European Journal of Protistology 94 (2024) 126080

Contents lists available at ScienceDirect

European Journal of Protistology

journal homepage: www.elsevier.com/locate/ejop

Research Paper

Pacific marine gregarines (Apicomplexa) shed light on biogeographic speciation patterns and novel diversity among early apicomplexans

Eric Odle [a], Siratee Riewluang [a], Kentaro Ageishi [a], Hiroshi Kajihara [b], Kevin C. Wakeman [c,d,*]

[a] Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Hokkaido, Japan
[b] Faculty of Science, Hokkaido University, Sapporo 060-0810, Hokkaido, Japan
[c] Institute for the Advancement of Higher Education, Hokkaido University, Sapporo, Hokkaido 060-0817, Japan
[d] Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

ARTICLE INFO

Keywords:
Evolution
Lecudinids
Morphology
Phylogenetics
Systematics
Taxonomy

ABSTRACT

Gregarines are the most biodiverse group of apicomplexan parasites. This group specializes on invertebrate hosts (e.g., ascidians, crustaceans, and polychaetes). Marine gregarines are of particular interest because they are considered to be the earliest evolving apicomplexan lineage, having subsequently speciated (and radiated) through virtually all existing animal groups. Still, mechanisms governing the broad (global) distribution and speciation patterns of apicomplexans are not well understood. The present study examines Pacific lecudinids, one of the most species-rich and diverse groups of marine gregarines. Here, marine polychaetes were collected from intertidal zones. Single trophozoite cells were isolated for light and electron microscopy, as well as molecular phylogenetic analyses using the partial 18S rRNA gene. The cytochrome *c* oxidase subunit 1 gene was used to confirm morphology-based host identification. This study introduces *Undularius glycerae* n. gen., n. sp. and *Lecudina kitase* n. sp. (Hokkaido, Japan), as well as *Difficilina fasoliformis* n. sp. (California, USA). Occurrences of *Lecudina* cf. *longissima* and *Lecudina* cf. *tuzetae* (California, USA) are also reported. Phylogenetic analysis revealed a close relationship between *L. pellucida*, *L. tuzetae*, and *L. kitase* n. sp. Additionally, clustering among North Atlantic and Pacific *L. tuzetae* formed a species complex, likely influenced by biogeography.

1. Introduction

Apicomplexans are single-celled parasites of animals (Levine, 1970). Several "core-apicomplexan" lineages that cause malaria (Kolawole et al., 2023), toxoplasmosis (Adem and Ame, 2023), and babesiosis (Kuibagarov et al., 2023) are studied because of their impact on public health and livestock (*Plasmodium*, *Toxoplasma*, and *Babesia*, respectively). Apicomplexa are a diverse group, possessing roughly 350 known genera (Adl et al., 2019). Morrison (2009) reasoned that given the host-specific nature of apicomplexans, only a small portion of their actual diversity, likely less than 1%, has been described. Thus, hundreds of thousands, if not millions of apicomplexans, may exist. Collectively, this group is an evolutionary success story, having radiated through major marine (Dubey et al., 2003), freshwater (Molla et al., 2013), terrestrial (McAllister et al., 1995), and extremophile (Moreira and López-García, 2003; Wakeman et al., 2018) animal groups. Apicomplexan lineages that specialize on invertebrates (especially marine lineages) have been under-studied compared to their vertebrate-infecting counterparts because they do not directly impact public health or livestock. Consequently, the mechanisms governing adaptation and speciation among some of the most diverse apicomplexan groups—marine apicomplexans—remain largely uncharted.

The largest diversity of apicomplexans comprises a group of invertebrate parasites called gregarines, consisting of at least 1600 described species (Desportes and Schrével, 2013; Levine, 1988). Gregarines are an important model for evolutionary studies because they are 1) early-branching in the tree of apicomplexans (Schrével et al., 2016); 2) comprised of lineages that have either maintained ancestral characteristics (Leander, 2008; MacGregor and Thomasson, 1965) or developed feeding/motility modifications (Paskerova et al., 2018; Valigurová et al., 2013); 3) biogeographically distributed in marine, freshwater, and terrestrial hosts; 4) found in virtually all invertebrate groups (Desportes and Schrével, 2013; Levine, 1977; Levine, 1979; Valigurová and Koudela, 2005) and reported in the developmental stage of some frogs (Chambouvet et al., 2016); 5) highly specific to a particular host species or group; and 6) largely homoxenous, requiring only one host to

* Corresponding author at: Institute for the Advancement of Higher Education, Hokkaido University, Sapporo, Hokkaido 060-0817, Japan.
E-mail address: wakeman.k@oia.hokudai.ac.jp (K.C. Wakeman).

https://doi.org/10.1016/j.ejop.2024.126080
Received 21 November 2023; Received in revised form 28 March 2024; Accepted 29 March 2024
Available online 30 March 2024

Table 1
Summary of marine gregarines and their hosts, as well as the sampling sites and GenBank accession numbers from the present study.

Isolate	Site	Isolate 18S (length)	Host sample	Host identity	Host COI
Undularius glycerae n. gen., n. sp.	Oshoro Bay, Hokkaido, Japan	OQ650267 (1704), OQ650268 (1638)	Oshoro_H13	*Glycera* sp.	OQ957055
Difficilina fasoliformis n. sp.	Crescent City, California, USA	OQ650265 (1559), OQ650266 (1497)	CC_H3	Terebellidae sp.	OQ957062
Lecudina kitase n. sp.	Oshoro Bay, Hokkaido, Japan	OQ650261 (1519)	Oshoro_H14	*Perinereis* sp.	OQ957061
		OQ650262 (1576), OQ650263 (1573), OQ650264 (1501)	Oshoro_H25		
Lecudina cf. *longissima*	Crescent City, California, USA	OQ650252 (1515), OQ650253 (1545), OQ650254 (1534), OQ650255 (1504), OQ650256 (1504)	CC_H4	*Lumbrineris* sp.	OQ957056
Lecudina cf. *tuzetae*	Crescent City, California, USA	OQ650257 (1633), OQ650258 (1616), OQ650259 (1633), OQ650260 (1640)	CC_H1	*Nereis* sp.	OQ957060

Oshoro Bay, Hokkaido, Japan (43°12′37.073″N 140°51′25.935″E), visited in November 2022.
Crescent City, California, USA (41°44′12.5766″N 124°11′40.2354″E), visited in January 2023.

complete their life cycle (Lee et al., 2000). Furthermore, recent work has highlighted the importance of gregarines in our overall understanding of early apicomplexan/alveolate parasitism, organelle development, and evolution (Gubbels and Duraisingh, 2012; Mathur et al., 2021; Templeton and Pain, 2016).

Marine gregarines are a fascinating group for their broad diversity. They are likely also the earliest of the gregarines to have evolved. In addition, marine gregarines are believed to have subsequently radiated, leading to the rise of other apicomplexan groups (Leander, 2008; Théodoridès, 1984). Among these, the genus *Lecudina* and its close relatives (i.e., lecudinids) hold particular significance because they encompass a broad biodiversity of marine gregarines stemming from ancestral forms (Iritani et al., 2018; Leander et al., 2006). Within this group, several lineages exhibit innovations in structural form and motor function. For instance, *Veloxidium leptosynaptae* exhibits unique thrashing motility driven by transverse membrane striations (Wakeman and Leander, 2012), while *Lecudina* possesses highly folded cell membranes used in gliding motility (Leander, 2008; Rueckert et al., 2010, 2011b). Other examples of derived lineages among marine gregarines include the genera *Pterospora*, *Lithocystis*, and *Lankesteria*, which have developed membrane features such as cross-hatches (Landers and Leander, 2005), crenulations (Coulon and Jangoux, 1987), and knob-like protrusions (Iritani et al., 2021; Rueckert et al., 2015). These innovations highlight the diverse ways in which such parasites have evolved to interact with their hosts and thrive in their specific ecological niches. Still, available molecular and geographical data on marine gregarines remain incomplete.

Descriptions of most marine gregarines are based on morphological observations, with only a small subset (less than 100) supported by molecular data (Wakeman et al., 2021). Consequently, some researchers have attempted to reconcile past morphological observations with more contemporary molecular datasets (Diakin et al., 2016; Iritani et al., 2021; Rueckert and Horák, 2017; Rueckert et al., 2011a; Schrével et al., 2016; Simdyanov et al., 2015; Wakeman et al., 2021). Ultimately, collecting marine gregarines for taxonomic and molecular observations will allow us to address basic questions surrounding apicomplexans—filling gaps in our knowledge of gregarine biogeographical distribution and host specificity, as well as the biotic and abiotic factors that drive these mechanisms.

To this end, the present study aims to improve our current understanding of marine gregarine biodiversity and speciation patterns across the northern Pacific Ocean, with a specific focus on lecudinids parasitizing polychaetes. Here, lecudinids from northern Japan and the western United States were studied with combined morphological observations (light and electron microscopy) and molecular phylogeny based on the nuclear small subunit (18S) rRNA gene.

2. Material and methods

2.1. Host collection and sample processing

Hosts were collected opportunistically from intertidal zones near Oshoro Marine Station, Hokkaido, Japan (43°12′37.073″N 140°51′25.935″E) and Crescent City, California, USA (41°44′12.5766″N 124°11′40.2354″E) between November 2022 and January 2023 (Table 1) from under intertidal rocks and inside tufts of seagrass. Individual hosts were isolated, photographed, then dissected. Host digestive tracts (intestines) were removed and shredded with forceps to separate gregarine parasites from host tissue. Individual trophozoites (feeding stages) were isolated under an inverted microscope (Olympus CKX53, Tokyo, Japan; Nikon Eclipse Ts2 Tokyo, Japan) using hand-drawn glass pipettes. Isolates were washed two or three times (until clean) in well slides containing filter-sterilized seawater. Some of these isolates were photographed using a Kiss X8i DSLR camera (Canon, Tokyo, Japan) mounted on the inverted microscope, and then placed in 0.2 ml PCR microcentrifuge tubes for single-cell DNA extraction. Other isolates were

transferred to a glass microscope slide for differential interference contrast (DIC) microscopy using a Kiss X8i DSLR camera (Canon, Tokyo, Japan) mounted on an Axioskop 2 Plus DIC microscope (Zeiss, Baden-Württemberg, Germany), or placed in 2.5 % glutaraldehyde for electron microscopy. Many isolate images were obtained from cells also used for molecular or other analyses, and cell polarity (anterior/mucron vs. posterior) was determined by the direction of cell movement. The remaining host tissue was preserved in 100 % ethanol for molecular (barcode) identification.

