

Identity of environmental DNA sequences using descriptions of four novel marine gregarine parasites, *Polyplacium* n. gen. (Apicomplexa), from capitellid polychaetes

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Abstract Environmental PCR surveys of small-subunit (SSU) rDNA sequences are powerful approximations for the overall diversity of microbial eukaryotes (protists) living in specific marine habitats. However, many environmental DNA sequences generated from these approaches have unknown cellular origins because they are not closely related to other sequences that were generated directly from fully characterized, identified organisms. The unidentified sequences from marine environments tend to belong to poorly understood groups of apicomplexan parasites, especially gregarines. Single-cell PCR (SC-PCR) approaches on newly discovered gregarines provide the evidence necessary for determining the cellular identities of SSU rDNA sequence clades. In this study, the trophozoites of four novel gregarine morphotypes were isolated from the intestines of two different species of capitellid polychaetes collected from the eastern Pacific Ocean (British Columbia, Canada). The trophozoites of each morphotype were characterized using light microscopy, scanning electron microscopy, and SSU rDNA sequences amplified from four, single-cell isolates from each of the four novel morphotypes described in this study (16 new SSU rDNA sequences in total). Molecular phylogenetic analyses demonstrated five robust subclades within a more inclusive clade that contained all 16 new sequences and 5 environmental SSU rDNA sequences. The combination of SC-PCR approaches, molecular

phylogenetic analyses, and comparative morphology (1) illustrate the utility of SC-PCR approaches for distinguishing between different gregarine species, (2) demonstrate the cellular identity of a previously unidentified environmental SSU rDNA sequence clade, and (3) enable us to establish four new species within one novel genus: *Polyplacium lacrimae* n. gen. et sp. (type species), *P. curvarae* n. gen. et sp., *P. translucidae* n. gen. et sp., and *P. citrusae* n. gen. et sp.

Keywords Alveolata · Apicomplexa · Capitellid polychaetes · Environmental DNA sequences · Marine gregarines · Molecular phylogeny · *Polyplacium*

Introduction

Marine gregarine apicomplexans are a diverse but poorly understood assemblage of endoparasites that infect the intestines and other extracellular spaces in a wide range of marine invertebrates (Grassé 1953; Levine 1971, 1976; Perkins et al. 2002). Only a tiny fraction of the known diversity of marine gregarines is represented in molecular phylogenetic datasets; the most widely explored marker so far has been small-subunit (SSU) rDNA sequences (Leander 2007; Leander and Keeling 2004; Leander et al. 2003, 2006; Rueckert et al. 2010; Rueckert and Leander 2008, 2009, 2010; Rueckert et al. 2011a, b; Wakeman and Leander 2012). Nonetheless, phylogenetic analyses of DNA sequences used in tandem with high-resolution microscopy of trophozoite stages has helped shape our understanding of gregarine diversity and evolutionary history (Leander 2008). This approach has also been vital for the delimitation and identification of different gregarine species and for establishing the cellular identities of ambiguous environmental

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DNA sequences generated from several different PCR surveys of marine biodiversity (Berney et al. 2004; Cavalier-Smith 2004; Dawson and Pace 2002; Edgcomb et al. 2002; Leander and Ramey 2006; López-García et al. 2007; Moreira and López-García 2003; Stoeck and Epstein 2003; Stoeck et al. 2007; Takishita et al. 2005, 2007a, b).

Environmental PCR surveys targeting SSU rDNA sequences are informative approximations for the overall composition of species in an ecosystem, especially when considering the vast assortment of uncultivated lineages of microbial eukaryotes present in these systems (e.g., intertidal areas, salt marshes, and deep sea hydrothermal vents). However, the cellular identities of numerous environmental sequences generated from marine environments remain ambiguous. This is mainly due to an inability to establish the sister lineages of highly divergent sequences using molecular phylogenetic datasets with only a limited sample of taxa that have also been characterized at the morphological level (Leander and Ramey 2006; Rueckert et al. 2011a, b). This situation has led some authors to conclude that the variation observed in some SSU rDNA sequences represents novel lineages of eukaryotic diversity that are largely or completely unknown (Dawson and Pace 2002; López-García et al. 2007; Stoeck and Epstein 2003; Stoeck et al. 2007). Other authors interpret ambiguous environmental sequences as representing known species or more inclusive taxonomic groups that have yet to be characterized at the molecular level (Cavalier-Smith 2004; Rueckert et al. 2011a, b). These contrasting interpretations are difficult to evaluate when the molecular phylogenetic relationships between lineages of interest are unresolved (Leander 2008).

The exploration of gregarine diversity using molecular phylogenetic data has established the cellular identities of several different environmental sequence clades within the Apicomplexa (Leander and Ramey 2006; Rueckert et al. 2011a, b). The lifecycle of marine gregarine apicomplexans includes a cyst stage that, in marine environments, is dispersed in the sediment and eventually ingested by a new individual host (Leander 2008; Vivier and Desportes 1990). The amplification of DNA sequences in environmental PCR surveys suggest that the cysts of marine gregarines are prevalent in marine sediments and show that the extreme divergence of some gregarine SSU rDNA sequences make them difficult to analyze (Cavalier-Smith 2004; Leander 2007; Leander and Ramey 2006; Takishita et al. 2005, 2007a, b). A study of gregarines isolated from the intestines of crustaceans, for instance, demonstrated that the highly divergent SSU rDNA sequences from these particular species were only identifiable in PCR surveys after establishing direct links between the DNA sequences and other cellular traits (e.g., trophozoite morphology). Therefore, species discovery surveys that aim to characterize novel organisms

using culture-independent methods to acquire data at both morphological and molecular levels provide the necessary context for identifying clades of ambiguous environmental sequences.

Like most groups of microbial eukaryotes (protists), gregarine parasites are particularly prone to having a low degree of morphological diversity between different species and a high degree of morphological plasticity within species (Rueckert et al. 2011b); moreover, like other parasites, gregarines have different life cycle stages that vary considerably at the morphological level (e.g., gametocysts, oocysts, and the developmental stages between sporozoites and trophozoites). Therefore, using extensive and tedious morphometric measurements to delimit one gregarine species from another is not only inadvisably time consuming for delimiting one species from another but also largely impenetrable, impractical, and misleading.

In this study, we discover and characterize the morphology and molecular phylogenetic markers of four novel species of Pacific marine gregarines isolated from the intestines of two capitellid polychaetes, *Notomastus tenuis* and *Heteromastus filiformis*. These combined data enabled us to establish a new genus of marine gregarines that provides the cellular identity of a clade of SSU rDNA environmental sequences isolated from various marine environments around the globe.

Materials and methods

Collection of organisms

The capitellid polychaete, *Notomastus tenuis* Moore, 1909, was collected at low tide from Boundary Bay, Tsawwassen (Vancouver), British Columbia, Canada in August 2011. A second capitellid polychaete, *Heteromastus filiformis* Claparède, 1864, was collected from the rocky intertidal area at Jericho Beach, Vancouver, British Columbia, Canada, in September 2011. No specific permits were required for the collection of worms in these field sites. Tide levels were estimated and acquired through Fisheries and Oceans Canada. Host material was transported and kept in chilled seawater prior to dissection. All dissections were completed within 24 h of collection. No fixatives were used either during dissections or while taking photographs of the trophozoites. Three different morphotypes of gregarine trophozoites (*Polyplacarium lacrimae* n. gen. et sp., *P. curvarae* n. gen. et sp., and *P. translucidae* n. gen. et sp.) were collected from the intestines of *N. tenuis*; one distinct morphotype of a gregarine trophozoite (*P. citruruae* n. gen. et sp.) was collected from the intestines of *H. filiformis*.

Light microscopy and single-cell DNA extraction, amplification and sequencing

Hand-drawn glass pipettes were used to collect individual trophozoites representing four distinct morphotypes, using an inverted microscope (Zeiss Axiovert 200; Carl-Zeiss, Göttingen, Germany). Four single-cell (SC) isolates were collected from each of the four distinct morphotypes (a total of 16 SC isolates) and prepared for light microscopy and DNA extraction. The SC isolates were washed three times (until clean) in chilled, autoclaved seawater and photographed either on glass slides with a Leica DC 500 color camera connected to a Zeiss Axioplan 2 microscope (Carl-Zeiss) or on well-slides with a Pixelink Megapixel color digital camera (PL-A662-KIT; Ottawa, Canada) connected to an inverted Zeiss Axiovert 200 microscope (Carl-Zeiss). Each of the SC isolates was then placed in a 1.5-ml Eppendorf tube containing cell lysis buffer. Genomic DNA was extracted with the standard protocol provided by the MasterPure complete DNA & RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). However, the final elution step was lowered to 4 μ l with the goal of concentrating extracted DNA prior to PCR amplification.

