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ORIGINAL ARTICLE

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

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ABSTRACT

In an effort to broaden our understanding of the biodiversity and distribution of gregarines infecting crustaceans, this study describes two new species of gregarines, *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp., parasitizing a deep sea amphipod (*Oradarea* sp.). Amphipods were collected using the ROV Hyper-Dolphin at a depth of 855 m while on a cruise in Sagami Bay, Japan. Gregarine trophozoites and gamonts were isolated from the gut of the amphipod and studied with light and scanning electron microscopy, and phylogenetic analysis of 18S rDNA. *Thiriotia hyperdolphinae* n. sp. was distinguished from existing species based on morphology, phylogenetic position, as well as host niche and geographic locality. *Cephaloidophora oradareae* n. sp. distinguished itself from existing *Cephaloidophora*, based on a difference in host (*Oradarea* sp.), geographic location, and to a certain extent morphology. We established this latter new species with the understanding that a more comprehensive examination of diversity at the molecular level is necessary within *Cephaloidophora*. Results from the 18S rDNA molecular phylogeny showed that *T. hyperdolphinae* n. sp. was positioned within a clade consisting of *Thiriotia* spp., while *C. oradareae* n. sp. grouped within the Cephaloidophoridae. Still, supplemental genetic information from gregarines infecting crustaceans will be needed to better understand relationships within this group of apicomplexans.

GREGARINES are a group of obligate unicellular eukaryotes (protists) that almost exclusively infect the intestines, other internal cavities, and tissues of invertebrates (Desportes and Schrével 2013a,b; Lee et al. 2000; Levine 1985). Gregarines can be found in a variety of environments (marine, freshwater, and terrestrial), but marine lineages are regarded as some of the most diverse and earliest evolving lineages among gregarines and apicomplexans as a whole (Leander 2008; Théodoridès 1984). Gregarines infecting crustaceans are equally as diverse as their counterpart hosts (Levine 1977, 1979). Molecular data collected from this group have shown that gregarines from marine crustaceans tend to group in a single clade and have extraordinary fast evolutionary rates (even among gregarines) (Diakin et al. 2017; Rueckert et al. 2011b; Simdyanov et al. 2015). While there are numerous studies that have described the morphological diversity of these gregarines lineages, molecular data associated with this group remain limited to a handful of 18S rDNA and a couple 28S rDNA sequences. By studying the biology and diversity of gregarines infecting crustaceans we aim to improve our understanding of their evolution, and relationships they have with their host groups.

The vast majority of apicomplexans infecting crustaceans appear to have been significantly modified from lineages of gregarines that have retained more ancestral characteristics ("archigregarines"); collectively, these gregarines that have diversified are pragmatically referred to as "eugregarines". Past and contemporary studies on the morphology and available molecular data have led to this group being divided into six families: Thiriotiidae (Desportes and Schrével

2013a), Porosporidae (Labbé 1899), Cephaloidophoridae (Kamm 1922), Cephalolobidae (Théodoridès and Desportes 1975), Ganymedidae (Huxley 1910), and Uradiophoridae (Grassé 1953) (Rueckert et al. 2011b). No molecular data are available for members of the Cephalolobidae or Porosporidae (from crustaceans). There is some small discrepancy whether the genus *Thiriotia* belongs within the Porosporidae (Rueckert et al. 2011b), or is its own distinct family (Desportes and Schrével 2013a). The trophozoites (or feeding stages) of *Thiriotia* are aseptate (Desportes et al. 1977; Rueckert et al. 2011b; Sano et al. 2017), compared to members of the Porosporidae where the septum is only absent in satellites (during syzygy) (Desportes and Schrével 2013a).

Apicomplexan parasites infecting crustaceans are diverse, containing approximately 150 species (across 15 genera) from crabs, shrimp, krill, barnacles, and amphipods (Desportes and Schrével 2013a; Levine 1977, 1979). The largest proportion of species described belong to a single genus, *Cephaloidophora*. This genus is commonly found infecting maxillipeds (barnacles) and decapods (particularly, amphipods). While the *Cephaloidophora* infecting barnacles have mostly been classified as a single species, namely *C. communis*, *Cephaloidophora* infecting amphipods have been divided into many different species (containing around 80 species). Many of these species have been established based on morphometric data (or discrete observations of cell shape) observed in trophozoites. This is in part problematic because recent molecular studies have shown that trophozoites of the same species can be quite plastic (Leander et al. 2003; Rueckert et al. 2011b). Currently, *C.* cf. *communis* is the only member of this genus for which sequence data are available (Rueckert et al. 2011b; Simdyanov et al. 2015), and so more molecular data are needed in order to evaluate species complexes, biodiversity, and distribution of *Cephaloidophora* infecting crustaceans.

In the present study, two new species of gregarine apicomplexans, *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp., are described using light and scanning electron microscopy, as well as molecular analysis of 18S rDNA from a deep sea amphipod (*Oradarea* sp.) in the Western Pacific.

MATERIALS AND METHODS

Collection of hosts and isolation of *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp

Oradarea sp. (Crustacean; Amphipoda) was collected from the methane seep at off-Hatsushima I stand, Sagami Bay (35°00′N, 139°13′E) at a depth of 855 m on the May 8, 2016. Amphipods were held in cool seawater until dissected. The amphipod intestines were dissected using forceps and razors. A total of 15 hosts were collected and 10 were dissected; all 10 were heavily infected with *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. in the digestive tract. Individual trophozoites of *T. hyperdolphinae* n. sp. and *C. oradareae* n. sp. were isolated using hand-drawn glass pipettes, and subsequently washed (until clean) in filtered, autoclaved seawater for further morphological and molecular analysis.