2.2. Electron microscopy

Gregarines isolated for electron microscopy were fixed in baskets containing 2.5 % glutaraldehyde (diluted in seawater) on ice for 30 min. Baskets were constructed from shortened pipette tips with Isopore 5.0 μm PC Membrane filters (Merck Millipore Ltd., Ireland) attached with silicone. Fixed cells were washed three times (until clean) in filter-sterilized seawater, then post-fixed in 1 % osmium tetroxide (diluted in seawater) for 30 min in the dark. Samples were then washed in filter-sterilized seawater (three times, 3 min each), desalted in deionized water (three times, 3 min each), then dehydrated using an ethanol series (70 %, 80 %, 90 %, 100 %, 100 %) for 3 min at each concentration. Finally, samples were dried using an HCP-2 815B critical point dryer (Hitachi, Tokyo, Japan) and sputter coated with gold for 400 sec at 15 μA. Scanning electron microscopy (SEM) was performed on an S-3000N scanning electron microscope (Hitachi, Tokyo, Japan). Images of larger isolates were taken in succession, stitched together, and then cropped from the background using Affinity Photo (Serif Ltd., Nottingham, United Kingdom).

Isolates taken for transmission electron microscopy (TEM) were fixed in 2.5 % glutaraldehyde on ice for 30 min, washed with filter-sterilized seawater (three times, 3 min each), then stained in 1 % osmium tetroxide for 1.5 hrs in the dark. Samples were then washed in filter-sterilized seawater (three times, 3 min each), desalted in deionized water (three times, 3 min each), then dehydrated using an ethanol series (70 %, 80 %, 90 %, 100 %, 100 %) for 3 min at each concentration. Cells were permeabilized in 1:1 ethanol:acetone for 5 min, 100 % acetone two times for 5 min, 1:1 acetone:resin (Agar Scientific Ltd., Essex, United Kingdom) for 30 min, then in 100 % resin (two times) for 5 hrs each. Finally, cells were polymerized at 68 °C for 48 hrs and shaped using an EM UC6 ultramicrotome (Leica, Wetzlar, Germany). A diamond knife was used to create 50 nm sections that were placed on formvar-coated grids and imaged on an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

2.3. Small subunit (18S) rRNA gene amplification and sequencing

Trophozoites were placed individually in 0.2 ml PCR micro-centrifuge tubes along with a small volume of seawater and then frozen for 1–5 days. Genomic DNA was extracted using a QuickExtract FFPE DNA Extraction Kit (Lucigen, Middlesex, United Kingdom), according to manufacturer protocols. The 18S gene was initially amplified using general primers (Table 2) and KOD One® PCR Master Mix (Toyobo, Osaka, Japan). Thermal cycling during initial amplification was run under the following parameters: denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 52 °C for 5 sec, and extension at 68 °C for 15 sec, then ending in a final extension at 68 °C for 1 min. The PCR products from the initial amplification were diluted 1:100 with ultrapure water (UltraPure™ DNase/RNase-Free Distilled Water, Thermo Fisher Scientific Inc., Massachusetts, USA) and used as a template for a second nested reaction with internal primers (Table 2). A nested amplification reaction was then run under the following parameters: denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 sec, annealing at 54 °C for 5 sec, and extension at 68 °C for 10 sec, then ending in a final extension at 68 °C for 1 min. Final PCR amplicons, which ranged from 600 to 1000 base pairs in size, were visualized by gel electrophoresis. Samples with single, sharp amplicons of the expected size were purified with polyethylene glycol (PEG) in preparation for sequencing. The sequencing reaction was performed using a SupreDye™ v1.1 Cycle Sequencing Kit (AdvancedSeq LLC, California, USA) on a 3730 DNA Analyzer (Applied Biosystems, Massachusetts, USA).

2.4. Phylogenetic analysis of 18S sequences

All sequencing reads were imported into Geneious Prime 2023.2.1 (https://www.geneious.com) for subsequent analysis. Within Geneious, reads were screened against the National Center for Biotechnology Information (NCBI) GenBank (Benson et al., 1993) database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990), and then trimmed using the modified Mott algorithm (Green and Ewing, 2002). Contigs were generated using the Geneious de novo assembler. A second NCBI BLAST screen was then performed on full 18S contigs followed by primer site visualization to avoid constructing

Table 2
List of primers with references.

Primer name	Sequence	Direction	Position	Citation
SR1	5′-TAC CTG GTT GAT CCT GCC AG-3′	forward	1	Nakayama et al. (1996)
SR12	5′-CCT TCC GCA GGT TCA CCT AC-3′	reverse	1721	Nakayama et al. (1996)
SR1B	5′-GAT CCT GCC AGT AGT CAT ATG CTT-3′	forward	2	Yamaguchi and Horiguchi (2005)
18SRF	5′-CCC GTG TTG AGT CAA ATT AAG-3′	reverse	1154	Mo et al. (2002)
SR4	5′-AGG GCA AGT CTG GTG CCA G-3′	forward	527	Yamaguchi and Horiguchi (2005)
SR9	5′-AAC TAA GAA CRG CCA TGC AC-3′	reverse	1245	Takano and Horiguchi (2005)
SR5	5′-ACT ACG AGC TTT TTA ACT GC-3′	reverse	590	Nakayama et al. (1996)
Lecu717MR*	5′-GTG CTG GCA CCA GAC TTT TCC-3′	reverse	528	Present study
LecudinoidR*	5′-GAA CAC GCC GAT TCA CTC-3′	reverse	724	Present study
Lecu401F*	5′-CAA AGT TTC TGA CCC ATC AG-3′	forward	275	Present study
Lecu1617R*	5′-CCA CGA ACT AAG AAC GGC-3′	reverse	1252	Present study
Lecu1272F*	5′-GAT CAA GAA CGA AAG TTA GGG G-3′	forward	942	Present study
Tuz3R*	5′-CGA TTC ACT CAA AGT ACA G-3′	reverse	715	Present study
Tuz4*	5′-CTC TCC AGT ACT TTC TGA G-3′	forward	1040	Present study
t1791	5′-CTC CGC CTA ACT CAT GAT AC-3′	reverse	1632	Iritani et al. (2021)
GenEukR1	5′-CGG TGT GTA CAA ACG GCA GGG AC-3′	reverse	1592	Iritani et al. (2021)
SR12B	5′-CGG AAA CCT TGT TAC GAC TTC TCC-3′	reverse	1720	Yamaguchi and Horiguchi (2005)
polyLCO	5′-GAY TAT WTT CAA CAA ATC ATA AAG ATA TTG G-3′	forward	n/a	Carr et al. (2011)
polyHCO	5′-TAM ACT TCW GGG TGA CCA AAR AAT CA-3′	reverse	n/a	Carr et al. (2011)

Note 1: Positions for 18S primers are based on the complete *Toxoplasma gondii* 18S sequence (accession number: L37415), measured from the 5′ terminus.
Note 2: Positions for primers absent on the *T. gondii* reference sequence are estimated by relative distance from the nearest present primer.
(*) denotes novel primer designed for this study.

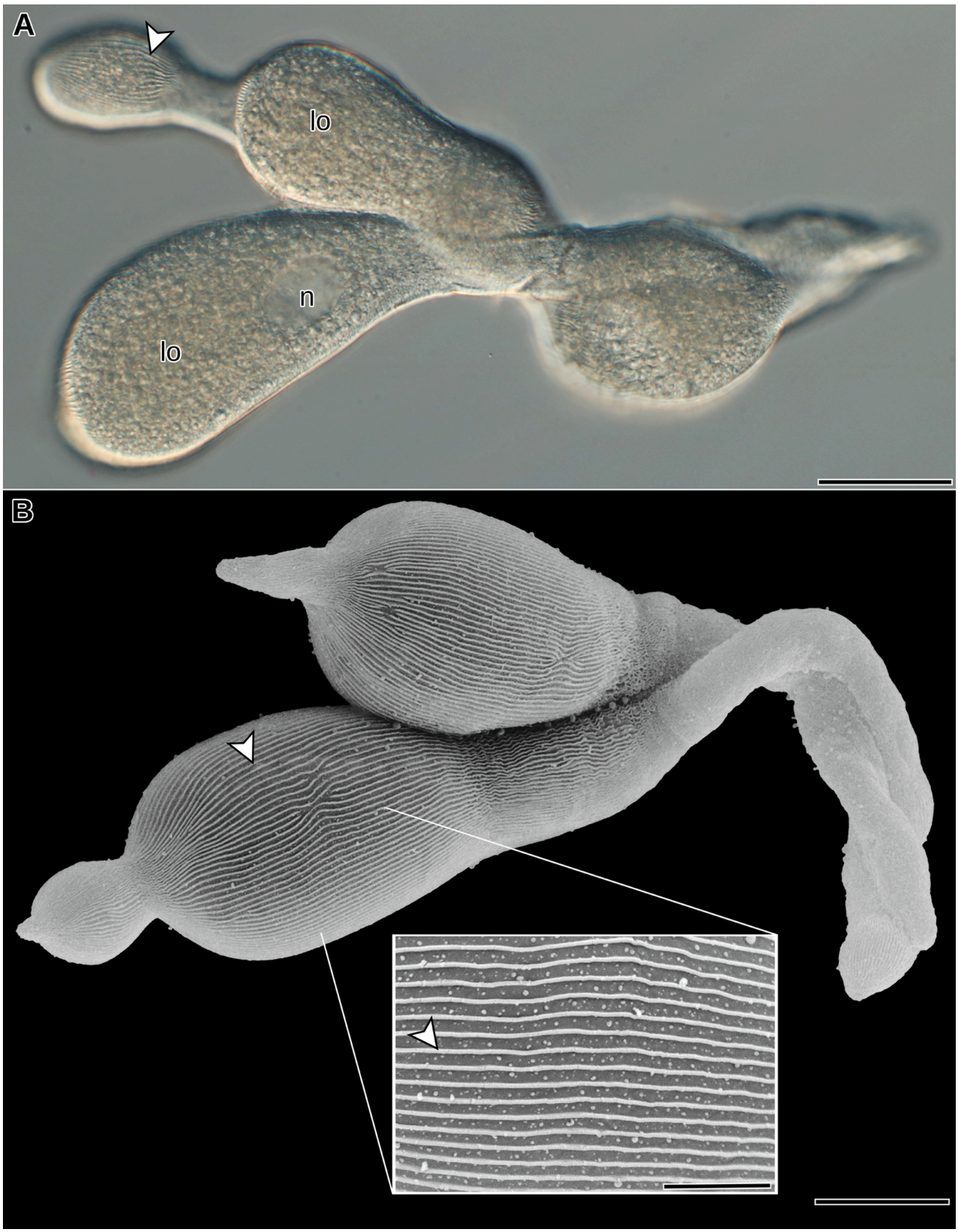

Fig. 1. Light and scanning electron micrographs of *Undularius glycerae* n. gen., n. sp. (**A**) Differential interference contrast (DIC) micrograph showing the nucleus (n). Peristaltic movement of the cells appears as bulging lobes (lo). Epicytic folds are also visible (white arrowhead). (**B**) Scanning electron micrograph (SEM) showing gamonts in lateral syzygy. Epicytic folds (white arrowhead) are also visible running the longitudinal length of the cell. (**B inset**) An SEM showing epicytic folds with a density of 1 fold/μm. Scale bars: 10 μm (B inset), 20 μm (A), and 30 μm (B).

chimeric sequences.