Sixteen novel SSU rDNA sequences were generated by nested PCR with primers specific for the gregarine parasite species (Table 1). Initially, outside primers PF1 and SSUR4 (Leander et al. 2003) were used in a 25- μ l PCR reaction with EconoTaq 2X Master Mix (Lucigen, Middleton, WI, USA). The following program was used on the thermocycler for the initial amplification: initial denaturation at 94 °C for 2:00 min; 35 cycles of denature at 94 °C for 0:30 s, anneal at 52 °C for 0:30 s, extension at 72 °C for 1:50 m., final extension 72 °C 9:00 m. Subsequently, a pair of internal primers, F1 and R2 (Table 1), were used in a nested PCR with 1 μ l of template DNA generated from the first PCR reaction in order to amplify a 1,000-bp region of the SSU rRNA gene using the following program on a thermocycler: initial denaturation for 94 °C for 2:00 min; 25 cycles of denature at 94 °C for 0:30 s., anneal at 51 °C for 0:30 s.,

extension at 72 °C for 1:20 min; final extension at 72 °C for 9:00 min.

From the initial sequences, specific primers were then designed and paired with outside (universal eukaryotic) primers (e.g., PF1-PlacrimaeR and PlacrimaeF-SSUR4) in semi-nested PCR reactions in order to attain the final SSU rDNA sequences (1,650–1,800 bp) (Table 1). All PCR products were separated on agarose gels and isolated using the UltraClean15 DNA Purification Kit (MO BIO, Laboratories, Carlsbad, CA, USA). All sequencing reactions were performed using ABI big dye reaction mix with appropriate primers (Table 1). Novel sequences were initially identified using the National Center for Biotechnology Information's (NCBI) BLAST tool and confirmed with molecular phylogenetic analyses. All unique sequences generated in this study were deposited in GenBank (Accession numbers JX535336–JX535351).

Scanning electron microscopy

Between 20 and 65 individual trophozoites representing each morphotype were pooled in 2 % glutaraldehyde in seawater on ice. A 10- μ l polycarbonate membrane filter was placed within a Swinnex filter holder (Millipore, Billerica, MA, USA). Trophozoites were then collected with a hand-drawn glass pipette and placed in the filter holder, which was then placed in a small beaker (4 cm diam. and 5 cm tall) that was filled with 2 % glutaraldehyde in seawater. Ten drops of 1 % OsO₄ were added to the opening of the filter holder, and the samples were post-fixed on ice for 30 min. A syringe was used to slowly run distilled water over all samples. A graded series of ethanol washes (30, 50, 75, 85, 95, and 100 %) was then used to dehydrate the fixed cells using the syringe system. Following dehydration, the polycarbonate membrane filters containing the trophozoites were transferred from the Swinnex filter holders into an aluminum basket submerged in 100 % ethanol in preparation for critical point drying with CO₂. The dried polycarbonate membrane filters containing the trophozoites were mounted on aluminum stubs, sputter coated with 5 nm gold

Table 1 Primers designed in this study to amplify small subunit rDNA sequences; the annealing regions refer to one of the sequences derived from *Polyplacarium lacrimae* n. gen. et sp. (GenBank accession no. JX535336)

Primer name	Direction	Sequence 5'–3'	Annealing region
F1	Forward	5'-GATTAAGCCATGCATGTCTAAG-3'	47 to 70
<i>P. lacrimae</i> F	Forward	5'-CGTTTCTACGATTATCAATTGG-3'	486 to 508
<i>P. curvarae</i> F	Forward	5'-CGTTTCTATGAGTACCCATTGG-3'	486 to 508
<i>P. translucidae</i> F	Forward	5'-CGTTTCTACGATTACCCATTGG-3'	486 to 508
<i>P. citrusae</i> F	Forward	5'-CTTTCTACGAGTACCAATTGG-3'	486 to 508
R1	Reverse	5'-CGGTGTGTACAAACGGCAGGGAC-3'	1762 to 1740
<i>P. lacrimae</i> R	Reverse	5'-CTGACAGGGCCGAGGTCCTATCG-3'	671 to 648
<i>P. curvarae</i> R	Reverse	5'-CGGATAAGACGGAAGTCCTATCG-3'	671 to 648
<i>P. translucidae</i> R	Reverse	5'-GGGATAGGACGGAAGTCCTATAG-3'	671 to 648

and viewed under a Hitachi S4700 scanning electron microscope (Nissei Sangyo America, Pleasanton, CA, USA). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA).

Phylogenetic analyses of molecular sequences

Two separate phylogenetic analyses were conducted in this study. A comprehensive 79-taxon dataset contained a representative SSU rDNA sequence from each of the four novel morphotypes described here: five closely related environmental DNA sequences, three dinoflagellate sequences (outgroup), and 67 sequences representing major clades of gregarines and other apicomplexans. The 79-taxon alignment was visually fine-tuned using MacClade 4 (Maddison and Maddison 2000); gaps and ambiguously aligned regions were excluded resulting in 1,007 unambiguously aligned sites. JModeltest (Guindon and Gascuel 2003; Posada and Crandall 1998) selected a GTR+I+ Γ model of evolution under AIC and AICc (proportion of invariable sites=0.1280, gamma shape=0.5250). Garli-GUI (Zwickl 2006) was used to generate a maximum likelihood (ML) tree, and ML bootstrap analysis (100 pseudoreplicates, one heuristic search per pseudoreplicate).

Bayesian posterior probabilities were calculated for the larger dataset using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We set our program for four Monte Carlo Markov Chains starting from a random tree (MCMC; default temperature=0.2), a gamma distribution and stop rule of 0.01 (i.e. when the average split deviation fell below 0.01, the program would terminate). A sum of 5,000,000 generations was calculated. Trees were sampled every 100 generations, with a prior burn-in of 500,000 generations. Burn-in was confirmed manually, and a majority-rule consensus tree was constructed. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

A more restricted phylogenetic analysis focused on the interrelationships between the 16 SSU rDNA sequences generated from four different single-cell isolates from each of the four different morphotypes described here; the alignment also contained the five closely related environmental DNA sequences identified in GenBank. This 21-taxon alignment was visually fine-tuned with MacClade 4, excluded gaps and ambiguous sites, and contained 1,407 sites. An unrooted ML tree and ML Bootstrap percent values were calculated using Garli-GUI under a GTR+I+ Γ model of evolution selected by JModeltest (proportion of invariable sites=0.4970, gamma shape=0.5830). Like the larger 79-taxon dataset, posterior probabilities were calculated using MrBayes 3.1.2. using the same criteria described previously. A sum of 700,000 generations was calculated with a prior

burn-in of 70,000 generations. PAUP 4.0 (Swofford 1999) was used to calculate percent differences between the 16 novel SSU rDNA sequences generated in this study.

Results

Morphological traits of the four new species

Polyplacarium lacrimae n. gen et sp. The trophozoites were teardrop-shaped (Fig. 1a–e). The anterior end of the cell was bulbous, having an average width of 54 μ m (range 38–66 μ m, $n=42$) at its widest part. The average length of the cell was 197 μ m (range 183–207 μ m, $n=42$). Cells appeared dark-brown from large amounts of amylopectin. The nucleus was circular to ovoid (17–22 μ m \times 15–21 μ m, $n=17$) and located in the central part of the bulbous anterior of the cell (Fig. 1a–e). The anterior end tapered slightly toward a blunt and otherwise inconspicuous mucron. The posterior end tapered to a point (Fig. 1a–e). Longitudinal epicytic folds covered the surface of the cell with a density of 4–5/ μ m (Fig. 1f, g). Gliding motility was present. A distinct region of shallow epicytic folds was observed on the surface of cells examined under SEM (Fig. 1g, h). This region was observed in two out of the six samples recovered for viewing.