Light microscopy and scanning electron microscopy

Differential interference contrast (DIC) images of the trophozoite stages of *T. hyperdolphinae* n. sp. and *C. oradareae* n. sp. were taken using a BX43 microscope (Olympus, Tokyo, Japan) equipped with a Sony HDR-CX 550V Handycam (Sony Corporation, Tokyo, Japan). For scanning electron microscopy, cells were transferred to 3 μm (Merck Millipore, Billerica, MA) filter submerged in 2.5% glutaraldehyde in seawater on ice for 15 min. After washing the cells 3 times for 5 min in seawater, the filter was placed in 1% OsO_4 for 30 min, and subsequently washed with distilled water and dehydrated through a graded series of ethanol washes (30–100%) for 5 min at each step. Samples were critical point dried with CO_2, sputter-coated with 5 nm gold and viewed using a Hitachi N-3000 (Hitachi, Ltd., Tokyo, Japan).

DNA extraction, PCR amplification, and sequencing of 18S rDNA

Single-cell isolates of *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. were placed in 1.5 ml Eppendorf tubes. Total genomic DNA was extracted following the manufacturer's protocol using an Epicentre MaterPure DNA and RNA purification kit (Epicentre Technologies Corporation, Chicago, IL). To amplify the 18S rDNA, an initial PCR using Econotaq 2X Mastermix (Lucigen Corporation, Middleton, WI) was performed with Outside primers PF1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and SSUR4 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander et al. 2003) in a 25 μl PCR. The following program was used on the thermocycler for the initial amplification: Initial denaturation at 94 °C for 2 min; 35 cycles of denature at 94 °C for 30 s, anneal at 52 °C for 30 s, extension at 72 °C for 1 min 50 s, final extension 72 °C 5 min. Subsequently, internal primers F2 5′-GCYTGAAAAGGTGACDATCTG-3′ and R2 5′-CATATCTGCTAAGGTTCTG-3′ (Diakin et al. 2017) were paired with outside primers in a nested PCR using the following program on a thermocycler: Initial denaturation for 94 °C for 2 min; 25 cycles of denature at 94 °C for 30 s, anneal at 52 °C for 30 s, extension at 72 °C for 1 min 30 s; final extension at 72 °C for 7 min. PCR products were purified using a Qiagen PCR purification kit (Hilden, Germany); 1 μl of purified product was used in a sequencing reaction with ABI BigDye Terminator v1.1 (Applied Biosystems, Foster City, CA) and subsequently purified with ethanol, before being eluted in 18 μl Hi-Di Formamide (Applied Biosystems) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems). The two novel 18S rDNA sequences from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. were deposited in NCBI's GenBank (*Thiriotia hyperdolphinae* n. sp. MG009199 and *Cephaloidophora oradareae* n. sp. MG009200).

Phylogenetic analyses

The newly obtained 18S rDNA sequences from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. were identified by BLAST. These sequences were then aligned to 93 additional sequences representing the diversity of gregarines, as well as other apicomplexan groups and dinoflagellates (outgroup) using MUSCLE 3.8.31 under the default settings (Edgar 2004). A second alignment containing 46 taxa was constructed from sequences representing the diversity of gregarines and environmental sequences from crustacean hosts. The latter alignment was constructed in the same way as the former. Both alignments were fine-tuned visually using Mesquite 3.04 (Maddison and Maddison 2015); gaps were omitted from the analysis, resulting in alignments that included 1,042 and 1,350 sites, respectively for the alignments containing 95 and 48 taxa.

Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html; Zwickl 2006) was used to run maximum-likelihood (ML) bootstrap analyses on both datasets. Jmodeltest 0.1.1 selected a general-time reversible (GTR) model of nucleotide substitutions under Akaike information criterion with correction (AICc; Posada and Crandall 1998) that incorporated invariable sites and a discrete gamma distribution (eight categories; GTR + Γ + I model: $\alpha = 0.5820$ and fraction of invariable sites = 0.0983, for the 18S alignment with 93 taxa, and $\alpha = 0.5720$ and fraction of invariable sites = 0.1720 for the 18S alignment with 48 taxa). ML bootstrap analyses were performed on 500 pseudo-replicates, with one heuristic search per pseudo-replicate (Zwickl 2006), using the same program set to the GTR + Γ + I. Bayesian analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck and 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 8,500,000 and 6,000,000 generations were run for the larger and smaller 18S alignments, respectively. 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 fell below 0.01, the program was set to terminate. All other parameters were left at the default setting. A majority rule consensus tree was constructed from 65,000 post-burn-in trees for the larger 18S rDNA dataset, while 40,000 trees were used for the 18S rDNA dataset with fewer taxa. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees.

RESULTS

Morphology of *Cephaloidophora oradareae* n. sp

Trophozoites of *Cephaloidophora oradareae* n. sp. were cylindrical and rigid, composed of an epimerite (average length × width = 2.5 μm × 4 μm; range = 2.0–3.5 μm × 3.5–5 μm, $n = 20$), the protomerite (average length × width = 10 μm × 13 μm; range = 9–13 μm × 11–14 μm, $n = 20$), and deutomerite (average length × width = 30 μm × 13 μm; range = 25–35 μm × 11–15 μm, $n = 20$); the protomerite and deutomerite were divided by a distinct septum. The nucleus was spherical (diameter = 5 μm, $n = 20$) and was located in the upper portion of the deutomerite (Fig. 1A–C). The posterior of the cell was rounded. Folds ran longitudinally along the surface from the epimerite to the posterior end, with a density of 4 folds/μm (Fig. 2). Some folds terminated along the surface of the cell (i.e. not at the epimerite or posterior end) (Fig. 2E). Syzygy was not observed in any hosts. Trophozoites were observed gliding.