Sequenced 18S regions from gregarine isolates were aligned along with GenBank sequences of related taxa using MAFFT ver. 7 (Katoh et al., 2002) with the following settings: E-INS-i model, 100PAM/κ = 2 scoring matrix, and gap penalty = 1.53. Alignments were masked in regions containing 70 % or greater gaps prior to phylogenetic analysis. An optimal substitution model (GTR + F + R4) was determined under the Akaike Information Criterion with Correction using the IQTREE ModelFinder program (Kalyaanamoorthy et al., 2017). A GTR substitution model with gamma rate variation was used during Bayesian analysis. Gregarine phylogeny was then inferred using maximum likelihood (ML) analysis and Bayesian inference. ML analysis was performed using IQTREE ver. 2.3.1 (Nguyen et al., 2015) with 1000 bootstrap pseudoreplicates. Bayesian posterior probabilities (PP) were calculated using MrBayes ver. 3.2.6 (Ronquist et al., 2012) via the Geneious plug-in. Markov Chain Monte Carlo was run for ten million generations with other settings kept as plug-in defaults. Additionally, the distance matrix from an unmasked alignment was used to make pairwise identity comparisons.

2.5. Host identification

Pieces of host tissue (remaining after gregarine isolation from the gut) were preserved in 100 % ethanol for host barcoding. Host DNA was extracted from samples using silica gel and a commercial DNA extraction kit (Qiagen, Maryland, USA). The cytochrome *c* oxidase subunit 1 (COI) gene was amplified using primers polyLCO and polyHCO (Table 2) with Ex Taq (Takara Bio Inc., Shiga, Japan). Thermal cycling during COI amplification was run under the following parameters: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 30 sec, extension at 72 °C for 1 min 15 sec, and a final extension at 72 °C for 7 min. During the annealing phase, the temperature was lowered by 1 °C after each of the first 10 cycles. Amplicons were approximately 650 base pairs in size. Following

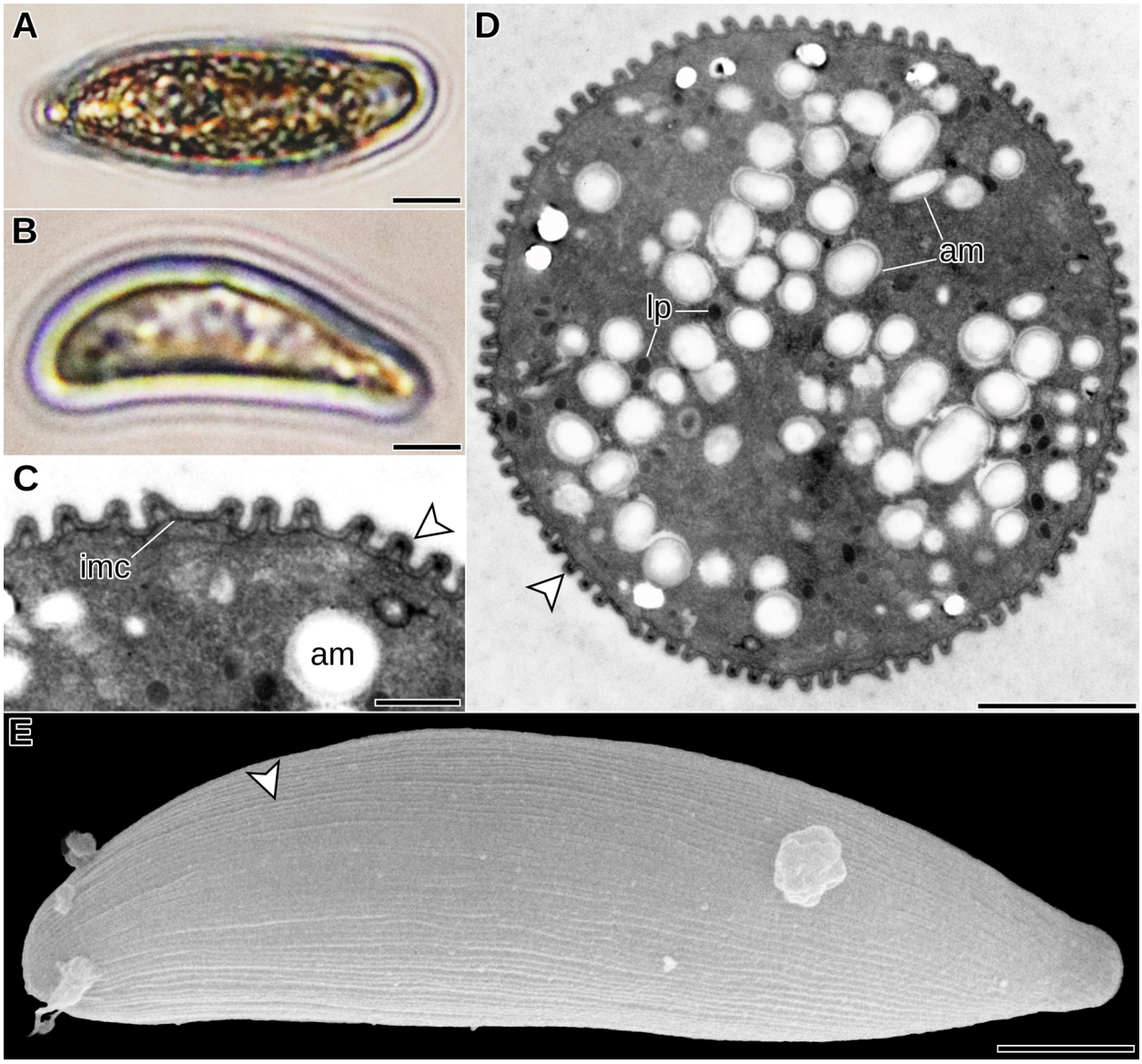

Fig. 2. Light and electron micrographs of *Difficilina fasoliformis* n. sp. Anterior ends (mucrons) are oriented to the right. (**A, B**) Light micrographs of trophozoite isolates. (**C**) Transmission electron micrograph (TEM) showing epicytic folds (white arrowhead) with underlying inner membrane complex (imc) and amylopectin granules (am). Fold density is 4 folds/μm. (**D**) A TEM cross-section showing the rounded shape of the trophozoites and epicytic folds (white arrowhead). Amylopectin granules (am) and lipid droplets (lp) are also shown. (**E**) Scanning electron micrograph showing general trophozoite morphology and epicytic folds (white arrowhead). Scale bars: 500 nm (C), 2 μm (D), 5 μm (E), and 10 μm (A, B).

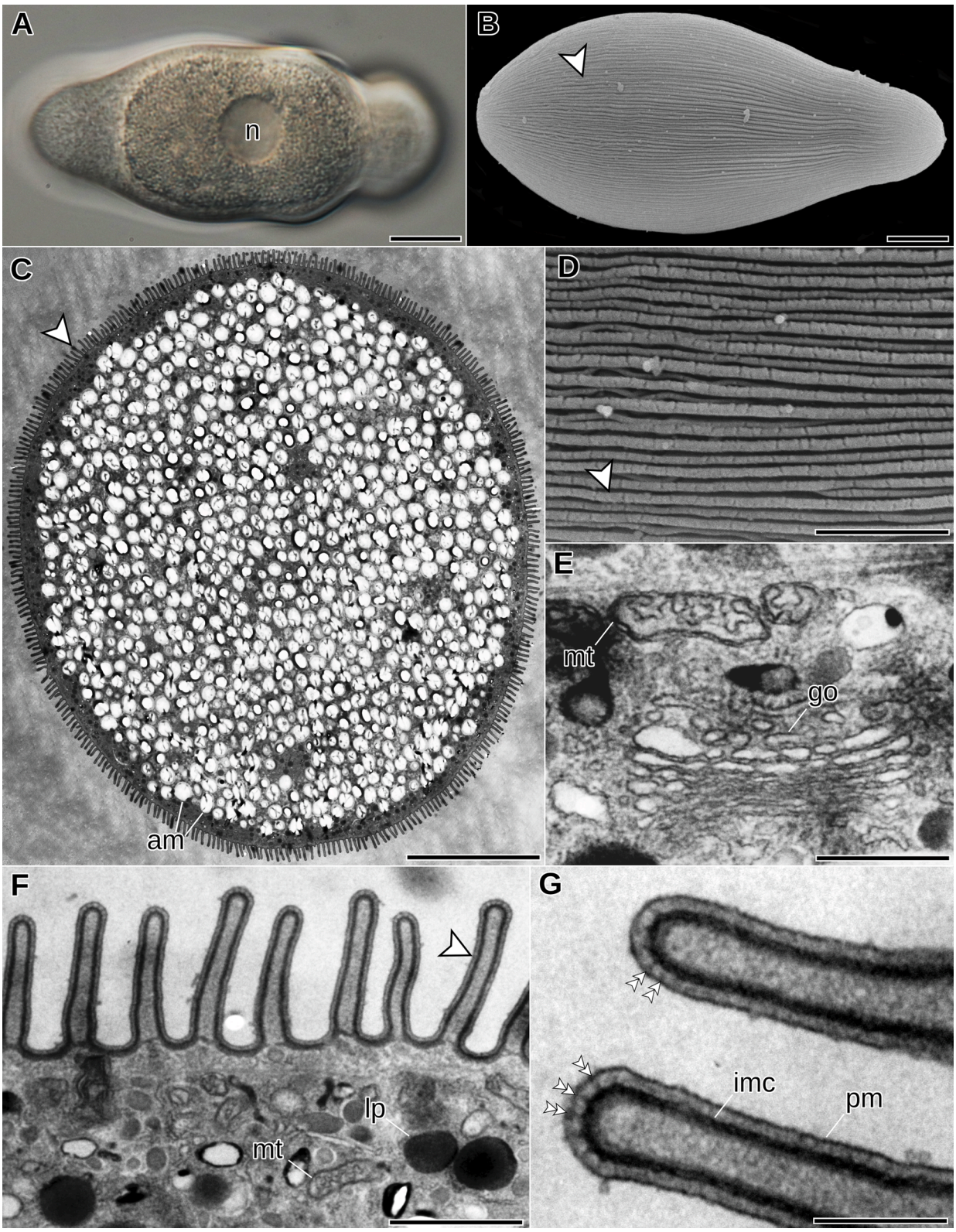

Fig. 3. Light and electron micrographs of *Lecudina kitase* n. sp. Anterior ends (mucrons) are oriented to the right. (**A**) Differential interference contrast (DIC) micrograph showing the nucleus (n). (**B**) Scanning electron micrograph (SEM) showing epicytic folds (white arrowhead). (**C**) Transmission electron micrograph (TEM) cross-section showing epicytic folds (white arrowhead) and amylopectin granules (am). (**D**) An SEM of epicytic folds (white arrowhead). (**E**) A TEM of a Golgi apparatus (go) and mitochondrion (mt). (**F**) A TEM of the cell membrane showing mitochondrion (mt), lipid droplets (lp), and epicytic folds at a density of 2 folds/μm. A single epicytic fold is indicated (white arrowhead). (**G**) A TEM showing rippled dense structures (double arrowheads), plasma membrane (pm), and inner membrane complex (imc). Scale bars: 200 nm (G), 500 nm (E), 1 μm (F), 5 μm (D), 10 μm (A, B), and 15 μm (C).

sequencing, COI host data were analyzed by BLAST. Then, neighbor-joining trees were generated to identify closely related NCBI sequences.