Polyplacarium curvarae n. gen. et sp. The trophozoites were slightly curved, 156 μ m long (range 98–167 μ m, $n=47$) and 42 μ m wide (range 34–53 μ m, $n=47$) (Fig. 2a–e). The nucleus was ovoid (16–21 μ m \times 10–12 μ m, $n=21$) and located in the posterior region of the cell. A conspicuous mucron was visible in cells under light microscopy (Fig. 2d); otherwise, the mucron appeared flat and inconspicuous (Fig. 2a–c). Cells appeared brown from the accumulation of amylopectin. SEMs demonstrated longitudinal epicytic folds covering the cell surface with a density of 4–5/ μ m (Fig. 2f). The posterior end of the trophozoites tapered slightly to a blunt and slightly compressed end (Fig. 2a–e). The trophozoites were capable of gliding motility. The surface of the cell contained a distinct region of 12–17 shallow epicytic folds that were 2–3 μ m wide and taller than the other epicytic folds (Fig. 2h). This pattern of epicytic folds was observed in 11 out of 20 cells observed under SEM.

Polyplacarium translucidae n. gen. et sp. Trophozoites were 163 μ m long (range 112–183 μ m, $n=59$) and 27 μ m wide (range 25–32 μ m, $n=59$) (Fig. 3a–e). The nucleus was circular to ovoid (12–15 μ m \times 7–13 μ m) and located in the middle to posterior end of the cell. The trophozoites were capable of gliding motility. The cells appeared translucent under light microscopy, having a low accumulation of

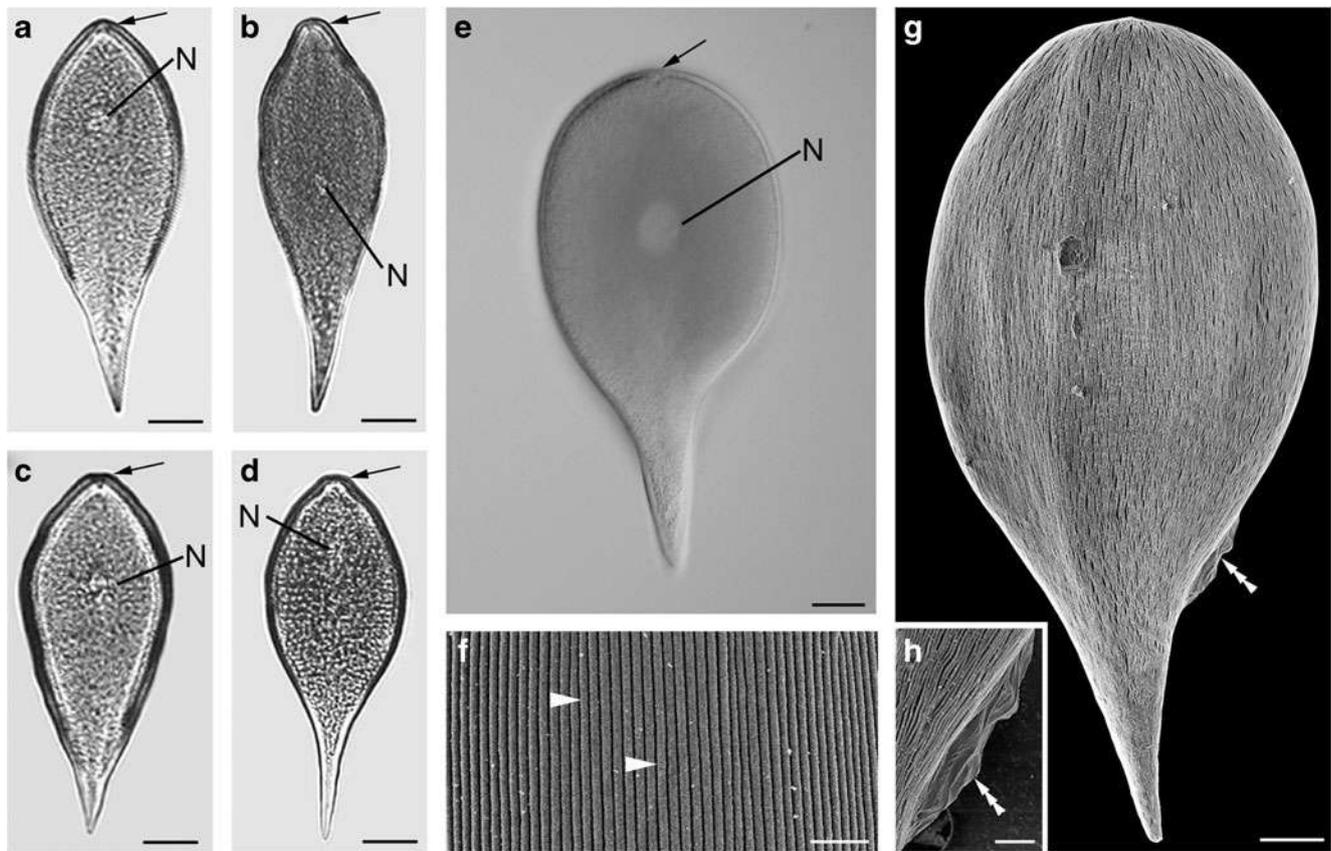


Fig. 1 Light micrographs (LM) and scanning electron micrographs (SEM) showing the trophozoite morphology and surface features of *Polyplacarium lacrimae* n. gen. et sp. **a–d** The LMs were taken of individual cells on a well-slide just before they were removed for DNA extraction and single-cell PCR. Individual trophozoites with a blunt mucron (arrow) and bulbous anterior containing a centrally located nucleus (N). The posterior region tapers to a point. **e** LM showing the general morphology of *P. lacrimae*, the inconspicuous mucron (arrow),

centrally located nucleus (N), and a pointed posterior end. **f** High-magnification SEM of the cell surface of *P. lacrimae* showing the epicytic folds (arrowhead). **g** SEM showing the general morphology, the inconspicuous mucron (arrow), the pointed posterior end of the cell, and the distinct region of wider folds on a trophozoite (triple-arrowhead). **h** High-magnification SEM of the distinct region of wider folds (triple-arrowhead) on trophozoites. Scale bars (a–d) 20.0 μm , (e) 25 μm , (f) 1 μm , (g) 15 μm , (h) 1.5 μm

amylopectin granules. The posterior end was slightly tapered and sometimes tapered to a nipple-like point (Fig. 3a–e). Longitudinal epicytic folds covering the surface had a density of 4–5/ μm (Fig. 3f, g). A distinct region of 10–13 shallow epicytic folds was observed on the surface of the cell (Fig. 3f, g). This pattern of epicytic folds was observed in 17 out of 30 cells observed under SEM.

Polyplacarium citrusae n. gen et sp. The trophozoites were extremely flat, ovoid to lemon-shaped, 47 μm long (range 39–53, $n=49$) and 32 μm wide (range 28–42, $n=49$) (Fig. 4a–d). The circular to ovoid nucleus (4–7 $\mu\text{m} \times 4$ –8 μm , $n=15$) was located in the central part of the cell. Cells appeared translucent to light-brown, depending on the amount of amylopectin granules present within the cell. Both the anterior and posterior ends were slightly tapered. The trophozoites were capable of gliding motility. The anterior end was differentiated from the posterior end

mainly by observing the direction of “forward” gliding motility. Syzygy was side-to-side (Fig. 4e). The cell surface was covered with longitudinal epicytic folds with a density of 4/ μm (Fig. 4e, f). No distinct region of shallow epicytic folds was present on the 32 cells observed under SEM.