Morphology of *Thiriotia hyperdolphinae* n. sp

Trophozoites of *Thiriotia hyperdolphinae* n. sp. were elongate (average length = 50 μm) and slender (average width = 4.5 μm, $n = 20$). The nucleus (diameter = 4 μm) was spherical and was located in the middle of the trophozoite (Fig. 1D). The epimerite was rounded or cup-like (possibly gamonts disassociated during syzygy); the posterior was rounded. Folds ran longitudinal surface of the cell, with a density of 5 folds/μm (Fig. 3). Syzygy was observed to be caudo-frontal (Fig. 3C). Trophozoites did not move (bend or twist) in a way that was readily observable. Gliding motility was not observed.

Phylogenetic analyses of 18S rDNA from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp

Phylogenetic analysis of the larger 18S rDNA dataset comprising *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. and 91 additional (closely related) taxa recovered clades of rhytidocystids, coccidians, cryptosporidians, two clades of terrestrial gregarines (I and II), *Selenidium* from tube-forming polychaetes, *Polyplicarium*, paralecudinids, Lecudinoidea, *Veloxidium*, gregarines from sipunculids, as well as a clade of gregarines from crustaceans (Fig. 4). Relationships between these major clades were uncertain throughout the deeper nodes in the tree; nodal support did improve in individual clades. The novel sequences generated from single-cell isolates of *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. were both well-supported within the clade of gregarines from crustaceans (Fig. 4). In this larger dataset, *T. hyperdolphinae* n. sp. was sister to a subclade of *Thiriotia* spp. and other environmental sequences with moderate support. The sequence from *C. oradareae* n. sp. was situated in a well-supported subclade containing *C.* cf. *communis* and other environmental sequences. The relationships between *C.* cf. *communis*, *C. oradareae* n. sp., and the environmental sequences in this subclade were not resolved (Fig. 4).

In the smaller rDNA dataset, gregarines from crustaceans formed four well-supported subclades, corresponding to the Thiriotiidae (Desportes and Schrével 2013a), Cephaloidophoridae, Ganymedidae, and

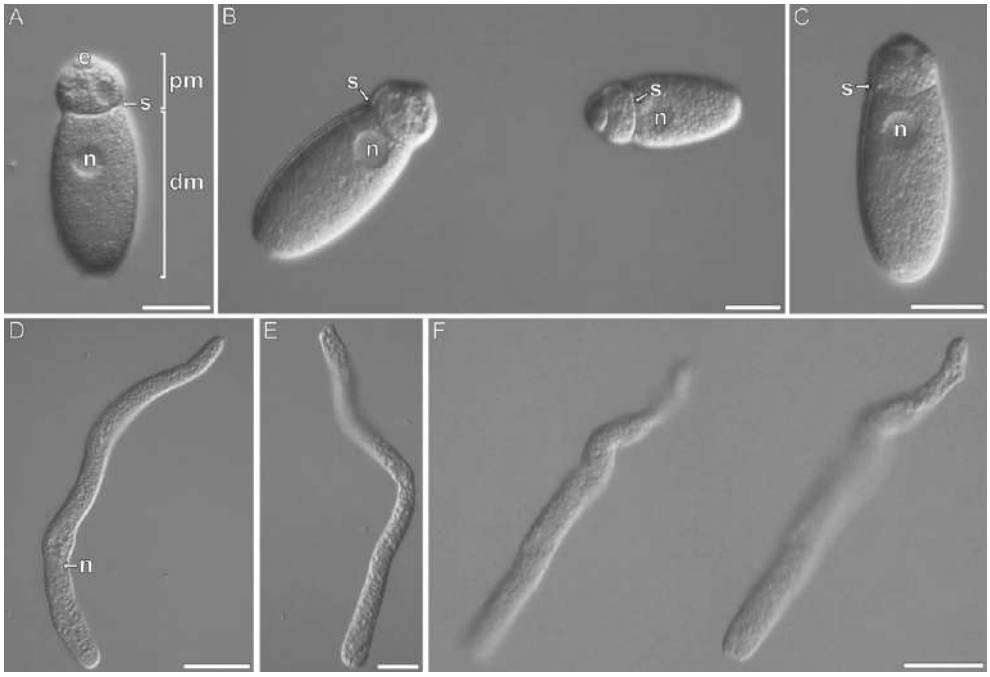

Figure 1 Light micrographs showing the general morphology of *Cephaloidophora oradareae* n. sp. and *Thiriotia hyperdolphinae* n. sp. trophozoites (feeding stages). (**A–C**) *Cephaloidophora oradareae* n. sp. trophozoites (feeding stages) showing the nucleus (n), septum (s) dividing the deutomerite (dm) and protomerite (pm), and epimerite (e). (**D–F**) *Thiriotia hyperdolphinae* n. sp. trophozoites showing the general narrow and elongated shape of this life stage. The nucleus (n) is located in the center or near the posterior (only visible in D). The mucron of the cell is oriented toward the top of the figure. Scales A–C = 10 μm; D, F = 20 μm; E = 10 μm.