3. Results

3.1. Morphology of Undularius glycerae n. gen., n. sp.

Undularius glycerae n. gen., n. sp. consisted of elongate trophozoite pairs in lateral syzygy (Fig. 1A, B); single trophozoites were not observed in the sample. Trophozoites actively moved in a dynamic wave-like motion (undulating) passing across the length of the trophozoite (Supplemental Video 1). Epicytic folds ran longitudinally along the cells (Fig. 1A, B, B inset). Folds were broadly spaced at the lobular bulge with a density of 1 fold/μm (Fig. 1B inset). Isolates measured 130–270 μm in length ($\bar{x}$ = 193 μm, n = 6) and 30–50 μm in width ($\bar{x}$ = 41 μm, n = 6). Spherical nuclei measured 10–15 μm in diameter ($\bar{x}$ = 11.25 μm, n = 4). The position of the nucleus within the cell was not fixed to a specific location but rather moved with the motion of the cell.

3.2. Morphology of Difficilina fasoliformis n. sp.

Difficilina fasoliformis n. sp. was oval to crescent-shaped (Fig. 2A, B, E) measuring 45–60 μm in length ($\bar{x}$ = 50 μm, n = 3) and 15–25 μm in width ($\bar{x}$ = 20 μm, n = 3). Nuclei were not visible. Cell mucrons had a papillary morphology (Fig. 2A–B, E). *Difficilina fasoliformis* was surrounded by low folds at the widest part of the cell with a density of 4 folds/μm (Fig. 2C, D).

3.3. Morphology of Lecudina kitase n. sp.

Lecudina kitase n. sp. had a rounded posterior and was narrow at the anterior end of the trophozoites (Fig. 3A). Light micrographs showed a centrally located, spherical nucleus and rounded mucron (Fig. 3A). Cells were either centrally wide, cascading, or pear-like in shape, and surrounded by longitudinal epicytic folds with a density of 2 folds/μm at the widest part of the cell (Fig. 3B–D, F; centrally wide morphotype not shown). *Lecudina kitase* measured 60–80 μm in length ($\bar{x}$= 67 μm, n = 4) and 33–40 μm in width ($\bar{x}$= 37 μm, n = 4) (Fig. 3B). Nuclei measured 10.0–12.5 μm in diameter ($\bar{x}$= 11.67 μm, n = 3). Cross-sections were circular with amylopectin granules concentrated towards the central

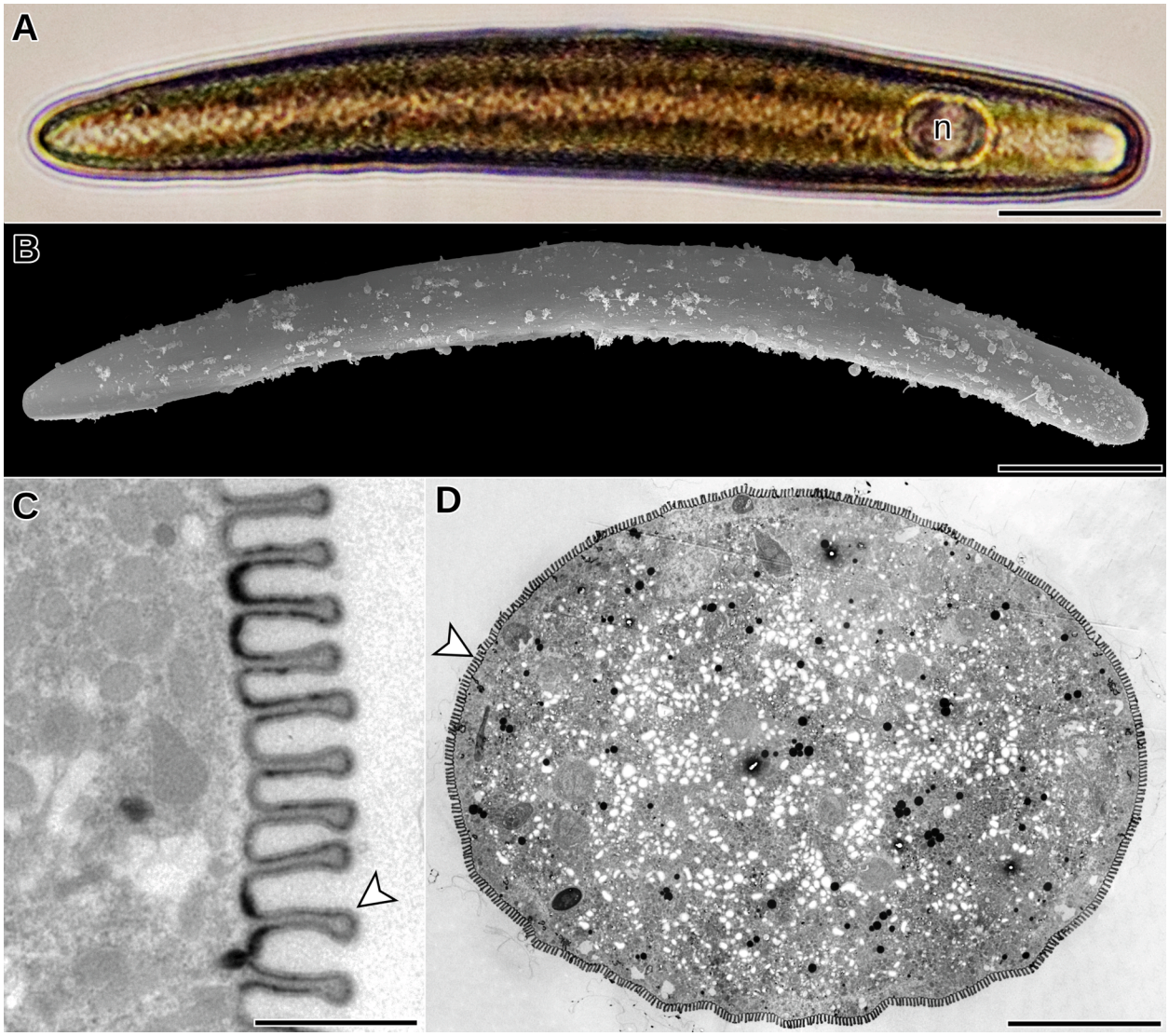

Fig. 4. Light and electron micrographs of *Lecudina* cf. *longissima*. Anterior ends (mucrons) are oriented to the right. (**A**) Light micrograph showing the nucleus (n). (**B**) Scanning electron micrograph showing a trophozoite. (**C**) Transmission electron micrograph (TEM) showing epicytic folds (white arrowhead) with a density of 4 folds/μm. (**D**) A TEM cross-section of trophozoite with folded membrane (white arrowhead). Scale bars: 1 μm (C), 10 μm (D), 30 μm (A), and 60 μm (B).