Molecular phylogenetic analyses of SSU rDNA sequences

Our phylogenetic analysis of the 79-taxon dataset recovered a clade of apicomplexans with moderate support. Our analyses also recovered groups of coccidians, piroplasmids, rhytidocystids, cryptosporidians, and terrestrial gregarines, ranging in support from moderate to robust (Fig. 5). Four major subgroups of marine gregarines were recovered in this analysis: crustacean gregarines, leucinids clade I, leucinids clade II plus urosporids, and the novel clade established here from capitellid hosts (Fig. 5). Species of *Selenidium*

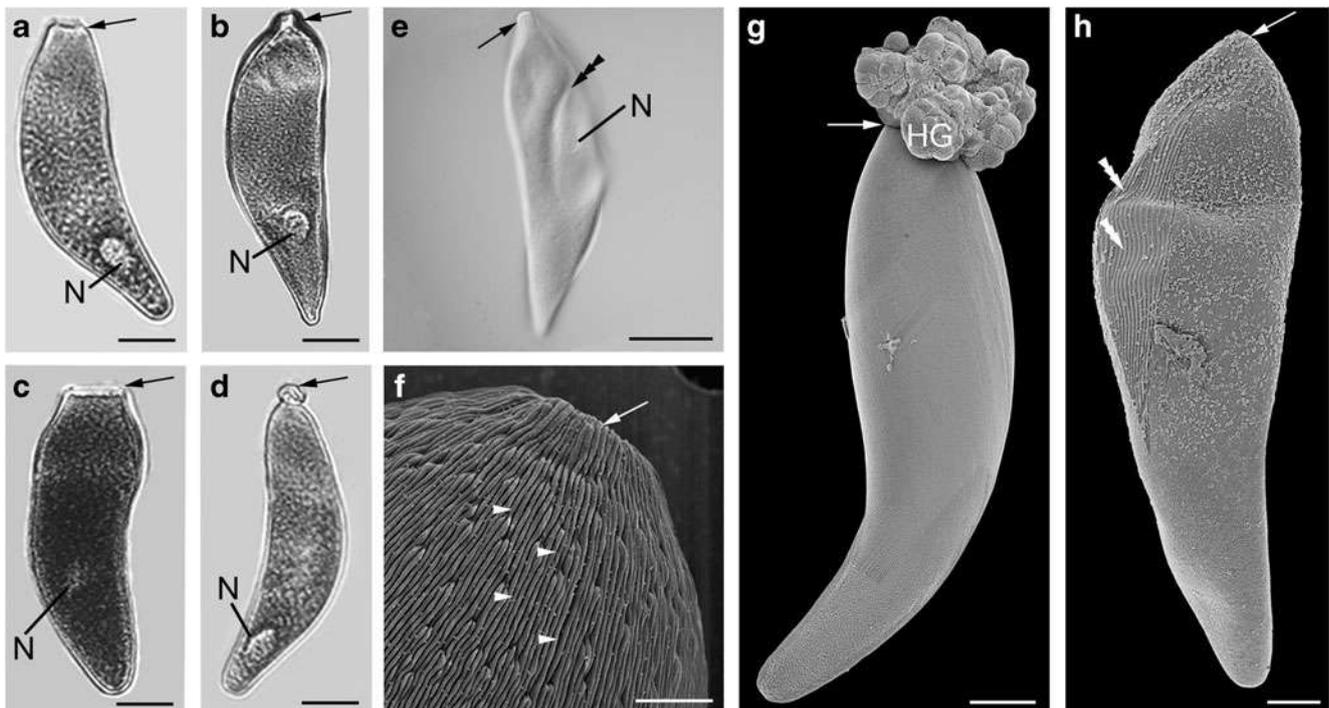


Fig. 2 Light micrographs (LM) and scanning electron micrographs (SEM) showing the trophozoite morphology and surface features of *Polyplacarium curvarae* n. gen. et sp. **a–d** The LMs were taken of individual cells on a well-slide just before they were removed for DNA extraction and single-cell PCR. General morphology of cup-like (**a–b**) or projected (**d**) mucrons (*arrow*). The nucleus (*N*) is located in the posterior or middle-posterior part of the cell. The posterior end tapers slightly to a blunt end. **e** LM showing the general morphology of a trophozoite with the mucron (*arrow*), nucleus (*N*), and a distinct fold

(*triple arrowhead*) in the center of the trophozoite. **f** High-magnification SEM showing the mucron (*arrow*) and dense epicytic folds (*arrowhead*) on surface of the cell. **g** SEM of a trophozoite attached to host gut (*HG*) material. The interface between the host gut and trophozoite is marked by an *arrow*. The posterior region of the cell is blunt and slightly compressed. **h** SEM showing the general morphology of the cell, the mucron (*arrow*), and a blunt posterior end. A distinct region of wider folds (*triple-arrowhead*) was observed on the cell surface. Scale bars (**a–d**) 30 μm , (**e**) 30 μm , (**f**) 3.5 μm , **g**, **h**) 10 μm

(i.e., archigregarines) branched from the unresolved apicomplexan backbone as three separate lineages. The four novel sequences representing *P. lacrimae*, *P. curvarae*, *P. translucidae*, and *P. citrusae* formed a well-supported clade with five environmental sequences of previously unknown origin within the Apicomplexa (AY179976, EF100216, EF100199, AB275013, and AY179975) (Fig. 5).

Molecular phylogenetic analyses with the 21-taxon dataset containing five environmental sequences and the 16 new SSU rDNA sequences (i.e., four from single-cell isolates of each of the four species in this study) are shown in Fig. 6. The analysis recovered four distinct, well-supported clades that represented the single-cell isolates from each species described in this study. A fifth clade consisted of four environmental sequences (AB275013, EF100199, EF100216, and AY179976), and environmental sequence AY179975 branched as the sister lineage to *P. citrusae* with strong statistical support (Fig. 6).

Intraspecific variation of the four SSU rDNA sequences generated from each of the four species ranged from 0.98 to 2.12 % (*P. lacrimae*), 0.22 to 1.46 % (*P. curvarae*), 0.68 to 1.73 % (*P. translucidae*), and 0.82 to 1.35 % (*P. citrusae*) (Table 2). Interspecific variation between isolates of *P.*

lacrimae, *P. curvarae*, *P. translucidae*, and *P. citrusae* ranged from 7.42 to 14.98 % (Table 2).

Formal taxonomic descriptions

Apicomplexa Levine, 1970

Gregarinea Bütschli, 1882, stat. nov. Grassé, 1953

Eugregarinorida Léger, 1900

Polyplacarium n. gen. Wakeman and Leander

Description Ovoid to elongate trophozoites with a blunt mucron. The posterior end is either blunt or tapers to a point. Longitudinal epicytic folds with density of 4–5/ μm ; most trophozoites also have a distinct region of wider, shallower epicytic folds. Gliding motility. The genus name, *Polyplacarium*, is latin, translates to “many folds”, and refers to the high density of epicytic folds on the surface of the trophozoite stages.

Type species *Polyplacarium lacrimae* Wakeman and Leander.