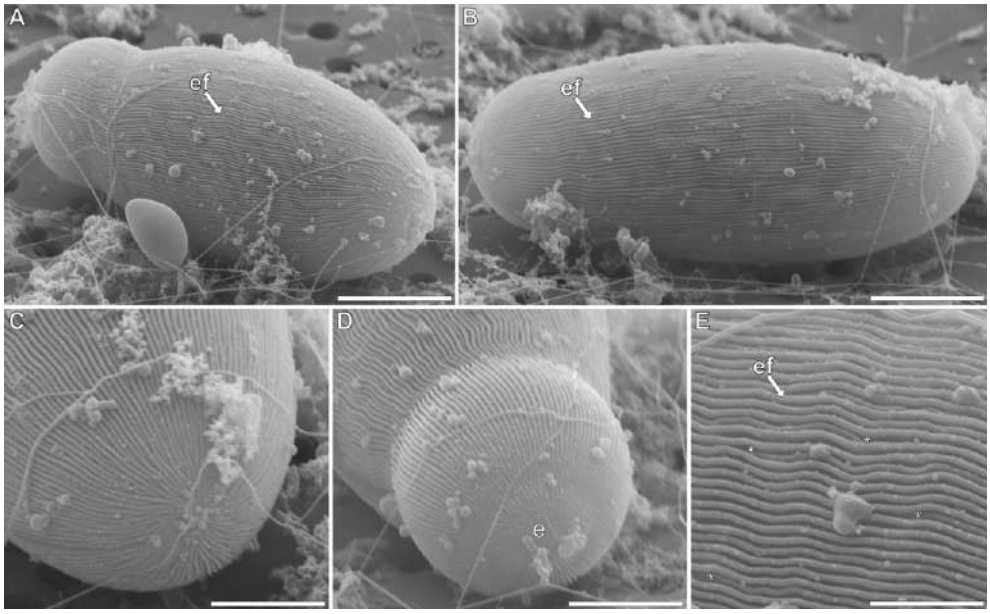

Figure 2 Scanning electron micrograph images highlighting the surface morphology of *Cephaloidophora oradareae* n. sp. trophozoites. (**A, B**) SEM micrographs showing the longitudinal-running epicytic folds (ef) on the surface of *C. oradareae* (the anterior ends of the cells are oriented toward the left). (**C**) SEM image showing the posterior end of *C. oradareae* n. sp. (**D**) SEM image of the epimerite (e) (anterior end of the cell). (**E**) High-magnification SEM showing the epicytic folds (ef) on the cell surface—note the ends (*). Scales A, B = 10 μm; C, D = 5 μm; E = 3 μm.

Uradiophoridae. Another clade of "unknown" environmental sequences was recovered at the base of the tree (Fig. 5). The relationships between Thiriotiidae, Cephaloidophoridae, Ganymedidae, and Uradiophoridae were not highly supported: sister relationships between the Thiriotiidae and Cephaloidophoridae were moderate;

