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Comparative Ultrastructure of Fornicate Excavates, Including a Novel Free-living Relative of Diplomonads: *Aduncisulcus paluster* gen. et sp. nov.

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The Fornicata (Excavata) is a group of microbial eukaryotes consisting of both free-living lineages (e.g., *Carpediemonas*) and parasitic lineages (e.g. *Giardia* and *Retortamonas*) that share several molecular and ultrastructural traits. *Carpediemonas*-like organisms (CLOs) are free-living lineages that diverged early within the Fornicata, making them important for inferring the early evolutionary history of the group. Molecular phylogenetic analyses of free-living fornicates, including sequences from environmental PCR surveys, have demonstrated that CLOs form six different lineages. Representatives from five of these lineages have been studied at the ultrastructural level. The sixth lineage has been labeled "CL2" but has yet to be described with ultrastructural data. Improved understanding of CL2 is expected to help elucidate character evolution within the Fornicata. Therefore, we comprehensively characterized CL2 (NY0171) in order to understand the ultrastructural traits in this lineage, especially the organization of the microtubular root system (i.e., the flagellar apparatus). CL2 shared several morphological features with other fornicates, including reduced mitochondria and an arched B fiber bridging flagellar roots 1 and 2. The molecular phylogenetic position combined with some distinctive ultrastructural traits (e.g., a curved ventral groove) in CL2 required us to establish a new genus and species, *Aduncisulcus paluster* gen. et sp. nov.

Key words: Culturing; cytoskeleton; evolution; flagellar apparatus; morphology; ultrastructure.

Introduction

Comparative ultrastructural data from unicellular eukaryotes (protists) within a molecular phylogenetic context has provided important insights into the diversity and evolution of eukaryotic cells. Traits associated with the microtubular root systems of protists (i.e., the flagellar apparatus) are particularly informative because they can be compared across the tree of eukaryotes and demonstrate broad patterns of morphological evolution within each major clade (Moestrup 2000; Yubuki and Leander 2013; Yubuki et al. 2016). The unity and diversity of the

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flagellar apparatus suggests that this cytoskeletal system was already complex in the most recent ancestor of all eukaryotes and was similar to those found in modern day excavates (Yubuki and Leander 2013). The Excavata is one of five eukaryotic supergroups, along with the Opisthokonta (Animalia, Fungi and their relatives), Amoebozoa, Archaeplastida and SAR (Stramenopile, Alveolata, Rhizaria) (Adl et al. 2012; Simpson 2003; Simpson and Roger 2004; Simpson et al. 2006). The Excavata includes single-celled organisms with different modes of nutrition, such as phototrophic microalgae (e.g., *Euglena*), human parasites (e.g., *Giardia* and *Naegleria*), free-living phagotrophic flagellates (e.g., *Carpediemonas*) and intestinal symbionts (e.g., *Trichonympha* and *Monocercomonoides*).

The diversity within the excavate lineage Fornicata has played a prominent role in reconstructing the earliest stages of eukaryote evolution. The traits associated with the Fornicata are particularly important for inferring the evolutionary transition(s) from free-living modes of life to parasitic/commensal modes of life. Fornicates are flagellates that inhabit low oxygen environments, such as aquatic sediments and the intestines of animals. All members of this group lack conventional mitochondria and instead have modified/reduced forms (e.g., hydrogenosomes and mitosomes) referred to as “mitochondrion-related organelles” (MROs) (Cavalier-Smith 1998; Simpson 2003; Stairs et al. 2015; Takishita et al. 2012). The Fornicata include several different free-living lineages, called “*Carpediemonas*-like organisms” (CLOs) (Kolisko et al. 2010), and mostly parasitic and commensal lineages within the Retortamonadida (*Retortamonas* and *Chilomastix*) and Diplomonadida (e.g., *Giardia*); however, there are also a few examples of free-living species within retortamonads and diplomonads, such as *Chilomastix cuspidata* and *Trepomonas agilis* (Silberman et al. 2002; Brugerolle and Lee 2000). Until recently, *Carpediemonas membranifera* was the only representative of free-living fornicates (Simpson and Patterson 1999; Simpson et al. 2006). Over the last decade, five additional CLO clades have been identified: *Dysnectes* (Yubuki et al. 2007), *Hicanonectes* (Park et al. 2009), *Ergobibamus c* (Park et al. 2010), *Kipferlia* (Kolisko et al. 2010; Yubuki et al. 2013) and an undescribed lineage called “CL2” (Kolisko et al. 2010; Takishita et al. 2012). The ultrastructure of the flagellar apparatus in CLOs, excluding CL2, has been characterized previously and encompasses a great deal of variability in traits that reflect phylogenetic relationships. Molecular phylogenetic analyses of small subunit (SSU) rDNA and six different protein gene sequences demonstrated that CLOs are distributed throughout the Fornicata within several different subclades: (i) *Dysnectes, Kipferlia,* retortamonads and diplomonads; (ii) *Hicanonectes, Ergobibamus* and CL2; and (iii) *Carpediemonas* (Takishita et al. 2012).

In order to understand the unity and diversity within the Fornicata more comprehensively, we characterized the ultrastructure of CL2 using the same strain (NY0171) included in previous molecular phylogenetic analyses (Kolisko et al. 2010; Takishita et al. 2012). Prior to this study, the morphology of CL2 was known only from two light micrographs. Our investigation of the ultrastructural traits in CL2 combined with its molecular phylogenetic position enabled us to establish a new genus and species, *Aduncisulcus paluster* gen. et sp. nov.

Results

The General Morphology of *Aduncisulcus paluster* gen. et sp. nov. (NY0171)

The cells were 8.9 μm (7.0-11.3 μm, n = 30) long, 5.4 μm (4.1-7.2 μm, n = 30) wide and had a broad ventral groove that extended from just below the anterior end of the cell and curved toward the left over the posterior end of the cell (Figs 1, 2). The cells had two flagella inserted at the anterior end of the cell (Figs 1, 2). Flagellum 1 (F1) extended posteriorly within the ventral groove, was twice the length of the cell, and moved with rapid sinusoidal waves (Fig. 1A-C). F1 was adorned with a long ventral vane and a diminutive dorsal vane (Figs 2, 3A-C). The ventral vane was supported by a densely striated fibrous lamellum (Fig. 3A-D) and terminated at the posterior end of the ventral groove (Fig. 2). The anterior flagellum 2 (F2) did not possess vanes, extended laterally to the left side of the cell and moved rhythmically from anterior to posterior (Figs 1A, B, 2, 4A). The cells often attached to substrates using the tip of the posterior F1 (Fig. 1D). When the culture was disturbed, the cells swam slowly in a spiral pattern. Food particles were drawn into the ventral groove in currents generated by F1 and subsequently ingested by a cytostome positioned at the posterior end of the groove.

The nucleus was situated immediately behind the flagellar apparatus (Fig. 3A, B). The cells contained several mitochondrion-related organelles (MROs) that lacked cristae and were enveloped by two membranes (Fig. 4B). These organelles were ca. 400 nm long and were positioned mostly within the anterior half of the cell (Figs 5, 6). The ER