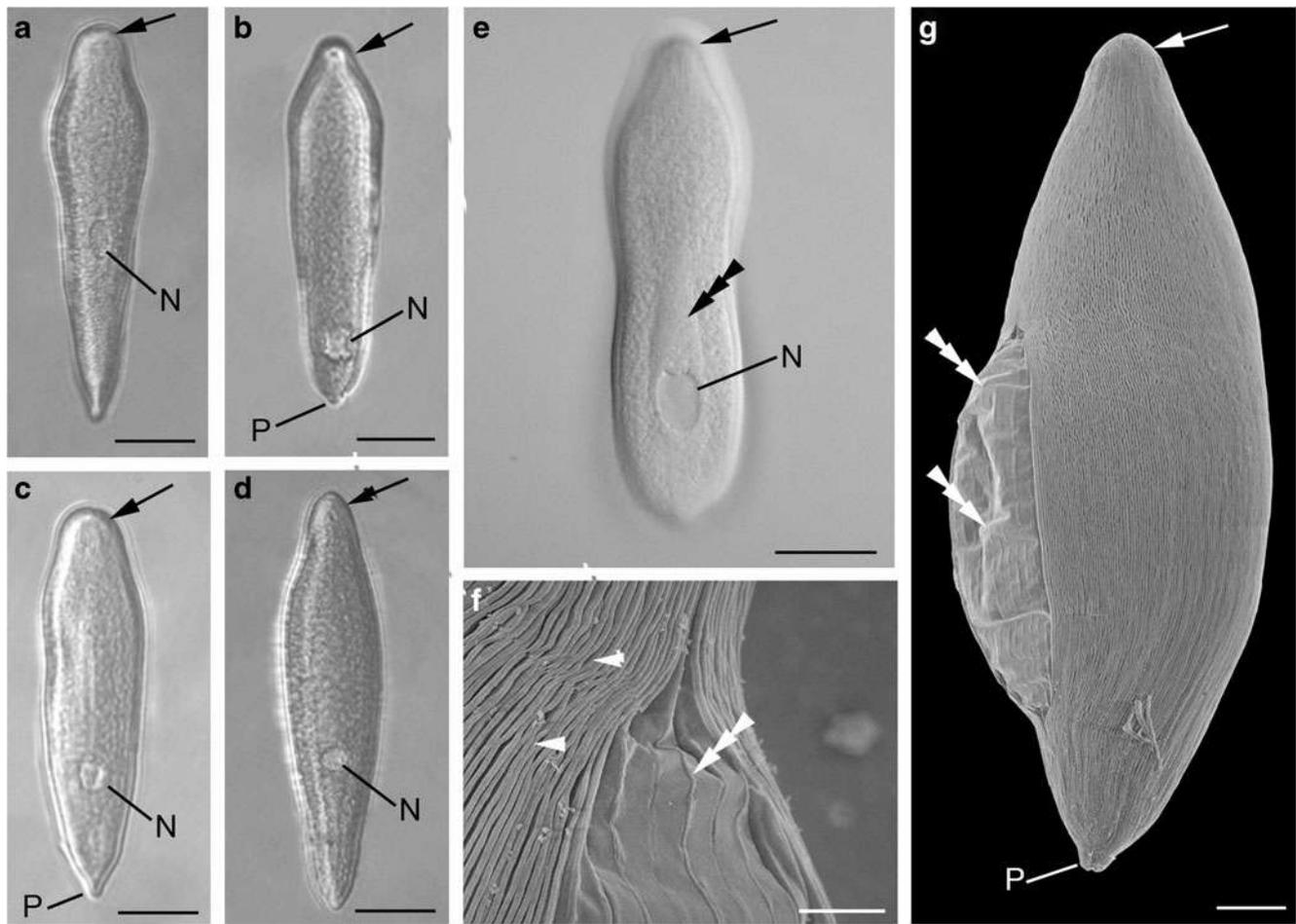


Fig. 3 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the trophozoite morphology and surface features of *Polyplacarium translucidum* n. gen. et sp. **a–d** The LMs were taken of individual cells on a well-slide just before they were removed for DNA extraction and single-cell PCR. Trophozoites have a posterior nucleus (*N*), a rounded mucron (*arrow*), and a posterior end that tapers slightly to a nipple-like point (*P*) (**b–c**). **e**

LM showing the general cell morphology, the mucron (*arrow*), the posterior nucleus (*N*), and a region of wider folds (*triple arrowhead*). **f** High-magnification SEM showing the dense epicytic folds (*arrowhead*) and a region of wider folds (*triple arrowhead*) on the cell surface. **h** SEM showing the mucron (*arrow*), the nipple-like posterior end of the cell, and the distinct region of wider folds (*triple-arrowhead*). Scale bars (**a–e**) 25 μm , (**f**) 2.0 μm , (**g**) 10 μm

Polyplacarium lacrimae n. sp. Wakeman and Leander

Description Trophozoites teardrop-shaped with a bulbous mucron. Average length and width, at the widest part, is 197 μm and 54 μm , respectively. Cells dark-brown. The posterior end tapered to a point; anterior end tapered slightly toward a blunt, inconspicuous mucron. Nucleus is circular to ovoid (17–22 μm \times 15–21 μm) and located in the anterior or central part of the cell. Gliding motility. Longitudinal epicytic folds with a density of 4–5/ μm . A distinct region of wider, shallower epicytic folds may be present on the surface of trophozoite stages.

DNA sequence SSU rRNA gene sequence (GenBank JX535336).

Type locality Boundary Bay (49°00′54.88″N, 123°02′12.72″W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sea level.

Type habitat Marine.

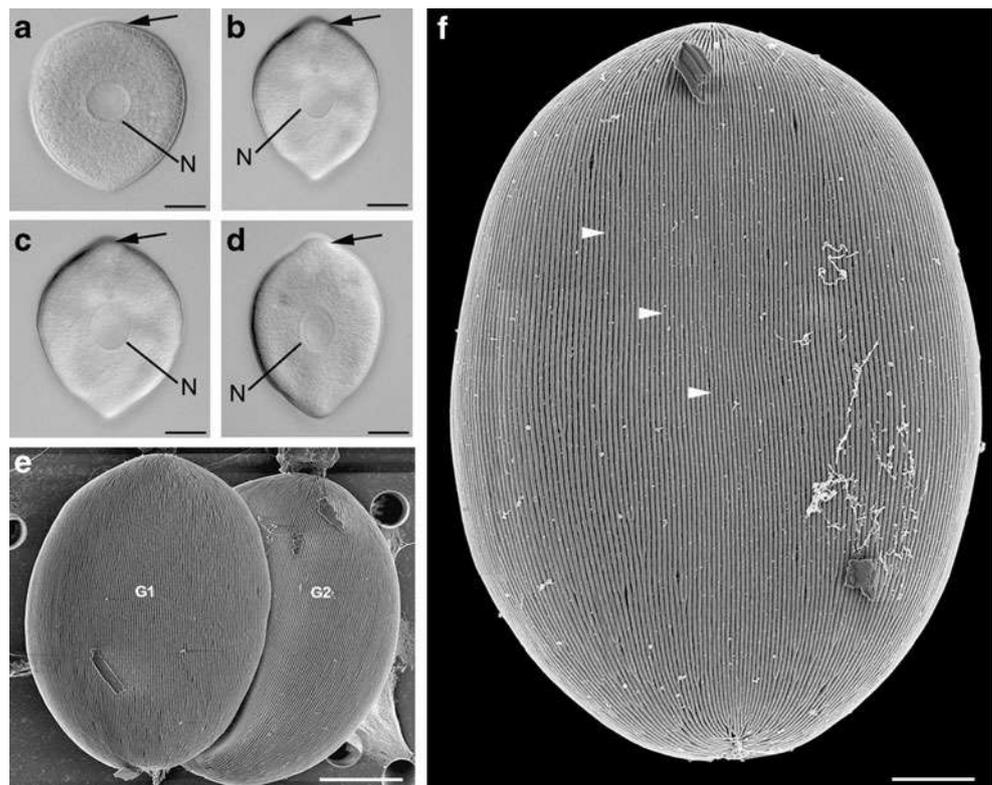
Type host *Notomastus tenuis* Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

Location in host Intestinal lumen.

Iconotype Figure 1g.

Hapantotype Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum

Fig. 4 Differential interference contrast light micrographs (LM) and scanning electron micrographs (SEM) showing the trophozoite morphology and surface features of *Polyplacium citrusae* n. gen. et sp. **a–d** The LMs were taken of individual cells just before they were removed for DNA extraction and single-cell PCR. Individual trophozoites were extremely flattened and had a centrally located nucleus (*N*). The mucron (*arrow*) was identified based on the forward direction of movement. **e** SEM showing two gamonts (*G1* and *G2*) in side-to-side syzygy and epicytic folds running along the longitudinal axis of the cell. **f** SEM of a single trophozoite showing the epicytic folds (*arrowhead*). The anterior and posterior ends of these cells was difficult to distinguish under SEM. Scale bars (**a–e**) 10 μm , (**f**) 5 μm



(Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR117.

Etymology The species name, *lacrimae*, stems from Latin meaning “tear” and refers to the “teardrop-shape” of the trophozoite stage.

Polyplacium curvarae n. sp., Wakeman and Leander

Description Trophozoites slightly curved with an average length and width of 156 μm and 42 μm , respectively. Cells brown. The posterior end of trophozoite slightly tapered and compressed. Ovoid nucleus located in the posterior region of the cell. Mucron usually flat and inconspicuous but sometimes pointed. Gliding motility. Longitudinal epicytic folds with a density of 4–5/ μm . A distinct region of 15–17 wider and shallower epicytic folds present on the trophozoite surface.

DNA sequence SSU rRNA gene sequence (GenBank JX535340).

Type locality Boundary Bay (49°00′54.88″N, 123°02′12.72″W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sea level.

Type habitat Marine.

Type host *Notomastus tenuis* Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

Location in host Intestinal lumen.

Iconotype Figure 2h.

