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Description of *Trichotokara nothriae* n. gen. et sp. (Apicomplexa, Lecudinidae) – An intestinal gregarine of *Nothria conchylega* (Polychaeta, Onuphidae)

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ABSTRACT

The trophozoites of a novel gregarine apicomplexan, *Trichotokara nothriae* n. gen. et sp., were isolated and characterized from the intestines of the onuphid tubeworm *Nothria conchylega* (Polychaeta), collected at 20 m depth from the North-eastern Pacific Coast. The trophozoites were 50–155 μm long with a mid-cell indentation that formed two prominent bulges (anterior bulge, 14–48 μm wide; posterior bulge, 15–55 μm wide). Scanning electron microscopy (SEM) demonstrated that approximately 400 densely packed, longitudinal epicytic folds (5 folds/μm) inscribe the surface of the trophozoites, and a prominently elongated mucron (14–60 μm long and 6–12 μm wide) was covered with hair-like projections (mean length, 1.97 μm; mean width, 0.2 μm at the base). Although a septum occurred at the junction between the cell proper and the mucron in most trophozoites, light microscopy (LM) demonstrated that the cell proper extended into the core of the mucron as a thin prolongation. A spherical nucleus (8–20 μm) was situated in the middle of the trophozoites, and gamonts underwent caudal syzygy. The small subunit (SSU) rDNA sequence and molecular phylogenetic position of *T. nothriae* was also characterized. The sequence from this species was the most divergent of all SSU rDNA sequences currently known from gregarines and formed a weakly supported clade with *Lecudina polymorpha*, which also possesses densely packed epicystic folds (3–5 folds/μm) and a prominently elongated mucron.

1. Introduction

Apicomplexans form a diverse group of unicellular parasites containing about 6000 described species and approximately 1.2–10 million undescribed species (Hausmann et al., 2003; Adl et al., 2007; Morrison, 2009). There are four major groups of apicomplexans recognized: (eu)coccidians, haemosporidians, piroplasmids and gregarines (Adl et al., 2005). Because some of these groups include pathogens of humans and life stock (e.g., *Plasmodium* – the causative agent of malaria), these species are better studied than groups that have no known medical or economic impact. Among the least understood apicomplexan parasites are gregarines, especially those inhabiting the oceans. Marine gregarines are dynamic extracellular parasites that inhabit the intestines and coeloms of invertebrates, and improved knowledge of these lineages will help elucidate the overall diversity and early evolutionary history of apicomplexans as a whole (Leander, 2008).

Gregarines have been conveniently lumped into three groups: archigregarines, eugregarines, and neogregarines (Grassé, 1953). The vast majority of described gregarine species are considered eugregarines, and, in large part, this is due to the fact that many eugregarines infect insects, which have garnered more attention from the parasitological community. Eugregarines, however, also occur in marine and freshwater habitats and have intestinal trophozoites (relatively large feeding stages) that are significantly different in morphology and behaviour from that of the infective sporozoites. Most eugregarine species that inhabit marine invertebrates have been classified within a poorly circumscribed family called the Lecudinidae Kamm, 1922 (25 genera) and within the genus *Lecudina* Mingazzini, 1899 (Levine, 1988). This genus has emerged as a "catch-all" taxon for marine aseptate gregarines that inhabit the intestines of polychaetes (and a few other host taxa). Consequently, the actual diversity of these parasites is greatly underestimated, and the identity and composition of *Lecudina* has become even more ill-defined within the context of modern molecular phylogenetic and comparative ultrastructural data (Leander et al., 2003b; Leander, 2008; Rueckert and Leander, 2009).

New species (i.e., morphotypes or phylotypes) of gregarines are generally discovered in previously unexplored host species, and closely related host species tend to be infected by closely related gregarine species (e.g., Levine, 1979; Perkins et al., 2000; Landers and Leander, 2005; Rueckert and Leander, 2008, 2009; Simdyanov,

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2009). Accordingly, the host taxon has been used as corroborating evidence for the delimitation of different gregarine species that are otherwise differentiated on the basis of molecular phylogenetic data and comparative morphology. A common morphological feature of the trophozoites of marine eugregarines is a cell cortex consisting of longitudinal epicytic folds that can vary considerably in overall number (Leander et al., 2003b; Leander, 2008). Some marine gregarines also possess conspicuous hair-like projections on the surface of the trophozoites, such as *Filipodium* (archigregarine), *Diplauxis* (eugregarine), *Urospora* (eugregarine) and *Cochleomeritus* (eugregarine). The hair-like projections in *Filipodium*, *Diplauxis* and *Urospora* cover the entire trophozoite surface (Hoshide and Todd, 1996; Dyakin and Simdyanov, 2005; Prensier et al., 2008; Rueckert and Leander, 2009), whereas the hair-like projections of *Cochleomeritus* are limited to the attachment apparatus, called the mucron (Levine, 1973). In each case, the longitudinal epicytic folds and hair-like projections are inferred to increase surface area, presumably for the acquisition of nutrients within the intestinal lumen of the host.

