gregarines with epicytic folds vary. Empty cells indicate that the sequence matches that of *L. ascidiae* at that position.

Sequence	165	166	167	168	...	415	416	417	418	612	613	614	615	717	718	719	720	737	738	739	740	741	1397	1398	1399	1400
Species with epicytic knobs																										
L. ascidiae	C	T	G	C		A	**T**	A	T	A	T	G	G	G	T	**G**	A	A	**A**	A	**C**	A	G	C	T	A
L. chelyosomae		G															T			G			T			
L. cystodytae		G															T			G			T			
L. cf. *ritterellae* 1		G									A		A				C	G		G			T			
L. cf. *ritterellae* 2		G									A		A				C	G		G			T			
L. didemni 2.2		G						G			G		T				T			G			T			
L. didemni 2.1		G						G			G		T				T			G			T			
L. didemni 1.1		G						G			G		T				T			G			T			
L. didemni 1.2		G						G			G		T				T			G		G	T			
Species with epicytic folds																										
L. hesperidiiformis		G	A			T	**A**			T	G	A				**A**	T	G	**G**	G	**T**		T		G	
L. hesperidiiformis cyst 1		G	A			T	**A**			T	G	A				**A**	T	G	**G**	G	**T**		T		G	
L. hesperidiiformis cyst 2		G	A			T	**A**			T	G	A				**A**	T	G	**G**	G	**T**		T		G	
L. metandrocarpae 1.1	T		A				**A**					C	C	A	A	**A**	C	G	**G**	G	**T**	C	T	G	C	
L. metandrocarpae 1.2	T		A				**A**					C	C	A	A	**A**	C	G	**G**	G	**T**	C	T	G	C	
L. halocynthiae 1.1	T	C	T				**A**					C	C	A	A	**A**	C	G	**G**	G	**T**	T	T	G	C	
L. halocynthiae 1.2	T	C	T				**A**					C	C	A	A	**A**	C	G	**G**	G	**T**	T	T	G	C	
L. herdmaniae		A	C	A		C	**A**	T	A		C	T	T	T		**A**	C		**G**	G	**T**	G	C		C	
L. abbotti		A	A			T	**A**			G	A	T	C	A		**A**	T		**G**		**T**		C	A	C	

(Mingazzini, 1891). These morphological characteristics clearly distinguish *Lankesteria hesperidiiformis* sp. nov. from *Lankesteria distapliae*, because the former is lemon-shaped and cells in syzygy are spherical. In the 21-sequence alignment, the three different isolates of *Lankesteria hesperidiiformis* sp. nov. (one trophozoite and two gamontocysts) clustered together with strong support and formed the sister group to a moderately supported clade containing *Lankesteria halocynthiae* sp. nov., *Lankesteria metandrocarpae* sp. nov. and *Lankesteria herdmaniae* sp. nov. The intraspecific sequence divergence in *Lankesteria hesperidiiformis* sp. nov. was 0.1–0.4 %, while the interspecific sequence divergence was a lot higher, at 7.8–12.5 %, showing that this gregarine is a different species at the molecular level. The interspecific sequence divergence of species of *Lankesteria* with epicytic knobs was generally lower (7.8–10.1 %) than that of species with surface folds (10.5–12.5 %).

The SSU rRNA gene sequence from *Lankesteria metandrocarpae* sp. nov. clustered strongly with that of *Lankesteria halocynthiae* sp. nov. Both species had a flattened peapod-like cell shape, but the latter species was distinctly longer, with a mean length of 277 μm, compared with 115 μm. The surfaces of both species were inscribed by four epicytic folds per μm. *Lankesteria abbotti* has a similar cell shape, but is intermediate in size (130–160 μm long). 'Pseudosepta' were described within *Lankesteria abbotti* (Levine, 1981; Leander *et al.*, 2006), but were not visible in either of the species described here. The SSU rRNA gene sequences from *Lankesteria metandrocarpae* sp. nov. and *Lankesteria halocynthiae* sp. nov. formed a strongly supported clade. The intraspecific sequence divergence was 0.7 % for *Lankesteria metandrocarpae* sp. nov. and 0.4 % for *Lankesteria halocynthiae*. The interspecific sequence divergence between those two species was 2.1–2.4 %, and it was 9.2–12.7 % in comparison with the remaining *Lankesteria* sequences. Although these two species are closely related, based on sequence divergence, different host species and differences in trophozoite length, we concluded that the establishment of two novel species is justified.