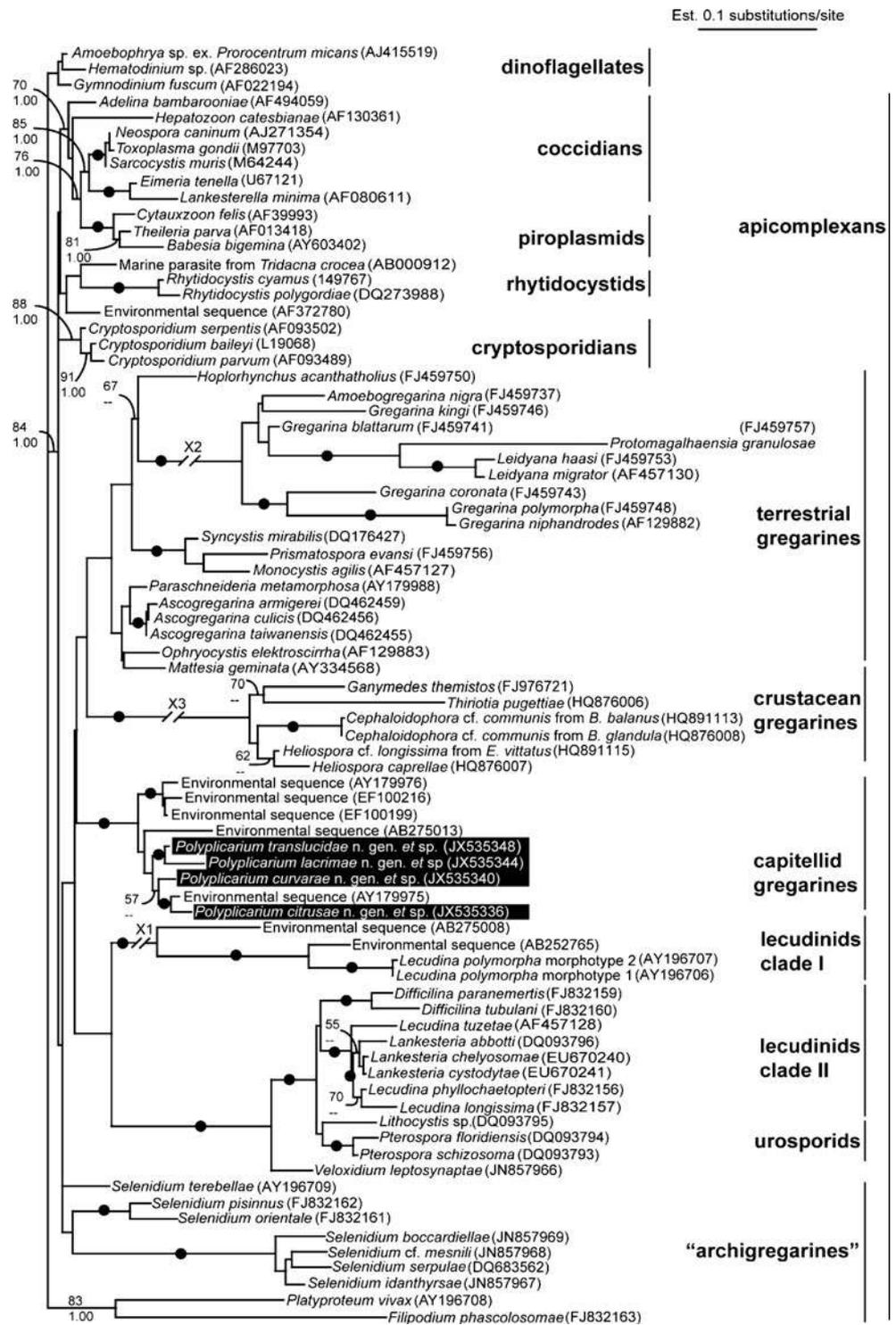
Hapantotype Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR118.

Etymology The species name, *curvarae*, stems from Latin meaning “curved” and refers to the curved cell shape of the trophozoites stage.

Polyplacium translucidae n. sp., Wakeman and Leander

Description Elongated trophozoites 163 μm long and 27 μm wide on average. The circular to ovoid nucleus was located in the posterior half of the cell. The posterior end tapered to a nipple-like tip. Gliding motility. Trophozoites with an inconspicuous mucron and distinctively translucent under light microscopy. Longitudinal epicytic folds with a density of 4–5/ μm over the trophozoite surface. A distinct swelling of 6–10 wider epicytic folds was present on one side of the trophozoite surface.

Fig. 5 Maximum likelihood (ML) tree based on 1,007 unambiguously aligned sites from 79 SSU rDNA sequences using the GTR+I+ Γ substitution model ($-\ln L=168,00.87694$, gamma shape=0.5250, proportion of invariable sites=0.1280). Bootstrap supports are given at the top of branches, and Bayesian posterior probabilities are given at the bottom. *Black dots* on branches represent bootstrap support values and Bayesian posterior probability 95/0.99 or greater. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree. Representative sequences from the four novel species described in this study are highlighted in *black boxes*



DNA sequence SSU rDNA gene sequence (GenBank JX535344).

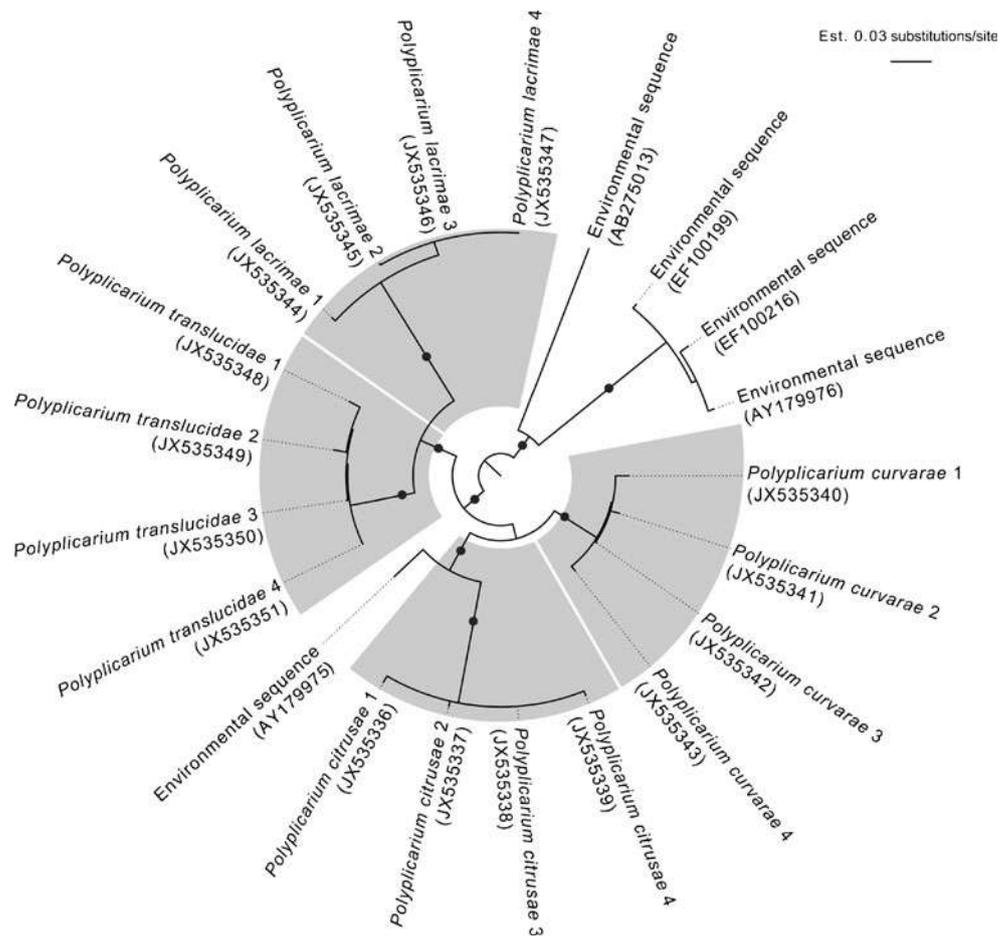
Type locality Boundary Bay (49°00'54.88"N, 123°02'12.72" W), Tsawwassen (Vancouver), British Columbia, Canada. Host in sand; upper intertidal; 0.30 m above mean sealevel.

Type habitat Marine.

Type host *Notomastus tenuis* Moore, 1909 (Annelida, Polychaeta, Scolecida, Capitellidae).

Location in host Intestinal lumen.

Fig. 6 Unrooted maximum likelihood (ML) tree of four single-cell isolates from each of the four novel species of *Polyplicarium* n. gen. described in this study, as well as five closely related environmental sequences. This tree is based on 1,407 unambiguously aligned sites from 21 SSU rDNA sequences using the GTR+I+ Γ substitution model ($-\ln L=16,800.87694$, gamma shape=0.5830, proportion of invariable sites=0.4970). Bootstrap supports are given at the top of branches, and Bayesian posterior probabilities are given at the bottom. *Black dots* on branches represent bootstrap support values and Bayesian posterior probability 95/0.99 or greater. Bootstrap and Bayesian values less than 55 and 0.95, respectively, were not added to this tree



Iconotype Figure 3g.