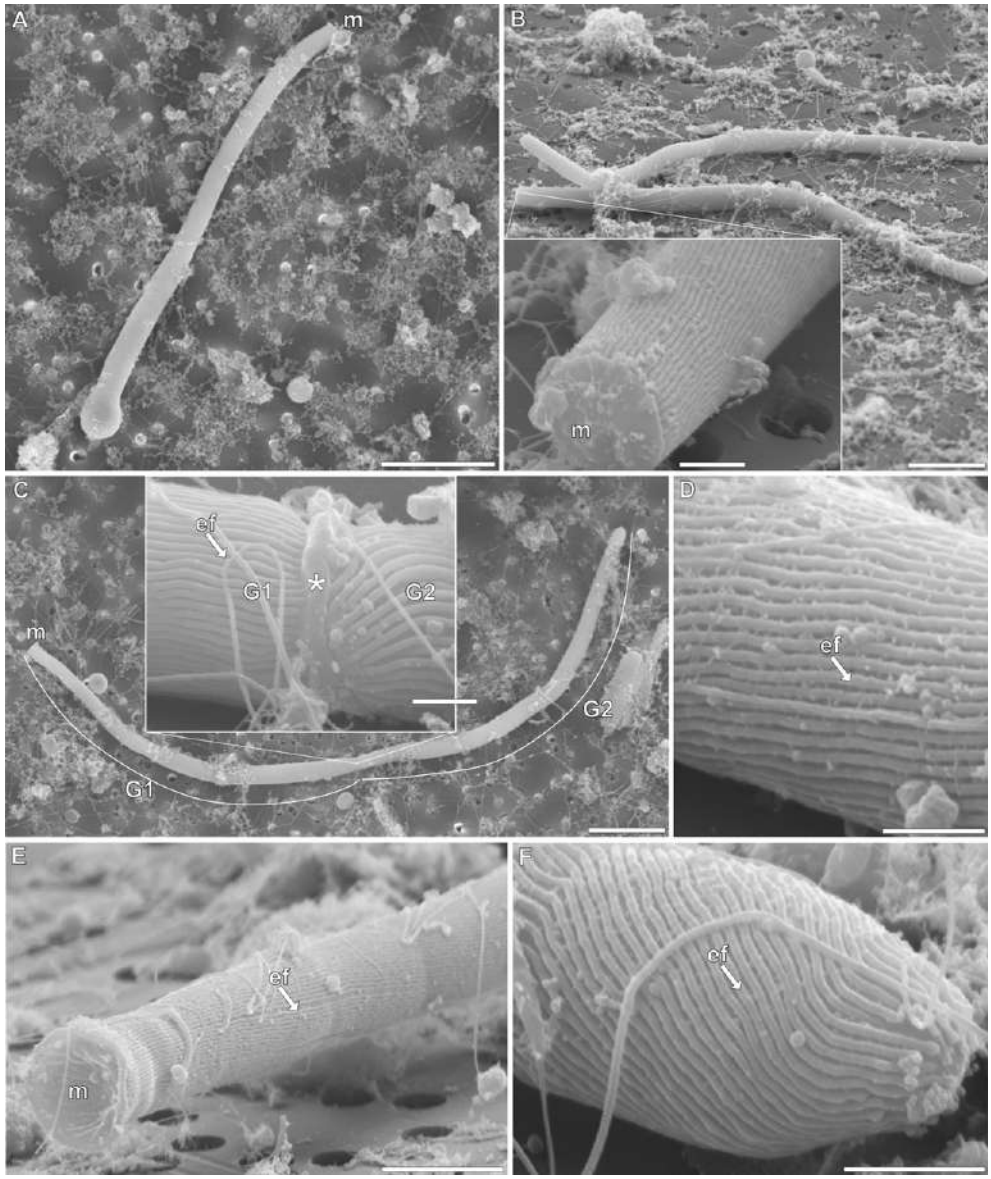

Figure 3 Scanning electron micrograph images highlighting the surface morphology of *Thiriotia hyperdolphinae* trophozoites. (**A, B**) SEM showing the generally thin and long morphology of *Thiriotia hyperdolphinae* n. sp. b (inset). Note the mucron of an isolate that is slightly rounded. (**C**) Two gamonts (G1 and G2) associated in a caudo-frontal syzygy (sexual reproduction). (C, inset). High-magnification SEM showing the syzygy interface (*) between the two gamonts (G1 and G2), as well as epicytic folds (ef). (**D**) High-magnification micrograph of the longitudinal-running epicytic surface folds (ef). (**E**) High magnification of the anterior end of the cell showing a cup-like mucron (m) and epicytic folds (ef). (**F**) High-magnification SEM of the rounded posterior end of the cell, also showing epicytic folds (ef). Scales A–C = 20 μm; C (inset) = 1 μm; D = 1 μm; E = 5 μm; F = 2 μm.

the relationships between Ganymedidae and Uradiophoridae to the other parts of the tree were uncertain. *Thiriotia hyperdolphinae* n. sp. formed a well-supported clade with the other *Thiriotia* spp. and environmental sequences. Nodes within this clade, particularly those connecting the environmental sequences and the clade containing *T. pugettiae* and *T. euchirellae,* were unresolved or had moderate support (Fig. 5). The position of *C. oradareae* n. sp. within a clade containing *C.* cf. *communis* was well-supported; however, nodes connecting these species and the environmental sequences were either robustly supported or carried no support (Fig. 5).

DISCUSSION

The classification and diversity within the genus *Cephaloidophora* is an inherently complicated situation. This genus has nearly 80 described species from decapods, maxillipods, euphausiids, and especially from amphipods (Desportes and Schrével 2013a). *Cephaloidophora oradareae* n.