The elongated shape of the trophozoites in *Lankesteria herdmaniae* sp. nov. was most similar to that of *Lankesteria siedleckii* Duboscq & Harant, 1923, described from *Ascidia mentula* and *Ascidia conchilega*, and *Lankesteria gigantea* Ormières, 1965, isolated from *Pyura microcosmus* (Ormières, 1965). The trophozoites of *Lankesteria siedleckii* are up to 260 μm long, while the trophozoites of *Lankesteria gigantea* are up to 800 μm long. The trophozoites of *Lankesteria herdmaniae* sp. nov. were isolated from a different host species and reach a length of 479 μm, setting the trophozoites of this isolate apart from the two species mentioned above. The interspecific sequence divergence between *Lankesteria herdmaniae* sp. nov. and the other species of *Lankesteria* was high, 10.8–13.8 %. Based on this high level of sequence divergence, the distinctive host species (*Herdmania momus*) and the morphological difference in our isolate compared with the two most similar species in the literature, there is little doubt that *Lankesteria herdmaniae* sp. nov. represents a novel species.

Lankesteria cf. *ritterellae* was isolated from *Ritterella rubra*. There are two species of *Lankesteria* described so far from *Ritterella*: *Lankesteria ritterellae* Levine, 1981, from *Ritterella pulchra*, and *Lankesteria pescaderoensis* Levine, 1981, from *Ritterella rubra* (Levine, 1981). The trophozoites we isolated from *Ritterella rubra* were nearly identical in morphology to those described for *Lankesteria ritterellae*. Even though the trophozoites we found were somewhat smaller than those described in the original species description of *Lankesteria ritterellae* and were isolated from a different host species collected in another ocean, we determined that these differences are not sufficient, at this time, to justify establishment of a novel species until molecular phylogenetic data can be gathered from the type specimen in the type locality. Therefore, we designated this isolate as *Lankesteria* cf. *ritterellae*. Although the host of *Lankesteria pescaderoensis* is closely related to the host of *Lankesteria* cf. *ritterellae*, the trophozoites of these two species of *Lankesteria* differ in morphology; the trophozoites of *Lankesteria pescaderoensis* are larger, wider, have a bigger nucleus and have a rectangular mucron (Levine, 1981). The interspecific sequence divergences between *Lankesteria* cf. *ritterellae* and the other *Lankesteria* species was 3.5–6.3 % for species with surface knobs and 7.8–11.1 % for species with surface folds; the intraspecific sequence divergence was only 0.1 %. This shows a clear differentiation of *Lankesteria* cf. *ritterellae* from the remaining species of *Lankesteria* described so far with molecular phylogenetic data.

The trophozoites of *Lankesteria didemni* sp. nov. were most similar to those of *Lankesteria* cf. *ritterellae*, but the epicytic knobs were individually more oval and altogether less dense in *Lankesteria didemni* sp. nov. than in *Lankesteria* cf. *ritterellae* (8–13 knobs per μm compared with 14–16 knobs per μm). The trophozoites in *Lankesteria didemni* sp. nov. were also larger than those in *Lankesteria* cf. *ritterellae*. The interspecific sequence divergence between *Lankesteria didemni* sp. nov. and the other species of *Lankesteria* was 3.5–3.9 %; the intraspecific variation for *Lankesteria didemni* sp. nov. was 0.4–0.8 %. Based on the molecular variation described above, differences in trophozoite traits and the differences in host species, we concluded that *Lankesteria didemni* sp. nov. represents a novel species.

Taxonomy

Phylum Apicomplexa Levine, 1970

Order Eugregarinorida Léger, 1900

Family Lecudinidae Kamm, 1922

Genus *Lankesteria* Mingazzini, 1891

Lankesteria hesperidiiformis Rueckert, Wakeman & Leander sp. nov. (Fig. 1a–k)

Description. Trophozoites lemon-shaped (hesperidiform); mean length 54 μm (35–80 μm), mean width 33 μm (25–42 μm); brownish in colour. Anterior end more pointed than posterior end; spherical nucleus (14 × 15 μm) in the middle of cell or shifted towards anterior end. Surface of trophozoites covered with longitudinal epicytic folds (two folds per μm). Gamonts in syzygy spherical; gametocyst spherical, mean diameter 89 μm (50–150 μm); oocysts oval (10 × 6 μm). Epicytic folds more like ridges (two folds per μm).

DNA sequence. SSU rRNA gene (GenBank accession nos KR024700–KR024702).

Holotype. Fig. 1(a–d).

Hapantotype. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (collection number MI-PR137).

Type locality. Wizard Islet (48° 51′ 06″ N 125° 09′ 04″ W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada.

Habitat. Marine, rocky sediment at 20 m depth.

Etymology. Refers to the lemon-like (hesperidiform) cell shape of the trophozoites (hes.pe.ri.di.i.for′mis.).

Type host. *Distaplia occidentalis* (Metazoa, Chordata, Ascidiacea, Holozoidae).

Location in host. Intestinal lumen.