Hapantotype Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR119.

Etymology The species name, *translucidae*, stems from Latin meaning “transparent”, and refers to the “see-through” quality of the trophozoites stages of this species.

Table 2 Summary of intraspecific divergences (along the diagonal) and interspecific divergences of small subunit rDNA sequences generated from four isolates of each of the four species of

	<i>Polyplicarium lacrimae</i> n. gen. et sp.	<i>P. curvarae</i> n. gen. et sp.	<i>P. translucidae</i> n. gen. et sp.	<i>P. citrusae</i> n. gen. et sp.
<i>Polyplicarium lacrimae</i> n. gen. et sp.	0.98–2.12 %	–	–	–
<i>P. curvarae</i> n. gen. et sp.	10.67–12.53 %	0.22–1.46 %	–	–
<i>P. translucidae</i> n. gen. et sp.	9.39–11.10 %	7.42–9.02 %	0.68–1.73 %	–
<i>P. citrusae</i> n. gen. et sp.	13.21–14.98 %	8.58–10.07 %	10.17–12.09 %	0.82–1.35 %

Polyplicarium citrusae n. sp., Wakeman and Leander

Description Trophozoites of *P. citrusae* extremely flattened and lemon-shaped with an average length and width of 47 and 32 μm , respectively. Trophozoites translucent to light-brown under light microscopy. Circular nucleus positioned in the center of trophozoites. Gliding motility. The anterior mucron region was inconspicuous and difficult to distinguish from the posterior end, without observing the direction of gliding motility. The cell surface was covered longitudinal epicytic folds with a density of 4/ μm . Syzygy side-to-side.

Polyplicarium n. gen. described in this study; percent divergences are based on comparisons of 1,678 nucleotides.

DNA sequence SSU rRNA gene sequence (GenBank JX535348).

Type locality Jericho Beach (49°16'24.39"N, 123°11'07.18" W), Point Grey (Vancouver), British Columbia, Canada. Host in black sediment; mid-low intertidal; 0.80 m below mean sea level.

Type habitat Marine.

Type host *Heteromastus filiformis* Eisig, 1887 (Annalida, Polychaeta, Scolecida, Capitellidae).

Location in host Intestinal lumen.

Iconotype Figure 4f.

Hapantotype Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada. Museum Code – MI-PR120.

Etymology The species name, *citrusae*, stems from Latin meaning “citrus”, and refers to the general “lemon-shape” of the trophozoites and gamonts.

Discussion

Species of marine gregarines have been established using a wide variety of criteria, including hosts affinity, geographical

distribution, detailed morphological dimensions of different life history stages, ultrastructural patterns on the surface of trophozoites, and, more recently, SSU rDNA sequence variability (Leander et al. 2003; Rueckert and Leander 2008; Rueckert et al. 2010, 2011a, b). Traditionally, new species were justified mainly on just one of these criteria (e.g., detailed morphological dimensions of different life history stages). More recent studies suggest that one criterion alone is inadequate to convincingly delimit different species of marine gregarines from one another (Rueckert et al. 2011a, b). For instance, the SSU rDNA sequences from very different morphotypes of *Lecudina polymorpha* were over 99 % identical (Leander et al. 2003; Rueckert et al. 2010). By contrast, the SSU rDNA sequences from morphologically similar gregarines isolated from two different species of nemerteans differed by 14.1 %, demonstrating two different species of *Difficilina* that were correlated with two different host species (Rueckert et al. 2010). A similar study described two different species of *Lankesteria* isolated from two separate species of tunicates (Rueckert and Leander 2008). Because the SSU rDNA sequences from these two species differed only by 2.1–3.1 %, the separation of the two species was also based on morphological variation (e.g., *L. chelyosomae* was over 10 times larger than *L. cystodytae*), different host affinities, and the fact that multiple isolates from each species clustered into two distinct clades (Rueckert and Leander 2008). Another study addressed the SSU rDNA sequence variation in several different morphotypes of *Lecudina* cf. *tuzetae* isolated from different hosts collected in two different geographical regions (Rueckert et al. 2011b). The range of variation in these sequences was 0.0–3.9 %; however, the sequences did not cluster into clades according to morphotype,

Table 3 Comparative morphology of the four new species of *Polyplacarium* n. gen. described in this study

	<i>Polyplacarium lacrimae</i> n. gen. et sp. (type species)	<i>P. curvarae</i> n. gen. et sp.	<i>P. translucidae</i> n. gen. et sp.	<i>P. citrusae</i> n. gen. et sp.
Host	<i>Notomastus tenuis</i>	<i>Notomastus tenuis</i>	<i>Notomastus tenuis</i>	<i>Heteromastus filiformis</i>
Host tissue	Intestines	Intestines	Intestines	Intestines
Locality	E. Pacific	E. Pacific	E. Pacific	E. Pacific
Trophozoite shape	Elongate, bulbous anterior, posterior end tapered to point	Elongate, cylindrical, posterior end slightly compressed	Elongate, compressed, posterior blunt	Round to ovoid, highly flattened
Trophozoite size				
L × W, μm	183–207 × 38–66	98–167 × 34–53	112–183 × 25–32	39–53 × 28–42
Nucleus shape	Ovoid	Ovoid	Ovoid	Ovoid
Nucleus size				
L × W, μm	17–22 × 15–21	16 × 21 × 10–12	12–15 × 7–13	4–7 × 4–8
Position of nucleus	Middle anterior	Middle posterior	Middle posterior	Middle
Motility	Gliding motility	Gliding motility	Gliding motility	Gliding motility
Density of epicytic folds	4–5/μm	4–5/μm	4–5/μm	4/μm
Shape of mucron	Simple, blunt	Simple, blunt	Simple, blunt	Simple, blunt
Region with alternative fold pattern	Present	Present	Present	Absent

location or host, suggesting that the degree of variation found in this study was intraspecific. Taken together, these studies illustrate the importance of considering multiple criteria to justify the establishment of new species and genera (Leander et al. 2003; Rueckert et al. 2010, 2011a, b). Arguably, the most pragmatic approach for describing new species of gregarines is to consider host affinity, morphological features of the most conspicuous life history stage (usually trophozoites), and a widely sampled molecular marker with sufficient interspecific variation (e.g., SSU rDNA sequences) using a single-cell PCR approach (Rueckert et al. 2011b).

A recently published list of “6 fundamental principles” for understanding species boundaries is a great testament to the shortcomings of delimiting gregarine species based on detailed morphometric data alone (Clopton 2012). According to Clopton (2012), gregarine systematics must include: (1) an ability to sort out mature life stages from immature life stages and all developmental stages in between; (2) an ability to observe all lifecycle stages; (3) an ability to sort out sexually dimorphic gamonts, if present; (4) large sample sizes of morphometric details that reflect the entire population; (5) measurements of morphology that are free of osmotic and other preparation artifacts; and (6) comparable and detailed morphometric data of all other closely related species. These goals not only position idealism above realism but reflect a pre-DNA worldview extrapolated from research on gregarines living in cockroaches and other insects. In practice, marine gregarines are encountered only as trophozoites and often in very low numbers (fewer than 10 specimens in an individual host) within a small percentage of individual hosts, which are also difficult to encounter and collect (e.g., the hosts might have been collected on a research cruise that was a one-time opportunity). More importantly, the challenges emphasized within the principles listed by Clopton (2012) are needless and overcome by efficient and pragmatic DNA-based approaches to systematics, and the literature is rich in excellent studies that demonstrate this in a wide variety of organisms and a broad range of contexts (e.g., biogeography, cryptic lifecycle stages, and cryptic species). Although reciprocal reinforcement of molecular data and other traits (e.g., trophozoite features and host affiliations) provide the most compelling arguments for species discrimination, a viewpoint that insists on an absolute set of morphometric details in gregarine systematics simply stifles the enterprise and provides a stark counterexample for why DNA-based approaches to biogeography, the delimitation of species, and phylogenetic reconstruction have become so predominant in advancing our understanding of biodiversity, especially within the context of protists.