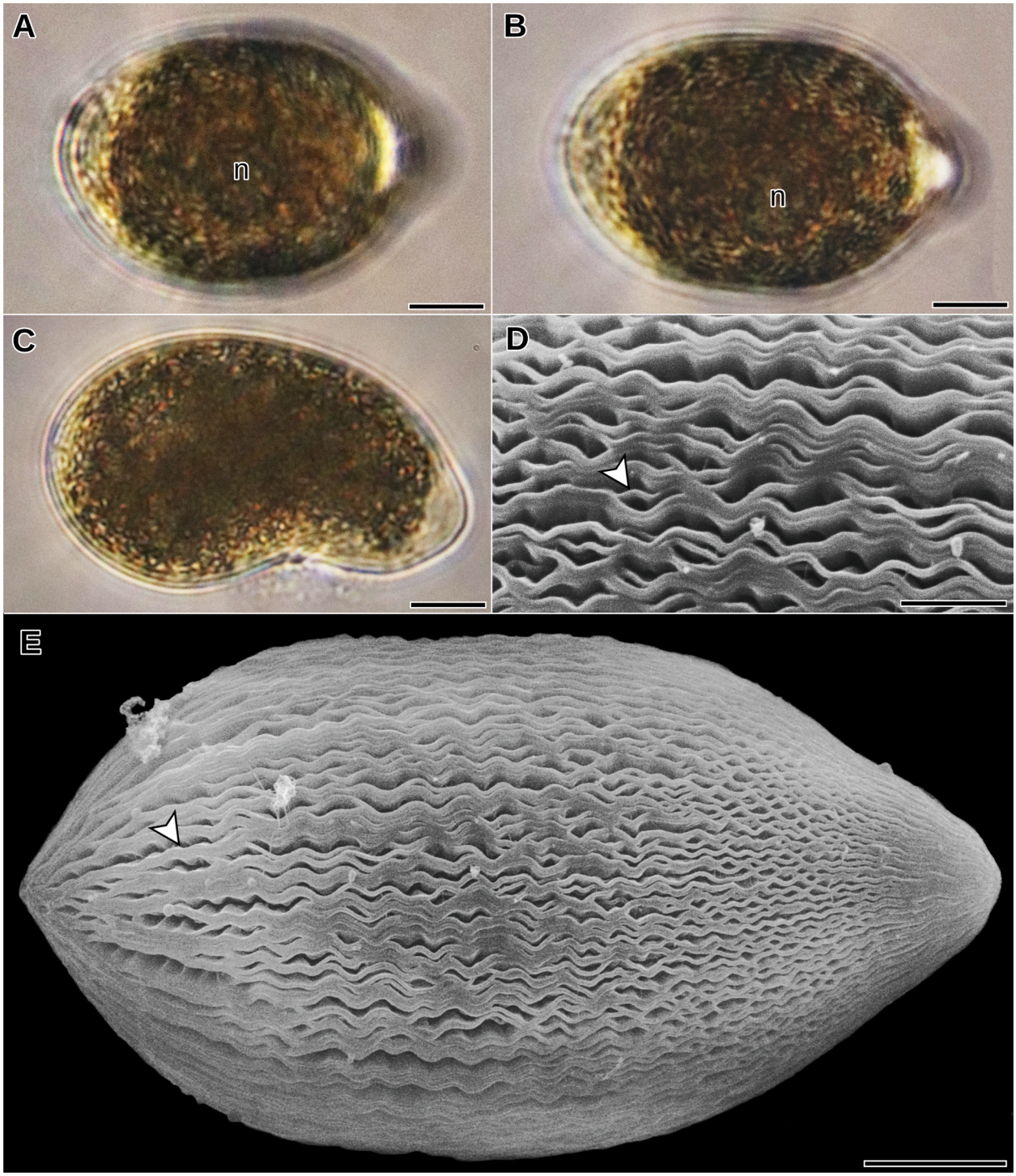

Fig. 5. Light and electron micrographs of *Lecudina* cf. *tuzetae* collected off the coast of northern California, USA. Anterior ends (mucrons) are oriented to the right. (**A–C**) Light micrographs of trophozoites cells with visible nuclei (n). (**D**) A scanning electron micrograph (SEM) showing epicytic folds (white arrowhead) and fold density of 2 folds/μm. (**E**) An SEM showing the trophozoite cell with epicytic folds (white arrowhead). Scale bars: 10 μm (D, E) and 15 μm (A–C).

axis of the cell (Fig. 3C). Organelles such as the Golgi apparatus, mitochondria, as well as lipid droplets were observed near the cell membrane (Fig. 3E, F). Additionally, rippled dense structures were visible at the terminal ends of epicytic folds (Fig. 3G).

3.4. Morphology of Lecudina cf. longissima and L. cf. tuzetae from northern California

Trophozoites of *Lecudina* cf. *longissima* were elongate, measuring 200–420 μm in length ($\bar{x}$= 270 μm, $n = 6$) and 30–70 μm in width ($\bar{x}$ = 43.3 μm, $n = 6$). The cells possessed a rounded mucron (Fig. 4A, B) and spherical nucleus located in the anterior third of the cell (Fig. 4A). Nuclei measured 18–22 μm in diameter ($\bar{x}$ = 20 μm, $n = 3$). Externally, *L.* cf. *longissima* isolates were covered in longitudinally-running epicytic folds with a density of 4 folds/μm at the widest part of the cell (Fig. 4C, D). The cell body was ovaloid in cross-section (Fig. 4D).

Lecudina cf. *tuzetae* trophozoites were 75–100 μm in length ($\bar{x}$ = 82 μm, n = 5), 45–60 μm in width ($\bar{x}$ = 51 μm, n = 5) and possessed a

spherical nucleus measuring 14–20 μm in diameter ($\bar{x} = 17$ μm, $n = 3$) (Fig. 5A–C). Externally, *L.* cf. *tuzetae* was covered in surface folds running the longitudinal length of the cell at a density of 2 folds/μm at the widest part of the cell (Fig. 5D, E).

3.5. Molecular phylogeny

Phylogenetic analysis of a 98-sequence 18S alignment was rooted with an outgroup of distantly related marine gregarines and marine alveolates (*Selenidium terebellae*, *Chromera velia*, and *Vitrella brassicaformis*). Many of the nodes within the tree were highly supported. This support corresponded with the different genera present in the analysis. The only exceptions were nodes connecting *Lecudina* Clade A, *Lankesteria*, and *Lecudina* Clade B. *Veloxidium* and *Undularius* n. gen. formed distinct lineages early in the phylogeny of lecudinids. *Difficilina fasoliformis* n. sp. sequences grouped with *D. tubulani*. These two lineages formed a sister group to a clade containing *D. paranemertis* and *Urospora*. Within *Lecudina* Clade B, *L.* cf. *longissima* sequences from northern California formed a subclade with *L.* cf. *longissima* from western Canada. Other *Lecudina* sequences from the current study grouped within *Lecudina* Clade A. *Lecudina kitase* sequences formed a fully supported clade to the exclusion of other *Lecudina* species (*L. tuzetae* from English Channel and *L.* cf. *tuzetae* from Crescent City and western Canada). All *L. tuzetae*/*L.* cf. *tuzetae* sequences formed a strongly supported clade and grouped into subclades by locality. The intraspecific pairwise identity between four *L. kitase* n. sp. isolates ranged from 99.8 % to 100 %, and the interspecific pairwise identity between *L. pellucida* and *L. kitase* n. sp. ranged from 88.9 % to 89.3 %. The interspecific pairwise identity between *L. tuzetae*/*L.* cf. *tuzetae* (English Channel, Crescent City, and western Canada) and *L. kitase* ranged from 88.3 % to 89.4 % (Supplemental Table 1, Supplemental File 1).

3.6. Molecular barcoding of host material

All marine gregarine isolates were collected from North Pacific polychaetes identified by pairwise identity to related COI sequences (Supplemental Table 2). *Undularius glycerae* n. gen., n. sp. host material was identified as *Glycera* sp. (pairwise identity: 86.0 %–88.5 %). *Difficilina fasoliformis* n. sp. host material was most closely related to the terebellid *Phyllocomus hiltoni* (pairwise identity: 85.1 %). *Lecudina kitase* n. sp. host material was identified as *Perinereis* sp. (pairwise identity: 90.5 %–97.1 %). *Lecudina* cf. *longissima* host material was identified as *Lumbrineris* sp. (pairwise identity: 88.6 %–88.8 %). Finally, *Lecudina* cf. *tuzetae* host material was identified as *Nereis* sp. (pairwise identity: 97.7 %–99.4 %).

4. Discussion

4.1. Novel gregarine biodiversity from the Pacific Ocean

4.1.1. Undularius glycerae n. gen., n. sp.

Undularius glycerae n. gen., n. sp. was recovered as one of the earliest diverging lineages of lecudinids. Comparing *U. glycerae* to its closest relatives (within the phylogenetic tree), namely *Pterospora* Labbé and Racovitza, 1897, *Urospora* Schneider, 1875, and *Lithocystis* Giard, 1876, we found their most apparent similarity to be peristaltic motility. Trophozoites of *U. glycerae* had a dynamic peristaltic movement that appeared as a lobular bulge passing in a repeating wave from anterior to posterior, then returning. *Pterospora* (Landers and Leander, 2005), *Urospora* (Diakin et al., 2016), and *Lithocystis* (Coulon and Jangoux, 1987), which inhabit the coeloms of hosts, also move by peristalsis. However, peristalsis in these genera is more sporadic than with *U. glycerae*. While *U. glycerae* was isolated from dissected gut tissue, its peristaltic motility suggests possible coelomic specialization. Epicytic folds on *U. glycerae* were broadly spaced (1 fold/μm). In contrast, *Pterospora* has cross-hatching patterns with bifurcating digits (Landers and Leander, 2005), *Urospora* has relatively moderately-spaced folds (2–3 fold/μm), and *Lithocystis* has membrane crenulations (Coulon and Jangoux, 1987) (Supplemental Table 3). Lastly, the host affiliation of *U. glycerae* with the *Glycera* bloodworm sets it apart from these other three genera. *Pterospora* is only known from maldanid hosts (Landers and Leander, 2005); *Urospora* is known from echinoderms, sipunculids, oligochaetes, nemerteans, and polychaetes (Desportes and Schrével, 2013; Diakin et al., 2016); and *Lithocystis* has only been reported from echinoderms (Coulon and Jangoux, 1987).

Other gregarines found in *Glycera* bloodworms include *Ceratospora* Schneider, 1982, *Gonospora* Schneider, 1875, *Lecudina legeri* Brasil, 1909, and *L. amphora* Hoshide, 1958. *Undularius glycerae* is distinct from these based on surface morphology, reproductive (syzygy) forms, and relative size. *Ceratospora* has distinct membrane protrusions and frontofrontal syzygy (Léger, 1892) rather than lateral syzygy observed in *U. glycerae*; *Gonospora* also exhibits larger trophozoites up to 1000 μm or more in length (Pixell-Goodrich, 1916). This contrasts with the bulbous shape and shorter length of *U. glycerae*, which ranged between 130 and 270 μm. *Ceratospora* and *Gonospora* have been reported in Europe from northern France (Léger, 1892) to the Mediterranean Sea (Pixell-Goodrich, 1916): an entirely different ocean basin from which *U. glycerae* was found (northwestern Pacific Ocean). Additionally, *Lecudina legeri* and *L. amphora* possess different morphologies and host preferences (Desportes and Schrével, 2013). Significantly, results from our phylogenetic analysis placed *U. glycerae* at the base of the clade, sister to the entire clade of lecudinids apart from the enigmatic *Veloxidium* (Wakeman and Leander, 2012), and distinct from known *Pterospora*, *Urospora*, and *Lithocystis* sequences. Together, our findings support *Undularius* as a novel marine gregarine genus.

4.1.2. Difficilina fasoliformis n. sp.

Difficilina fasoliformis n. sp. shared gliding motility with other *Difficilina* Simdyanov, 2009 species but were otherwise phylogenetically and morphologically distinct. Phylogenetically, *D. fasoliformis* formed a clade with other *Difficilina* and *Urospora* species with strong support. Although 18S sequences were not available for type species *D. cerebratuli* Simdyanov, 2009, the morphology of *D. fasoliformis* sets it apart from other *Difficilina* species. *Difficilina fasoliformis* had a papillary mucron and blunt posterior, distinguishing it from *D. cerebratuli* (tapered posterior), as well as *D. paranemertis* and *D. tubulani* (rounded mucrons, tapered posteriors) (Supplemental Table 4). Biogeographically, *D. fasoliformis* (northern California) does share a similar region and latitude with *D. paranemertis* and *D. tubulani* (western Canada). However, it is quite distant from the type locality of *D. cerebratuli* described from northwestern Russia (White Sea) (Simdyanov, 2009). Of note, this is the first time a *Difficilina* species has been described from an annelid polychaete (isolated from the gut). To date, all *Difficilina* species have been isolated from ribbon worms (Nemertea) (Rueckert et al., 2010; Simdyanov, 2009).