In this study, we describe a novel marine eugregarine with a prominent elongated mucron covered with hair-like projections: *Trichotokara nothriae* n. gen. et sp. This species was isolated from the intestines of *Nothria conchylega*, a tube-forming polychaete (Onuphidae) collected from the North-eastern Pacific Ocean. We characterized the morphological features of the trophozoites in this novel species with light and scanning electron microscopy (LM and SEM, respectively) and established a DNA signature (i.e., barcode) for the new lineage by generating its small subunit (SSU) rDNA sequence. Molecular phylogenetic analyses of the new sequence from *T. nothriae* also enabled us to re-evaluate and discuss the systematics of *Lecudina polymorpha*, another gregarine with a prominent elongated mucron.

2. Materials and methods

2.1. Collection and isolation of organisms

A dredge haul was conducted at Wizard Islet (48°51′6″N, 125°09′4″W) in June 2008 and June 2009 at a depth of 20 m during two trips on the research vessel MV/Alta from the Bamfield Marine Science Centre, British Columbia, Canada. Tubes of the onuphid polychaete *N. conchylega* (Sars, 1835) were collected from these samples.

The intestines of the host organism were dissected with fine-tipped forceps under a low magnification stereomicroscope (Leica MZ6) in order to extract the trophozoites of *T. nothriae*. Gut contents containing trophozoites were examined with an inverted compound microscope (Zeiss Axiovert 200 or Leica DM IL), and individual trophozoites were isolated by micromanipulation. Before being prepared for microscopy and DNA extraction, individual trophozoites were washed three times in filtered and autoclaved seawater.

2.2. Light and scanning electron microscopy

Differential interference contrast (DIC) light micrographs of the trophozoites of *T. nothriae* were taken with a compound microscope (Zeiss Axioplan 2) connected to a colour digital camera (Leica DC500). Individual trophozoites of *T. nothriae* ($n = 21$) were prepared for scanning electron microscopy (SEM) using the OsO_4 vapour protocol described previously (Rueckert and Leander, 2008, 2009). Isolated cells were deposited directly into the threaded hole of a Swinnex filter holder, containing a 5 μm polycarbonate membrane filter (Millipore Corp., Billerica, MA), that was submerged in 10 ml of seawater within a small canister (2 cm diameter and 3.5 cm tall). A piece of Whatman filter paper was mounted on the inside base of a beaker (4 cm dia. and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4% OsO_4 and the beaker was turned over the canister. The parasites were fixed by OsO_4 vapours for 30 min. Ten drops of 4% OsO_4 were added directly to the seawater and the parasites were fixed for an additional 30 min on ice. A 10 ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with CO_2. Filters were mounted on stubs, sputter-coated with 5 nm gold, and viewed under a scanning electron microscope (Hitachi S4700). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

2.3. DNA isolation, PCR amplification, cloning, and sequencing

DNA was extracted from two different isolates of trophozoites collected at different times. Seventeen individual trophozoites (isolate 1) and 18 individual trophozoites (isolate 2) were manually isolated from dissected hosts, washed three times in filtered and autoclaved seawater, and deposited into a 1.5 ml Eppendorf tube. Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification Kit (EPICENTRE, Madison, WI, USA). Small subunit rDNA sequences were PCR amplified using puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada) and the following eukaryotic PCR primers: F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander et al., 2003a). The following internal primers, designed to match existing eukaryotic SSU sequences, were used for nested PCR: F2 5′-AAGTCTGGTGCCAGCAGCC-3′, F3 5′-TGCGCTACCTGGTTGATCC-3′ and R2 5′-GCCTYGCGACCATACTCC-3′. PCR products corresponding to the expected size (~1806 bp) were gel isolated and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Eight cloned plasmids, for each PCR product, were digested with *Eco*RI, and inserts were screened for size using gel electrophoresis. Two identical clones were sequenced with ABI Big-dye reaction mix using vector primers and internal primers oriented in both directions. The SSU rDNA sequences were identified by BLAST analysis and molecular phylogenetic analyses (GenBank Accession number GU592817).

2.4. Molecular phylogenetic analysis

The new SSU rDNA sequence from *T. nothriae* was incorporated into two alignments using MacClade 4 (Maddison and Maddison, 2000) and visual fine-tuning: (1) a 42-sequence alignment representing all major groups of eukaryotes and (2) a 64-sequence alignment representing the diversity of gregarines. The 42-sequence alignment enabled us to more confidently establish the relationship of the highly divergent sequence from *T. nothriae* with gregarine apicomplexans (Supplementary file). The 64-sequence alignment enabled us to more thoroughly establish the relationship of *T. nothriae* within gregarine apicomplexans. The program PhyML (Guindon and Gascuel, 2003; Guindon et al., 2005) was used to analyze the 42-sequence alignment (928 unambiguously aligned positions; gaps excluded) and the 64-sequence alignment (1019 unambiguously aligned positions; gaps excluded) with maximum-likelihood (ML) using a general-time reversible (GTR) model (Posada and Crandall, 1998) incorporating the fraction of invariable sites and a discrete gamma-distribution with eight rate categories (GTR + I + Γ + 8 model: $\alpha = 0.450$ and $I = 0.068$ for the 64-sequence alignment; $\alpha = 0.371$ and $I = 0.000$ for the 42-sequence alignment). ML bootstrap analyses were performed on 100 re-sam-