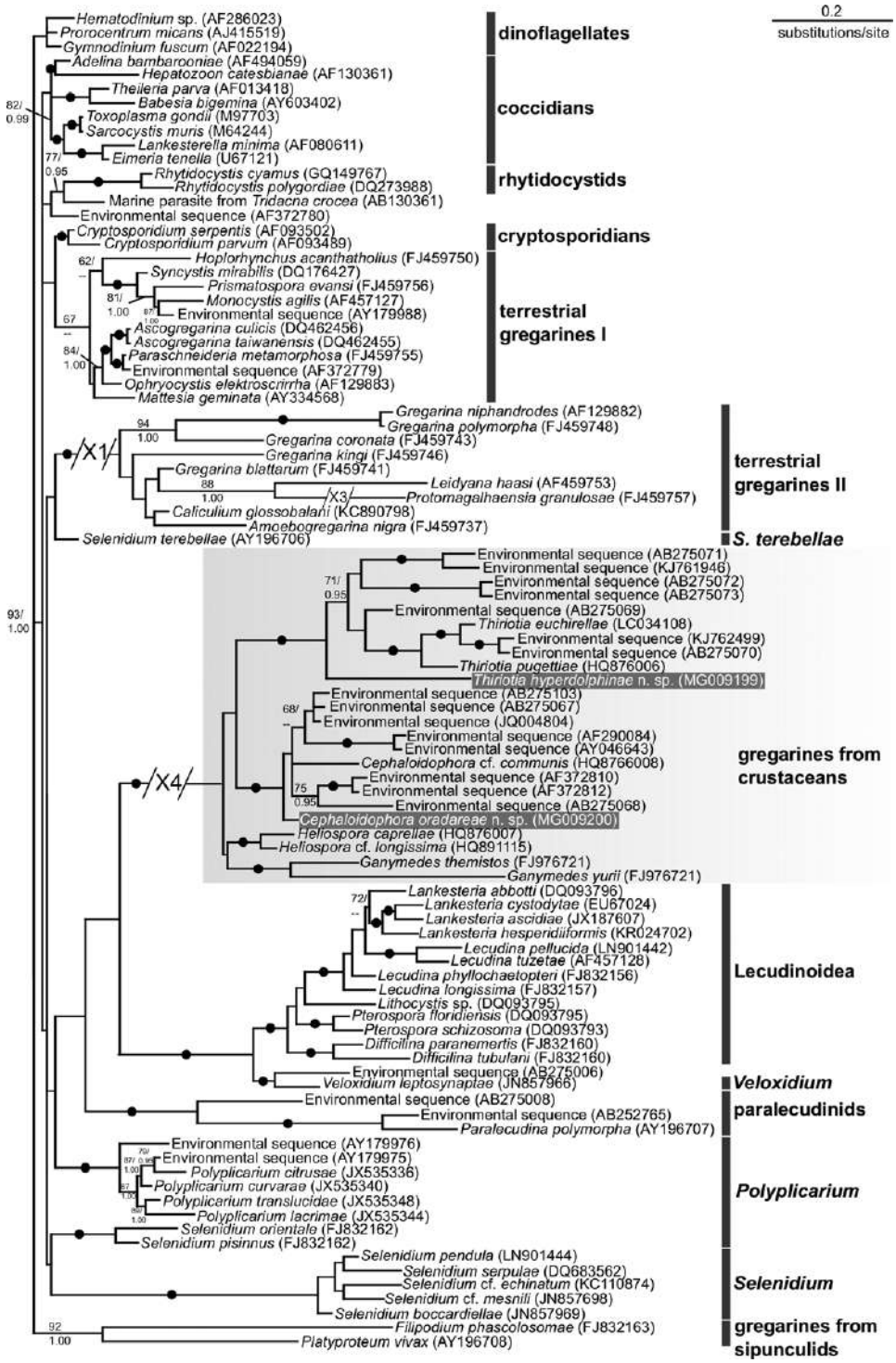

Figure 4 Maximum likelihood (ML) and Bayesian inference derived from the phylogenetic analyses of 18S rDNA from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. and 91 other taxa representing the diversity of gregarine apicomplexans, core apicomplexans, and dinoflagellates (outgroup) over 1,042 unambiguously aligned sites. This tree was inferred using the GTR + Γ + I substitution model (−ln L = 29,314.3827, gamma shape = 0.5820, proportion of invariable sites = 0.0983). Numbers at the nodes denote the ML bootstrap percentage and Bayesian posterior probabilities, respectively. Black dots on branches denote instances where bootstrap support values and Bayesian posterior probabilities were 95/0.95 or higher. Bootstrap and Bayesian values less than 55 and 0.95 were not added to this tree. The novel sequences from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. generated in this study are highlighted in a black box. Some branches were shortened by multiples of the length of the substitutions/site scale bar (e.g. X1).