4.1.3. Lecudina kitase n. sp.

Trophozoites of *Lecudina kitase* n. sp. closely resembled those of *L. pellucida* Mingazzini, 1891 (Vivier, 1968) as well as *L.* cf. *tuzetae* isolates from northern California (present study), western Canada (Rueckert et al., 2011b), and *L. tuzetae* from the English Channel (Schrével, 1964) (Supplemental Table 5). *Lecudina kitase* isolates also exhibited gliding motility similar to other *Lecudina* species (Desportes and Schrével, 2013). In our phylogenetic analyses (Fig. 6, Supplemental Tables 6–7, and Supplemental Fig. 1), *L. kitase* grouped with *L. pellucida*, and both formed a group sister to the other *L. tuzetae*/*L.* cf. *tuzetae* isolates. Even though *L. kitase* and *L. pellucida* come from distant localities (northern Japan and the English Channel, respectively), their close affiliation can be explained by their overlapping host preference: both can be found in the genus *Perinereis* (Schrével, 1963; Schrével et al., 2016; Vivier, 1968). Two other lecudinids inhabiting *Perinereis*, i.e. *L. pelmatomorpha* and *L. caudata*, have been described from the English

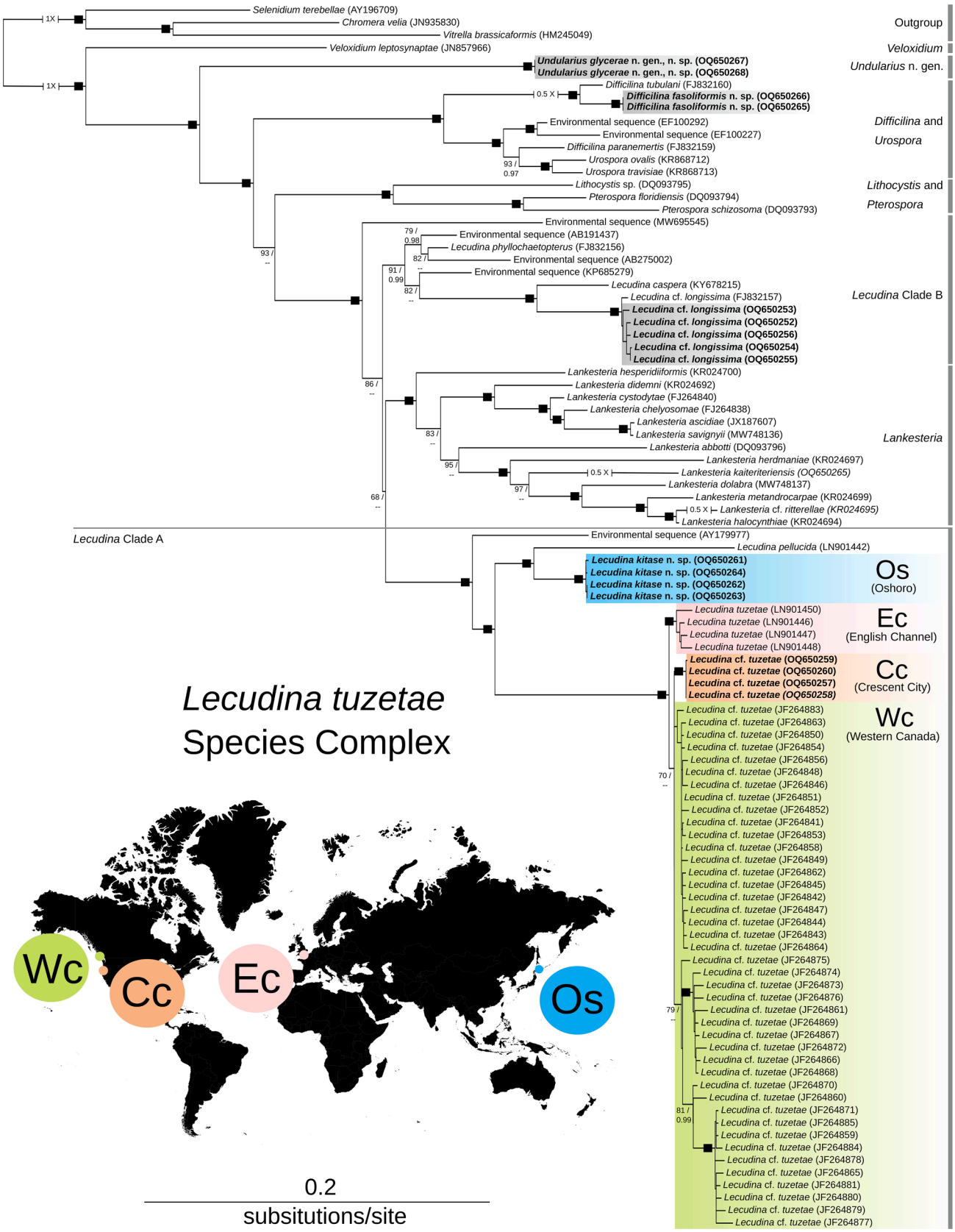

Fig. 6. Maximum likelihood (ML) tree inferred from small subunit 18S rRNA gene sequences. Bootstrap values > 50 and Bayesian posterior probabilities (PP) > 0.95 are shown adjacent to nodes (ML/PP). Black squares indicate statistical support ≥ 95/0.99. The scale bar represents the inferred evolutionary distance as a rate of base substitutions per site. The *Lecudina tuzetae* species complex is color-coded according to source localities: Oshoro, Hokkaido (blue); English Channel (pink); Crescent City, California (orange); and western Canada (light green). Novel sequences presented in this study are in bold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Channel (Schrével, 1963) and southern Japan (Hoshide, 1977), respectively. However, a lack of molecular data for these species prevents their phylogenetic analysis. Compared to these *Perinereis* parasites, *L. tuzetae/L.* cf. *tuzetae* appears to specialize on the family Nereididae, particularly the genus *Nereis* (Desportes and Schrével, 2013; Rueckert et al., 2011b) and other close relatives. Morphologically, the trophozoites of *L. kitase* were distinguished from *L. pellucida* by their rounded rather than cupped mucron (Vivier, 1968). *Lecudina caudata* (papillary mucron) and *L. pelmatomorpha* (conical mucron) also had different mucron morphologies from *L. kitase* (Supplemental Table 5). Morphological differences were supported in our phylogenetic analysis that grouped four single-cell isolates of *L. kitase* to the exclusion of *L. pellucida*. The intraspecific pairwise similarity between the *L. kitase* isolates ranged from 99.8 % to 100 %. On the other hand, the interspecific pairwise similarity between these isolates and *L. pellucida* ranged from 88.9 % to 89.3 %. For these reasons, it was concluded to establish *L. kitase* as a novel species.

4.2. Novel biogeographical insights on the distribution of Lecudina

The present study contains some of the first molecular data from lecudinids in the western Pacific. Other lecudinid data from around Japan focused on the genus *Lankesteria* inhabiting tunicates (Mita et al., 2012; Rueckert et al., 2015). The addition of data generated in the current study allowed for a broader comparison between members of the *Lecudina*, particularly *L. tuzetae/L.* cf. *tuzetae*. *Lecudina pellucida*, *L. tuzetae/L.* cf. *tuzetae*, and *L. kitase* n. sp. are all found in nereid polychaetes (Desportes and Schrével, 2013). The close relationship between lecudinid parasites from distant locations suggests that these lecudinid parasites could have moved to new locations and lost connectivity with other lecudinid populations. Jump dispersal may explain how these *L.* cf. *tuzetae* populations were founded and why their phylogenies do not necessarily show a diffuse dispersal pattern. For example, freight shipping across the Panama Canal has been implicated in the dispersal of protists (Lohan et al., 2017).

Nereid polychaetes are a cosmopolitan group and can be found in a variety of marine habitats (Özpolat et al., 2021). Therefore, it is easy to imagine that this particular host group can move to new locations with relative ease. Still, it is difficult to comprehensively compare hosts and parasites without molecular data that can conclusively delineate species. In this study, it was difficult to separate the clades of *L. tuzetae/L.* cf. *tuzetae* even though their clustering, based on biogeography, suggested that their grouping could be comprised of cryptic species. Moreover, assigning host identity by pairwise COI distance can be unclear at times. Therefore, future goals should include host barcoding using multiple genes and gregarine phylogenetic analyses that include faster evolving gene regions (e.g., ITS). This would provide insight into how cryptic diversity amongst lecudinids (especially closely related species), corresponds to host-species diversity and biogeographic distribution. It is also interesting to note that *L. tuzetae/L.* cf. *tuzetae* have been extensively found in environmental sequence surveys, including around Hokkaido in northern Japan (Hirai et al., 2017). While environmental sampling does not account for random DNA dispersal (e.g., cyst dispersal), our discovery of *Lecudina kitase* n. sp. (a close relative to *L. pellucida* and *L. tuzetae/L.* cf. *tuzetae*) hints at a broader biogeographic range for *Lecudina*, and lecudinids in general, that extends at least into East Asia.

In this study, we also examined isolates presumed to be *L. longissima* Tsugawa, 1944. Our samples were taken from a site in northern California and were identified through host ecology, molecular phylogeny, and light micrographs. Morphologically, these isolates were indistinguishable from type species *L. longissima* collected in Japan (Hoshide, 1958; Tsugawa, 1944), as well as from *L.* cf. *longissima* collected from southern California (Levine, 1974) and western Canada (Rueckert et al., 2010). *Lecudina* cf. *longissima* from northern California (this study) did form a clade, which was sister to *L.* cf. *longissima* from western Canada (Rueckert et al., 2010). However, we concluded that more samples would be needed to conclusively delineate these isolates. This is because the type locality of *L. longissima* is in southern Japan (Hoshide, 1958; Tsugawa, 1944), and molecular data is not available for the type species. Although the clustering of *L.* cf. *longissima* may be suggestive of a species complex, we decided to treat our samples as *L.* cf. *longissima* until more molecular data is available from western Canada, northern and southern California, and from the type locality in southern Japan.

5. Taxonomic summary

ZooBank registration number of the present work is urn:lsid: zoobank.org:pub:6A27B98D-0D7F-47F5-82D5-2B32091F00FC.

5.1. Undularius E. Odle and K. Wakeman, n. gen.

ZooBank registration number. urn:lsid:zoobank.org:act:329549FB-63AE-43FD-8A36-2289357C4B2E.