Lankesteria metandrocarpae Rueckert, Wakeman & Leander sp. nov. (Fig. 2a–d)

Description. Trophozoites shaped like a flattened peapod, mean length 115 μm (93–150 μm), mean width 37 μm (27–50 μm), brownish in colour. Rounded mucron with a pointed tip shifted laterally. Nucleus ellipsoidal (15 × 21 μm) and positioned in the middle or in the anterior half of the trophozoite. Surface of trophozoites covered with longitudinal epicytic folds (four folds per μm). Trophozoites rigid and capable of gliding motility.

DNA sequence. SSU rRNA gene (GenBank accession nos KR024698 and KR024699).

Holotype. Fig. 2(a, b).

Hapantotype. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (collection number MI-PR138).

Type locality. Bamfield Marine Science Centre (48° 50′ 03″ N 125° 08′ 13″ W), Vancouver Island, Canada.

Habitat. Marine, wooden piles at the dock in 5–10 m depth.

Etymology. Refers to the genus of the tunicate type host, *Metandrocarpa taylori* (me.tan.dro.car′pae.).

Type host. *Metandrocarpa taylori* (Metazoa, Chordata, Ascidiacea, Styelidae).

Location in host. Intestinal lumen.

Lankesteria halocynthiae Rueckert, Wakeman & Leander sp. nov. (Fig. 3a–d)

Description. Trophozoites shaped like a flattened peapod, mean length 227 μm (range 78–360 μm), mean width 53 μm (range 25–70 μm), brownish in colour. Cone-shaped mucron, free of amylopectin, bent to one side. Half-moon-shaped nucleus (18 × 26 μm) situated in the anterior part of the cell. Surface of trophozoites covered with longitudinal epicytic folds (four folds per μm). Cells rigid except for mucron area, capable of gliding motility.

DNA sequence. SSU rRNA gene (GenBank accession nos KR024693 and KR024694).

Holotype. Fig. 3(a, b).

Hapantotype. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (collection number MI-PR139).

Type locality. Bamfield Marine Science Centre (48° 50′ 03″ N 125° 08′ 13″ W), Vancouver Island, Canada.

Habitat. Marine, wooden piles at the dock in 5–10 m depth.

Etymology. Refers to the genus of the tunicate type host, *Halocynthia aurantium* (ha.lo.cyn′thi.ae.).

Type host. *Halocynthia aurantium* (Metazoa, Chordata, Ascidiacea, Pyuridae).

Location in host. Intestinal lumen.

Lankesteria herdmaniae Rueckert, Wakeman & Leander sp. nov. (Fig. 4a–e)

Description. Trophozoite cell shape long and slender, mean length 276 μm (range 198–478 μm), mean width 13 μm (range 7–17 μm), brownish in colour. Cone-shaped mucron with rounded tip free of amylopectin. Posterior end rounded. Nucleus oval (21 × 10 μm), situated in the anterior half of the cell. Surface of trophozoites covered with longitudinal epicytic folds. The posterior half of the cell rigid, anterior half able to bend and curl. Capable of gliding motility.

DNA sequence. SSU rRNA gene (GenBank accession no. KR024697).

Holotype. Fig. 4(a, b).

Hapantotype. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (collection number MI-PR140).

Type locality. Shimoda Marine Research Centre, Shimoda, Japan (34° 40′ 02″ N 138° 56′ 08″ E).

Habitat. Marine, drained holding tank.

Etymology. Refers to the genus of the tunicate type host, *Herdmania momus* (herd.ma′ni.ae.).

Type host. *Herdmania momus* (Metazoa, Chordata, Ascidiacea, Pyuridae).

Location in host. Intestinal lumen.

Lankesteria didemni Rueckert, Wakeman & Leander sp. nov. (Fig. 6a–h)

Description. Trophozoites club- to peanut-shaped, mean length 59 μm (range 31–100 μm), mean width 31 μm (range 19–42 μm), brownish in colour. Broadly rounded mucron with pointy, rounded tip, free of amylopectin. Nucleus half-moon shaped (12 × 15 μm), situated in anterior part of the cell. Free of epicytic folds, but covered in dense rows of oval epicytic knobs (76 × 58 nm, 8–13 knobs per μm). Cells rigid and capable of gliding motility.

DNA sequence. SSU rRNA gene (GenBank accession nos KR024689–KR024692).

Holotype. Fig. 6(a–c).

Hapantotype. Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada (collection number MI-PR141).

Type locality. Bamfield Marine Science Centre (48° 50′ 03″ N 125° 08′ 13″ W), Vancouver Island, Canada.

Habitat. Marine, wooden piles at the dock in 5–10 m depth.

Etymology. Refers to the genus of the tunicate type host, *Didemnum vexillum* (di.dem′ni.).

Type host. *Didemnum vexillum* (Metazoa, Chordata, Ascidiacea, Didemnidae).

Location in host. Intestinal lumen.

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