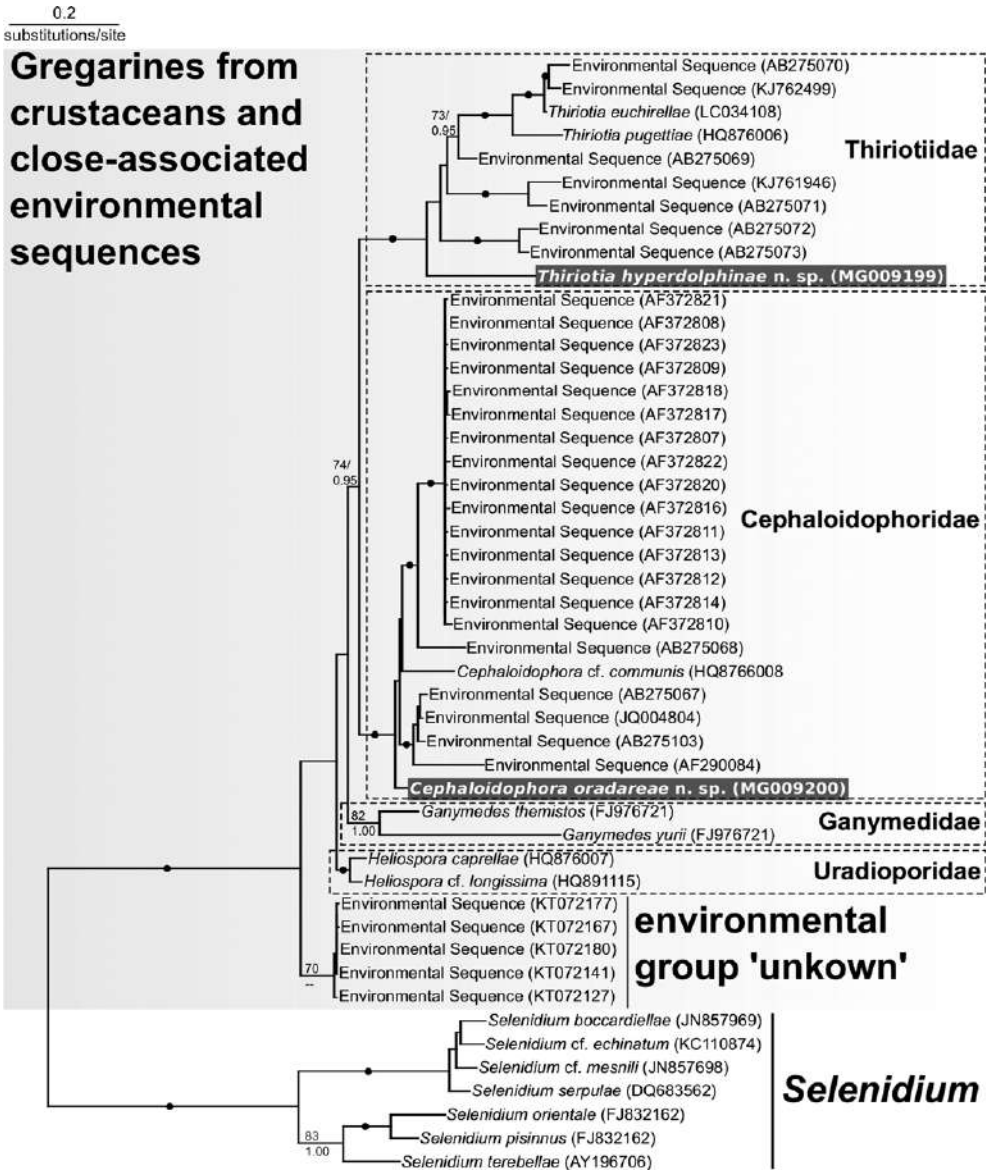

Figure 5 Maximum likelihood (ML) and Bayesian inference derived from the phylogenetic analyses of 18S rDNA from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. and 46 other taxa representing the diversity of *gregarines from crustaceans* over 1,350 unambiguously aligned sites. This tree was inferred using the GTR + Γ + I substitution model (-ln L = 16,519.4001, gamma shape = 0.5720, proportion of invariable sites = 0.1720). Numbers at the nodes denote the ML bootstrap percentage and Bayesian posterior probabilities, respectively. Black dots on branches denote instances were bootstrap support values and Bayesian posterior probabilities of 95/0.95 or higher were recorded. Bootstrap and Bayesian values less than 55 and 0.95 were not added to this tree. The novel sequences from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. generated in this study are highlighted in a black box. Some branches were shortened by multiples of the length of the substitutions/site scale bar (e.g. X1).

sp. was isolated from *Oradarea* sp., which represents a new host group (family level) of amphipod from which gregarines have been described. While there are eight species of *Cephaloidophora* that have been isolated from Amphipods in the West Pacific (Hoshide 1956, 1969a,b, 1971), trophozoites within this genus are superficially similar, with few (or no) discernible characteristics separating one species from another. *Cephaloidophora oradareae* n. sp. was on average smaller than the previously described Western Pacific *Cephaloidophora*, having an average length of around 43 μm and width of 10 μm; somewhat smaller than the next smallest, *C. warekara* (Hoshide 1969a), which measured 50 μm long and 25 μm wide. Still, size and shape of cells (alone) can be a cumbersome metric for species classification among gregarines.

While marine gregarines tend to have pronounced/distinct trophozoite stages that can aid in streamlining general classification, morphological characteristics alone

cannot be used to reliably delineate species, due to the plasticity of trophozoite stages (Leander et al. 2003; Rueckert et al. 2011b). In particular, 18S rDNA has already helped to identify and distinguish closely related gregarine apicomplexans from tunicates (Rueckert et al. 2015), tube-forming polychaetes (Wakeman and Leander 2013), and nemerteans (Rueckert et al. 2010). At the moment, the availability of molecular data from cephaloidophorid gregarines is also quite low. *Cephaloidophora* is represented in SSU rDNA datasets by a single species, *C.* cf. *communis* from *Balanus balanus* and *B. glandula* (barnacles). The sequence of *C.* cf. *communis* was 7.2% different from that of *C. oradareae* n. sp., and they do not group in their own clade in the phylogenetic analyses. Considering this, we have concluded to give a new species name to the *Cephaloidophora* isolate in our study, *Cephaloidophora oradareae* n. sp., with the understanding that a broader survey is needed that incorporates molecular data and morphology from type host material, in order to reconcile new and previously-established data and clarify species complexes among *Cephaloidophora*.

To date, the genus *Thiriotia* has three described species: *T. pisae* (the type species), *T. pugettiae*, and *T. euchirellae* (Desportes et al. 1977; Rueckert et al. 2011b; Sano et al. 2017). Trophozoite of *Thiriotia hyperdolphinae* n. sp. had an anterior end that was rounded, aseptate, and the trophozoites as a whole were extremely thin (4.5 μm) and long (50 μm). Both these characteristics have been proposed to unite the genus (and family) (Desportes and Schrével 2013a). *Thiriotia hyperdolphinae* n. sp. shared an elongated shape with *T. pisae*, *T. pugettiae*, and *T. euchirellae*. Nonetheless, the average dimensions of *T. hyperdolphinae* n. sp. (4.5 μm × 50 μm) differed from these other previously described isolates (*T. pisae* (25 μm × 130 μm), *T. pugettiae* (25 μm × 1,025 μm), and *T. euchirellae* (31 μm × 174 μm)). Furthermore, both *T. pugettiae* and *T. pisae* exhibited latero-frontal syzygy, while *T. hyperdolphinae* n. sp. was observed to have caudo-frontal associations; syzygy associations in *T. euchirellae* were not reported.

Both the larger and smaller molecular datasets in this study that examined the relationship between *Thiriotia hyperdolphinae* n. sp. and other gregarines from crustaceans placed *T. hyperdolphinae* n. sp. at the base of a well-supported clade (i.e. Thiriotiidae). *Thiriotia pugettiae* and *T. euchirellae* did form a smaller subclade, to the exclusion of *T. hyperdolphinae* n. sp. However, there is currently a lack of information regarding developmental life stages (e.g. cyst and oocyst morphology and structure) from the type species (and species for which molecular data are available) that could be used to differentiate *T. hyperdolphinae* n. sp. from other species in this genus. Based on this, and due to the novelty with regard to the host and its geographic locality, as well as a distinct trophozoite morphology that differs from other *Thiriotia*, it was concluded to place *T. hyperdolphinae* n. sp. within *Thiriotia*, but establish a new species.