Diagnosis. Two trophozoite cells joined in lateral syzygy. Lobular bulges undulate along cell by peristaltic action. Cell surface surrounded by longitudinal epicytic folds with density of 1 fold/μm at lobular bulge.

Type species. *Undularius glycerae* E. Odle and K. Wakeman, n. sp.

Etymology. This new generic name is masculine in gender, referring to the undulating peristaltic motility of the type species trophozoite.

5.2. Undularius glycerae E. Odle and K. Wakeman, n. gen., n. sp.

ZooBank registration number. urn:lsid:zoobank.org:act:29ECD663-12EB-4EDD-811A-CA1826365FD8.

Diagnosis. Trophozoite peristalsis generates lobular bulges that move across length of cell. Cells measure 130–270 μm in length and 30–50 μm in width. Nuclei measure 10–15 μm in diameter. Syzygy is lateral. Epicytic folds with density of 1 fold/μm at lobular bulge run longitudinally.

DNA sequences. The 18S rRNA gene sequences of the paratype specimens have been deposited in GenBank under accession numbers OQ650267 and OQ650268.

Holotype. Fixed and dried isolate mounted to SEM stub (specimen shown in Fig. 1B, archive label: 20221118-S7), held at the Hokkaido University Graduate School of Science Biodiversity Group Facility, Japan.

Type locality. Oshoro Bay, Hokkaido, Japan (43°12′37.073″N 140°51′25.935″E).

Type habitat. Marine.

Type host. *Glycera* sp. The COI gene sequence of the type host has been deposited in GenBank under accession number OQ957055. Host catalog code: Oshoro_H13.

Location in host. Gut.

Distribution. The type host was often found under large rocks or entangled within seagrass roots off the Sea of Japan coast around northwestern Hokkaido, specifically in the intertidal zone surrounding Cape Poromai.

Etymology. The specific name is a noun in the genitive case, from the genus of the host polychaete, *Glycera* sp., within which the new species was discovered.

5.3. Difficilina fasoliformis E. Odle and K. Wakeman, n. sp.

ZooBank registration number. urn:lsid:zoobank.org:act:BBDC730C-A79C-4376-AE2C-82C3C0F875B8.

Diagnosis. Trophozoites ovaloid with papillary mucron. Cells measure 45–60 μm in length and 15–25 μm in width. Cells surrounded by low folds with density of 4 folds/μm at widest part of cell.

DNA sequences. The 18S rRNA gene sequences of the paratype specimens have been deposited in GenBank under accession numbers OQ650265 and OQ650266.

Holotype. Fixed and dried isolate mounted to SEM stub (specimen

shown in Fig. 2E, archive label: 202301-CC-H5), held at the Hokkaido University Graduate School of Science Biodiversity Group Facility, Japan.

Type locality. Crescent City Jetty, California, USA (41°44′12.5766″N 124°11′40.2354″E).

Type habitat. Marine.

Type host. Terebellidae sp. The COI gene sequence of the type host has been deposited in GenBank under accession number OQ957062. Host catalog code: CC_H3.

Location in host. Gut.

Distribution. The type host was collected from the intertidal zone of the northwestern Pacific coast of California, USA, often under large rocks or entangled within seagrass roots.

Etymology. The Latin adjective *fasoliformis* refers to the legume-like shape of trophozoite stages.

5.4. *Lecudina kitase E. Odle and K. Wakeman, n. sp.*

ZooBank registration number: urn:lsid:zoobank.org:act:E92B3907-CADA-480D-8A25-F48EFB0E924F.

Diagnosis. Trophozoites either centrally wide, cascading, or pear-like in shape with distinct longitudinal epicytic folds and visible anterior mucrons. Cells translucent with opaque cytoplasm encompassing a spherical nucleus 10.0–12.5 μm in diameter. Cells measure 60–80 μm in length and 33–40 μm in width. Longitudinal epicytic folds have density of 2 folds/μm at widest part of cell. Trophozoites contain amylopectin granules centrally and organelles (Golgi apparatus, mitochondria, and lipid droplets) peripherally. Rippled dense structures are present at the ends of epicytic folds.

DNA sequences. The 18S rRNA gene sequences of the paratype specimens have been deposited in GenBank under accession numbers OQ650261, OQ650262, OQ650263, and OQ650264.

Holotype. Fixed and dried isolate mounted to SEM stub (specimen shown in Fig. 3B, archive label: 20221118-S6), held at the Hokkaido University Graduate School of Science Biodiversity Group, Japan.

Type locality. Oshoro Bay, Hokkaido, Japan (43°12′37.073″N 140°51′25.935″E).

Type habitat. Marine.

Type host. *Perinereis* sp. The COI gene sequence of the type host has been deposited in GenBank under accession number OQ957061. Host catalog code: CC_H14.

Location in host. Gut.

Distribution. The type host was found under large rocks or entangled within seagrass roots off the Sea of Japan coast around northwestern Hokkaido, specifically in the intertidal zone surrounding Cape Poromai.

Etymology. The species epithet is derived from the Japanese word for “North” (*kita*), the region of Japan from which the new species was discovered. The epithet is to be treated as a noun in apposition.

CRediT authorship contribution statement

Eric Odle: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Siratee Riewluang:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Kentaro Ageishi:** Writing – review & editing, Methodology, Investigation, Data curation. **Hiroshi Kajihara:** Writing – review & editing, Supervision, Methodology, Investigation. **Kevin C. Wakeman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We would like to thank Angela Wakeman for assistance in collecting samples in Crescent City, California. We would also like to thank the Oshoro Marine Station (Hokkaido University) and the Electron Microscopy Facility in the Agricultural Department at Hokkaido University allowing us to use their facilities, as well as Samuel Kahng and Shaun Cunningham for taking the time to proofread our manuscript. Furthermore, we would like to thank Professor Timur Simdyanov for assistance in obtaining rare and hard-to-get reference literature. Lastly, we would like to express a special thank you to Professor Kazuhiro Kogame for providing academic guidance and support. This work was supported by DX Fellowship to Eric Odle through Hokkaido University (Code PH8D230001) and a grant to Kevin Wakeman through the Japanese Society for the Promotion of Science (No. 18K14774). Kevin Wakeman was also supported by the ISP program at Hokkaido University. There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2024.126080.

References

Adem, D.M., Ame, M.M., 2023. Toxoplasmosis and its significance in public health: a review. J. Biomed. Biol. Sci. 2, 1–20.

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., 2019. Revisions to the classification, nomenclature, and diversity of eukaryotes. J. Eukaryot. Microbiol. 66, 4–119.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Benson, D., Lipman, D.J., Ostell, J., 1993. GenBank. Nucleic Acids Res. 21, 2963–2965.

Carr, C.M., Hardy, S.M., Brown, T.M., Macdonald, T.A., Hebert, P.D., 2011. A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes. PLoS One 6, e22232. https://doi.org/10.1371/journal.pone.0022232.

Chambouvet, A., Valigurová, A., Pinheiro, L.M., Richards, T.A., Jirků, M., 2016. *Nematopsis temporariae* (Gregarinasina, Apicomplexa, Alveolata) is an intracellular infectious agent of tadpole livers. Environ. Microbiol. Rep. 8, 675–679.

Coulon, P., Jangoux, M., 1987. Gregarine species (Apicomplexa) parasitic in the burrowing echinoid *Echinocardium cordatum*: occurrence and host reaction. Dis. Aquat. Org. 2, 135–145.

Desportes, I., Schrével, J., 2013. Treatise on Zoology—Anatomy, Taxonomy, Biology. The Gregarines: The Early Branching Apicomplexa. Volumes 1 and 2. Brill, Leiden.

Diakin, A., Paskerova, G.G., Simdyanov, T.G., Aleoshin, V.V., Valigurová, A., 2016. Morphology and molecular phylogeny of coelomic gregarines (Apicomplexa) with different types of motility: *Urospora ovalis* and *U. travisiae* from the polychaete *Travisia forbesii*. Protist 167, 279–301.

Dubey, J.P., Zarnke, R., Thomas, N.J., Wong, S.K., van Bonn, W., Briggs, M., Davis, J.W., Ewing, R., Mense, M., Kwok, O.C.H., Romand, S., 2003. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. Vet. Parasitol. 116, 275–296.

Green, P., Ewing, B., 2002. Phred, version 0.020425 c. [www document]. URL http://www.phrap.org/phredphrap/phred.html [accessed on September 01, 2023].

Gubbels, M.J., Duraisingh, M.T., 2012. Evolution of apicomplexan secretory organelles. Int. J. Parasitol. 42, 1071–1081.

Hirai, J., Katakura, S., Kasai, H., Nagai, S., 2017. Cryptic zooplankton diversity revealed by a metagenetic approach to monitoring metazoan communities in the coastal waters of the Okhotsk Sea, northeastern Hokkaido. Front. Mar. Sci. 4, e379. https://doi.org/10.3389/fmars.2017.00379.

Hoshide, H., 1958. Studies on the cephaline gregarines of Japan. Bull. Fac. Educ. Yamaguchi Univ. 6, 97–157.

Iritani, D., Wakeman, K.C., Leander, B.S., 2018. Molecular phylogenetic positions of two new marine gregarines (Apicomplexa)—*Paralecudina anankea* n. sp. and *Lecudina caspera* n. sp.—from the intestine of *Lumbrineris inflata* (Polychaeta) show patterns of co-evolution. J. Eukaryot. Microbiol. 65, 211–219.

Iritani, D., Banks, J.C., Webb, S.C., Fidler, A., Horiguchi, T., Wakeman, K.C., 2021. New gregarine species (Apicomplexa) from tunicates show an evolutionary history of host switching and suggest a problem with the systematics of *Lankesteria* and *Lecudina*. J. Invertebr. Pathol. 183, e107622. https://doi.org/10.1016/j.jip.2021.107622.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K., Von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589.

Katoh, K., Misawa, K., Kuma, K.i., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Kolawole, E.O., Ayeni, E.T., Abolade, S.A., Ugwu, S.E., Awoyinka, T.B., Ofeh, A.S., Okolo, B.O., 2023. Malaria endemicity in sub-Saharan Africa: past and present issues in public health. Microb. Infec. Dis. 4, 242–251.

Kuibagarov, M., Makhamed, R., Zhylkibayev, A., Berdikulov, M., Abdrakhmanov, S., Kozhabayev, M., Akhmetollayev, I., Mukanov, K., Ryskeldina, A., Ramankulov, Y., Shustov, A., 2023. *Theileria* and *Babesia* infection in cattle—first molecular survey in Kazakhstan. Ticks Tick Borne Dis. 14, e102078 https://doi.org/10.1016/j.ttbdis.2022.102078.