Justification for establishing the new genus and species

Nearly 1,700 species of gregarines have been formally described, often with only very limited morphological information; only a tiny fraction of these have been examined with molecular phylogenetic data (Leander 2008; Perkins et al. 2002; Levine 1971, 1976, 1977a, b, 1979). Molecular markers like SSU rDNA sequences that have been obtained from manually isolated trophozoites have been helpful in our understanding of gregarine species boundaries and phylogenetic relationships (Leander 2007; Leander et al. 2006; Rueckert and Leander 2008, 2009, 2010; Rueckert et al. 2010, 2011a, b; Wakeman and Leander 2012). Analyses of SSU rDNA sequences derived directly from known species have also been able to establish the cellular identities of ambiguous environmental DNA sequences that have accumulated from PCR surveys of biodiversity (Cavalier-Smith 2004; Rueckert et al. 2011a, b). Nonetheless, the absence of molecular data from the vast majority of described gregarine species severely constrains the comparative power needed to place newly discovered species within the context of known gregarine species.

In this study, we established four novel species within *Polyplacarium* n. gen. using comparative morphology and SSU rDNA sequences from four different single-cell isolates of each of the four different species. The trophozoite morphology of *Polyplacarium* species was most similar to the following genera within the Lecudinidae: *Hyperidion*, *Ancora*, *Ulvina*, *Zygosoma*, and *Lecudina*. The trophozoites in all of these genera have “simple mucrons” but differ from one another in several ways (Lee et al. 2000; Perkins et al. 2002). For instance, the trophozoites of *Hyperidion* are 175–350 μm long, contractile, have longitudinal folds with a density of about 2–3/ μm , and have a textured projection from the mucron (Lee et al. 2000; Mackinnon and Ray 1931). The trophozoites of *Polyplacarium* are shorter and almost twice as wide and have longitudinal folds with a density of 4–5/ μm . The trophozoites of *Ancora* have two–three lateral processes running from the mucron towards the posterior end (Hoshide 1998; Levine 1977a, b). Like *Polyplacarium*, two species of *Ancora* (*A. sagitatta* and *A. lutzi*) were isolated from a capitellid polychaete (*Capitella capitata*); however, *Polyplacarium* species do not have the lateral processes that define *Ancora* (Hasselmann 1918). The morphology of *Polyplacarium* is also different from *Ulvina* and *Zygosoma* (Lee et al. 2000; Mackinnon and Ray 1931). Unlike *Polyplacarium*, the trophozoites of *Ulvina* have an incomplete septum dividing the cell into a “pseudoprotomerite” and “pseudodeutomerite”, and the trophozoites/gamonts of *Zygosoma* are covered with nipple-like projections (Lee et al. 2000). *Lecudina* is the largest and most widely studied genus within the Lecudinidae (Clausen 1993; Hasselmann 1918; Leander et

al. 2003; Lee et al. 2000; Levine 1976). Several species of *Lecudina*, including the type species *L. pellucida*, have trophozoites with a density of epicytic folds in the range of 2–3/ μm (Leander et al. 2003; Rueckert et al. 2011a, b; Vivier 1968), which is about half the density of folds on the surface of trophozoites in *Polyplacarium*. Moreover, all species of *Lecudina* that have been examined with SSU rDNA sequences do not cluster with or within the *Polyplacarium* clade in molecular phylogenetic analyses.

Aside from the molecular phylogenetic data, the main features that delimit the trophozoites of *Polyplacarium* from other gregarine genera within the Lecudinidae are longitudinal epicytic folds with density of 4–5/ μm , a distinct region of wider epicytic folds (usually), and host affiliation. The four different species of *Polyplacarium* n. gen. described here can be distinguished from one another based on details of trophozoites morphology and SSU rDNA sequence variation (Tables 2, 3). Unlike the other species, the trophozoites of *P. lacrimae* have a bulging region in the anterior-middle region of the cell, a centrally located nucleus, and a posterior end that tapers to a distinct point. A previously described species by Lankester (1866), namely *Lecudina eunicae*, had a similar cell shape (boulbous anterior and a pointed posterior) and size (254 μm) to that of *P. lacrimae*. However, the species described by Lankester (1866) was isolated from a different host, *Eunice harassii*, and the original drawing of the trophozoite of *L. eunicae* shows a distinct bulbous anterior region that is about half the cell's total length. In contrast, the anterior region of the trophozoites of *P. lacrimae* appear to be about 2/3 the total length of the cell. The trophozoite stage and host affinity of *L. eunicae* is most similar to that of *Trichotokara eunicae*, recently described by Rueckert et al. (2012), and most likely represents a close relative those gregarines isolated from Eunicid polychaetes (Rueckert et al. 2012).

The trophozoites of *P. curvarae* had a posteriorly positioned nucleus and were distinctly curved and cylindrical, compared to *P. lacrimae*, and *P. translucidae* (Table 3). The trophozoites of *P. translucidae* lacked a large number of amylopectin granules within the cytoplasm, giving this species a characteristic transparent appearance under light microscopy. In contrast to the other species of *Polyplacarium*, *P. citrusae* is highly flattened and relatively small with an average length and width of only 53 and 29 μm , respectively. The trophozoites of *P. citrusae* were also isolated from the intestines of different capitellid host, namely *Heteromastus filiformis*. The general outline shape of *P. citrusae* was distinctly ovoid, reminiscent of a lemon (Table 3).

In contrast to *P. citrusae*, the surface of the trophozoites in *P. lacrimae*, *P. curvarae*, and *P. translucidae* had a distinct region of wider and shallower epicytic folds. Although the functional significance of this particular region of epicytic folds in these three species is uncertain, it is

plausible that the folds facilitate the acquisition of nutrients by expanding and contracting, thereby moving contents in the host gut around the cell. The consistent presence of this region of shallow folds in repeated observations of three different species, each prepared multiple times, minimized the chance that this distinct feature reflects a preparation artifact.

Our molecular phylogenetic analyses of SSU rDNA grouped the four single-cell isolates from each of the four species into four separate and corresponding clades (Fig. 6). Intraspecific variation of the SSU rDNA sequence within each clade was low, ranging from 0.22 to 2.12 % (Table 2). Interspecific variation between the four clades was relatively high, ranging from 7.42 to 14.98 % (Table 2). The morphological features of the trophozoites combined with the phylogenetic pattern of SSU rDNA sequence variation provided strong evidence for the delimitation of all four species of *Polyplacarium* from one another.

Environmental SSU rDNA sequences and the *Polyplacarium* clade

The four species of *Polyplacarium* that we described here grouped strongly with five SSU rDNA environmental sequences of similar branch length that were retrieved from GenBank. Environmental sequences AY179975 and AY179976 were generated from a PCR survey of sediment in a salt marsh near Cape Cod, Massachusetts, USA; environmental sequences EF100199 and EF100216 generated from a PCR survey of sediment from a marine tidal flat off the coast of Greenland (Stoeck and Epstein 2003); environmental sequence AB275013 was generated from sediment from a deep sea methane cold seep near Sagami Bay, Japan (Takishita et al. 2007a, b). Until now, the cellular identity of these environmental sequences was either considered uncertain within gregarine apicomplexans (Cavalier-Smith 2004; Leander 2007; Leander et al. 2006; Rueckert and Leander 2008, 2009, 2010; Rueckert et al. 2010, 2011a, b) or entirely misinterpreted (e.g., novel jacobid-like sequences) (López-García et al. 2007; Stoeck and Epstein 2003). Nonetheless, the vastly different geographical locations from which the environmental DNA sequences were generated indicate that the *Polyplacarium* clade has a global distribution and that we are at an early stage of understanding the total composition of this clade.

Concluding remarks

This study represents the first molecular phylogenetic data gathered from gregarines isolated from capitellid polychaetes. The combination of SC-PCR approaches, molecular phylogenetic analyses of SSU rDNA sequences, and comparative morphological data demonstrated the cellular

identity of a previously unidentified environmental SSU rDNA sequence clade and enabled us to establish four new species within one novel genus: *Polyplacarium lacrimae* n. gen. et sp. (type species), *P. curvarae* n. gen. et sp., *P. translucidae* n. gen. et sp., and *P. citrusae* n. gen. et sp. These data highlight significant limitations of environmental PCR surveys of biodiversity, mainly that accurate interpretations of the resulting DNA sequences require a comprehensive sample of reference species that have also been characterized at the cellular level. Hopefully, an appreciation for this organismal context will inspire future exploration into the overall diversity of marine gregarines using an approach that combines single-cell PCR, molecular phylogenetic analyses, and comparative morphology.

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