Results from our phylogenetic analyses of the novel sequences from *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. were largely similar to previous work which identified four major subgroups (clades) of gregarines from crustaceans (Rueckert et al. 2011b). Deeper relationships between these groups remained unresolved in both the larger and smaller datasets. It seems that more genetic information, possibly from 28S rDNA (Simdyanov et al. 2015), could further resolve this issue. The smaller molecular analysis which included more environmental sequences showed that *Thiriotia hyperdolphinae* n. sp. and *Cephaloidophora oradareae* n. sp. have a number a environmental sequences that are associated with these gregarine lineages; this is something that has been shown in previous work (Rueckert et al. 2011a). Many of these environmental sequences were generated from environmental samples taken from a diverse set of ecosystems including the shallow and deep sea (Lie et al. 2014), anoxic sediments (Dawson and Pace 2002), and marine methane seeps (Takishita et al. 2007). A novel clade of sequences referred to as "environmental group 'unknown'" was recovered in our analysis. These sequences were recovered from the NCBI database, and were apparently generated from a sulfide karst spring in Slovenia. The branching pattern recovered in the smaller molecular analysis suggests perhaps that this could represent a novel molecular clade of gregarines infecting crustaceans. However, while these sequences are available to the public, they are not linked to any published article, and so further information on the origin of these environmental sequences is not available. It is also interesting to point out that the Thiriotiidae and Cephaloidophoridae appear to have significantly more environmental sequences associated with their clades, compared to the Uradioporidae and Ganymedidae which have only a few associated with their respective clades (Rueckert et al. 2011a). Whether this carries any ecological significance, or is simply an artifact of some sampling bias is uncertain. Extrapolating on the ecology of gregarine parasites given only the presence of environmental sequences should be treated with caution, as proliferating cyst stages of the parasite can conceivably float/sink to extreme depths and geographic locations.

With regard to the sampling site and the ecology of gregarines, the off-Hatsushima I stand is known for its composition of chemosynthetic bathyal communities of invertebrates including worms and clams (Sakai et al. 1987). Within the scope of this work, it is interesting to mention that gregarines, at least those from crustaceans, appear to be highly amenable to their environment, even under "extreme" conditions where the host organisms have become highly specialized, typically resorting to a form of symbiosis with bacteria in order to acquire nutrients and survive (Bright and Lallier 2010). While we did not collect data related the mechanisms that allow gregarines to exploit these environments, gregarines can be found in arthropods from freshwater, marine (Rueckert et al. 2011a), and terrestrial habitats (Criado-Fornelio et al. 2017), highlighting the extent to which this group of apicomplexan parasites is able to adapt.

TAXONOMIC SUMMARY

Cephaloidophora oradareae n. sp. Wakeman, Yabuki, Fujikura, Tomikawa & Horiguchi 2017.

Description. Trophozoites of *Cephaloidophora oradareae* n. sp. were cylindrical and rigid, with a density of 4 folds/μm. Epimerite (average length × width = 2.5 μm × 4 μm), protomerite (average length × width = 10 μm × 13 μm), and deutomerite (average length × width = 30 μm × 13 μm) divided by a distinct septum. Nucleus spherical (diameter = 5 μm) and located in the upper portion of the deutomerite. Syzygy not observed. Trophozoites exhibit gliding motility.
DNA sequence. SSU rDNA sequences (GenBank MG099200).
Type locality. Methane seep at off-Hatsushima I stand, Sagami Bay (35°00.9525′N, 139°13.3294′E), 855 m in depth.
Type habitat. Marine.
Type host. A species of genus *Oradarea* Walker 1903 (Crustacea; Amphipoda; Oradarea) inhabiting the off-Hatsushima methane seep.
Location in host. Intestinal lumen.
Type material: Parasites on a gold sputter-coated SEM stub (KCW_deepcrust_Thiriotia) and hosts material (KCW_deepcrust_host) preserved in ethanol have been deposited in the Biodiversity Collection at Hokkaido University.
Etymology. The species name *Cephaloidophora oradareae* refers to the host from which the parasites were isolated, *Oradarea* sp.

Thiriotia hyperdolphinae n. sp. Wakeman, Yabuki, Fujikura, Tomikawa & Horiguchi 2017.

Description. Trophozoites of *Thiriotia hyperdolphinae* n. sp. elongate (average length = 50 μm) and slender (average width 4.5 μm). Nucleus (diameter = 4 μm) spherical and located centrally in trophozoite. Mucron rounded or cup-like (possibly from gamonts); posterior rounded. Folds run along longitudinal surface of the cell, with a density of 5 folds/μm. Syzygy caudo-frontal. Trophozoites not moving (bending or twisting) in a way readily observable. Gliding motility not observed.
DNA sequence. SSU rDNA sequences (GenBank MG099199).
Type locality. Methane seep at off-Hatsushima I stand, Sagami Bay (35°00.9525′N, 139°13.3294′E), 855 m in depth.
Type habitat. Marine.
Type host. A species of genus *Oradarea* Walker 1903 (Crustacea; Amphipoda; Oradarea) inhabiting the off-Hatsushima methane seep.
Location in host. Intestinal lumen.
Type material: Parasites on a gold sputter-coated SEM stub (KCW_deepcrust_Cephaloidophora) and hosts material (KCW_deepcrust_host) preserved in ethanol have been deposited in the Biodiversity Collection at Hokkaido University.
Etymology. The species name, *Thiriotia hyperdolphinae* n. sp., refers to the ROV Hyper-Dolphin that collected samples during the cruise.

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