Landers, S.C., Leander, B.S., 2005. Comparative surface morphology of marine coelomic gregarines (Apicomplexa, Urosporidae): *Pterospora floridiensis* and *Pterospora schizosoma*. J. Eukaryot. Microbiol. 52, 23–30.

Leander, B.S., 2008. Marine gregarines: evolutionary prelude to the apicomplexan radiation? Trends Parasitol. 24, 60–67.

Leander, B.S., Lloyd, S.A., Marshall, W., Landers, S.C., 2006. Phylogeny of marine gregarines (Apicomplexa)—*Pterospora*, *Lithocystis* and *Lankesteria*—and the origin(s) of coelomic parasitism. Protist 157, 45–60.

Lee, J.J., Leedale, G.F., Bradbury, P., 2000. The Illustrated Guide to the Protozoa, 2nd edn. Allen Press Inc, Lawrence, Kansas.

Léger, L., 1892. Recherches sur les grégarines. Typographie Oudin et cie, 3, 1–183.

Levine, N.D., 1974. Gregarines of the genus *Lecudina* (Protozoa, Apicomplexa) from Pacific Ocean polychaetes. J. Protozool. 21, 10–12.

Levine, N.D., 1977. Checklist of the species of the aseptate gregarine family Urosporidae. Int. J. Parasitol. 7, 101–108.

Levine, N.D., 1979. New genera and higher taxa of septate gregarines (Protozoa, Apicomplexa). J. Protozool. 26, 532–536.

Levine, N.D., 1988. Progress in taxonomy of the apicomplexan Protozoa. J. Protozool. 35, 518–520.

Levine N.D., 1970. Taxonomy of the Sporozoa. In: Proceedings of the Second International Congress of Parasitology. Allen Press Inc, Lawrence, Kansas. pp. 208–209.

Lohan, K.M.P., Fleischer, R.C., Torchin, M.E., Ruiz, G.M., 2017. Protistan biogeography: a snapshot across a major shipping corridor spanning two oceans. Protist 168, 183–196.

MacGregor, H.C., Thomasson, P.A., 1965. The fine structure of two archigregarines, *Selenidium fallax* and *Ditrypanocystis cirratuli*. J. Protozool. 12, 438–443.

Mathur, V., Wakeman, K.C., Keeling, P.J., 2021. Parallel functional reduction in the mitochondria of apicomplexan parasites. Curr. Biol. 31, 2920–2928.

McAllister, C.T., Upton, S.J., Trauth, S.E., Dixon, J.R., 1995. Coccidian parasites (Apicomplexa) from snakes in the southcentral and southwestern United States: new host and geographic records. J. Parasitol. 81, 63–68.

Mita, K., Kawai, N., Rueckert, S., Sasakura, Y., 2012. Large-scale infection of the ascidian *Ciona intestinalis* by the gregarine *Lankesteria ascidiae* in an inland culture system. Dis. Aquat. Org. 101, 185–195.

Mo, C., Douek, J., Rinkevich, B., 2002. Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence. Mar. Biol. 140, 883–889.

Molla, S.H., Bandyopadhyay, P.K., Gürelli, G., 2013. On the occurrence of a Haemogregarinae (Apicomplexa) parasite from freshwater turtles of south 24 Parganas, West Bengal. India. Türk. Parazitol. Derg. 37, 118–122.

Moreira, D., López-García, P., 2003. Are hydrothermal vents oases for parasitic protists? Trends Parasitol. 19, 556–558.

Morrison, D.A., 2009. Evolution of the Apicomplexa: Where are we now? Trends Parasitol. 25, 375–382.

Nakayama, T., Watanabe, S., Mitsui, K., Uchida, H., Inouye, I., 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. Phycol. Res. 44, 47–55.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol. Biol. Evol. 32, 268–274.

Özpolat, B.D., Randel, N., Williams, E.A., Bezares-Calderón, L.A., Andreatta, G., Balavoine, G., Bertucci, P.Y., Ferrier, D.E.K., Gambi, M.C., Gazave, E., Handberg-Thorsager, M., Hardege, J., Hird, C., Hsieh, Y.W., Hui, J., Mutemi, K.N., Schneider, S. Q., Simakov, O., Vergara, H.M., Vervoort, M., Jékely, G., Tessmar-Raible, K., Raible, F., Arent, D., 2021. The Nereid on the rise: *Platynereis* as a model system. EvoDevo 12, e10. https://doi.org/10.1186/s13227-021-00180-3.

Paskerova, G.G., Miroliubova, T.S., Diakin, A., Kováčiková, M., Valigurová, A., Guillou, L., Aleoshin, V.V., Simdyanov, T.G., 2018. Fine structure and molecular phylogenetic position of two marine gregarines, *Selenidium pygospionis* sp. n. and *S. pherusae* sp. n., with notes on the phylogeny of Archigregarinida (Apicomplexa). Protist 169, 826–852.

Pixell-Goodrich, H.L., 1916. Memoirs: the gregarines of *Glycera siphonostoma*. J. Cell. Sci. 2, 205–216.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Rueckert, S., Chantangsi, C., Leander, B.S., 2010. Molecular systematics of marine gregarines (Apicomplexa) from north-eastern Pacific polychaetes and nemerteans, with descriptions of three novel species: *Lecudina phyllochaetopteri* n. sp., *Difficilina tubulani* n. sp. and *Difficilina paranemertis* n. sp. Int. J. Syst. Evol. Microbiol. 60, 2681–2690.

Rueckert, S., Horák, A., 2017. Archigregarines of the English Channel revisited: new molecular data on *Selenidium* species including early described and new species and the uncertainties of phylogenetic relationships. PLoS One 12, e0187430. https://doi.org/10.1371/journal.pone.0187430.

Rueckert, S., Simdyanov, T.G., Aleoshin, V.V., Leander, B.S., 2011a. Identification of a divergent environmental DNA sequence clade using the phylogeny of gregarine parasites (Apicomplexa) from crustacean hosts. PLoS One 6, e18163. https://doi.org/10.1371/journal.pone.0018163.

Rueckert, S., Villette, P.M., Leander, B.S., 2011b. Species boundaries in gregarine apicomplexan parasites: a case study—comparison of morphometric and molecular variability in *Lecudina* cf. *tuzetae* (Eugregarinorida, Lecudinidae). J. Eukaryot. Microbiol. 58, 275–283.

Rueckert, S., Wakeman, K.C., Jenke-Kodama, H., Leander, B.S., 2015. Molecular systematics of marine gregarine apicomplexans from Pacific tunicates, with descriptions of five novel species of *Lankesteria*. Int. J. Syst. Evol. Microbiol. 65, 2598–2614.

Schrével, J., 1963. Grégarines nouvelles de Nereidae et Eunicidae (Annélides Polychètes). C. R. Seances. Soc. Biol. 157, 814–816.

Schrével, J., 1964. Contribution à l'étude de trois Grégarines parasites d'Annélides Polychètes: *Lecudina elongata* Mingazzini, 1891; *Lecudina tuzetae* Schrével, 1963; *Gonospora varia* Léger, 1892. Archs. Zool. Exp. Gen. 104, 125–142.

Schrével, J., Valigurová, A., Prensier, G., Chambouvet, A., Florent, I., Guillou, L., 2016. Ultrastructure of *Selenidium pendula*, the type species of archigregarines, and phylogenetic relations to other marine Apicomplexa. Protist 167, 339–368.

Simdyanov, T., 2009. *Difficilina cerebratuli* gen. et sp. n. (Eugregarinida: Lecudinidae)—a new gregarine species from the nemertean *Cerebratulus barentsi* (Nemertini: Cerebratulidae). Parazitologiia 43, 273–287 (in Russian).

Simdyanov, T., Diakin, A.Y., Aleoshin, V.V., 2015. Ultrastructure and 28S rDNA phylogeny of two gregarines: *Cephaloidophora* cf. *communis* and *Heliospora* cf. *longissima* with remarks on gregarine morphology and phylogenetic analysis. Acta. Protozool. 54, 241–262.

Takano, Y., Horiguchi, T., 2005. Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. J. Phycol. 42, 251–256.

Templeton, T.J., Pain, A., 2016. Diversity of extracellular proteins during the transition from the 'proto-apicomplexan' alveolates to the apicomplexan obligate parasites. Parasitology 143, 1–17.

Théodoridès, J., 1984. The phylogeny of the Gregarinia (Sporozoa). Orig. Life 13, 339–342.

Tsugawa, H., 1944. Nihonsan tamourui ni kisei suru shinzokuchuurui ni tsuite (On eugregarine parasites of polychaetes from Japan). Bull. Fac. Sci. Hiroshima Univ. 6, 218–230 (in Japanese).

Valigurová, A., Koudela, B., 2005. Fine structure of trophozoites of the gregarine *Leidyana ephestiae* (Apicomplexa: Eugregarinida) parasitic in *Ephestia kuehniella* larvae (Lepidoptera). Eur. J. Protistol. 41, 209–218.

Valigurová, A., Vaškovicova, N., Musilová, N., Schrével, J., 2013. The enigma of eugregarine epicytic folds: where gliding motility originates? Front. Zool. 10, e57. https://doi.org/10.1186/1742-9994-10-57.

Vivier, E., 1968. L'organisation ultrastructurale corticale de la gregarine *Lecudina pellucida*; ses rapports avec l'alimentation et la locomotion. J. Protozool. 15, 230–246.

Wakeman, K.C., Hiruta, S., Kondo, Y., Ohtsuka, S., 2021. Evidence for host jumping and diversification of marine cephaloidophorid gregarines (Apicomplexa) between two distantly related animals, viz., crustaceans and salps. Protist 172, e125822. https://doi.org/10.1016/j.protis.2021.125822.

Wakeman, K.C., Leander, B.S., 2012. Molecular phylogeny of Pacific archigregarines (Apicomplexa), including descriptions of *Veloxidium leptosynaptae* n. gen., n. sp., from the sea cucumber *Leptosynapta clarki* (Echinodermata), and two new species of *Selenidium*. J. Eukaryot. Microbiol. 59, 232–245.

Wakeman, K.C., Yabuki, A., Fujikura, K., Tomikawa, K., Horiguchi, T., 2018. Molecular phylogeny and surface morphology of *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. (Gregarinasina, Apicomplexa) isolated from a deep sea *Oradarea* sp. (Amphipoda) in the West Pacific. J. Eukaryot. Microbiol. 65, 372–381.

Yamaguchi, A., Horiguchi, T., 2005. Molecular phylogenetic study of the heterotrophic dinoflagellate genus *Protoperidinium* (Dinophyceae) inferred from small subunit rRNA gene sequences. Phycol. Res. 53, 30–42.