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Placement of the unclassified *Cyranomonas australis* Lee 2002 within a novel clade of Cercozoa

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Abstract

Two heterotrophic flagellate strains were isolated from marine sediment samples off eastern Canada and Korea. These new isolates are indistinguishable by light microscopy from the unclassified protist *Cyranomonas australis*. The organisms are ovoid-shaped cells, 3.5–6 μm long, laterally compressed, and somewhat flexible. They have two unequal flagella, about 1.1–2.5 times body length. Typically, the cells show a gliding motility and do not exhibit any amoeboid form or pseudopodia. 18S rDNA phylogenies clearly indicate that the isolates can be assigned to the taxon Filosa, within Cercozoa. The isolates are closest to an environmental sequence (CYSGM-16; 99% identity). *Cyranomonas*, CYSGM-16, and uncultured eukaryote RM1-SGM46 form a clade with strong statistical supports, here called novel clade CU (*Cyranomonas* plus Uncultured eukaryotes). This clade may be sister to the order Marimonadida. The novel clade CU and the Marimonadida have been detected only in marine habitats. Our findings suggest that *C. australis* may not belong to any previously described family within Filosa and Cercozoa.

Keywords: Cercozoa; *Cyranomonas*; Marine sediment; Protist; Taxonomy; Unclassified Filosa

Introduction

Heterotrophic nanoflagellates (HNFs, <20 μm in size) are diverse organisms that exhibit some of the highest densities among eukaryotes in aquatic environments (Laybourn-Parry, 1992). The term "HNFs" is considered to refer to a single functional group, which is usually responsible for ~50% of prokaryote mortality in aquatic environments (Fuhrman and Noble, 1995). In addition, organisms from higher trophic levels (i.e., ciliates) feed preferentially on HNFs, although ciliates and HNFs are also compete in their grazing on prokaryotes (Epstein and Shiaris, 1992). HNFs previously described in light microscopy observations of marine, freshwater, and extreme habitats, have been insufficiently studied using modern techniques that could clarify their taxonomic position (Adl et al., 2012; Park et al., 2007; Park and Simpson, 2010, 2015; Šlapeta et al., 2005; Takishita et al., 2007). In addition, many environmental sequences suspected to come from HNFs still belong to phylogenetic groups with no well-characterized representatives (i.e. species that have been cultured and/or studied carefully by microscopy), and this raises the question of their classification at the species/genus

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level (Alexander et al., 2009; Howe et al., 2011b; Takishita et al., 2007). Consequently, knowledge of the biodiversity and taxonomic affinities among HNFs is still rather limited.

The unclassified organism *Cyranomonas australis* is one of the many HNFs that is occasionally observed in natural seawater samples (Aydin and Lee, 2012; Lee, 2001, 2002, 2015). It has been documented with light microscopy from marine habitats, primarily marine sediments, off Australia, Korea, Turkey, and the United Kingdom (Aydin and Lee, 2012; Lee, 2001, 2002, 2015; Tong, 1994). *Cyranomonas* was first recorded as "Cyrano" by Tong (1994), and was formally named by Lee (2002), with *Cyranomonas australis*, being the sole species included in the genus to date. *Cyranomonas australis* has rounded cells ~5 μm in length, and two unequal flagella 1–1.5 times the body length (Lee, 2002). Although this species is somewhat similar to *Rhynchomonas nasuta*, *Ancyromonas*, and *Amastigomonas* species (Lee, 2001, 2002), and to the Glissomonadida in the Cercozoa (Howe et al., 2009, 2011a), the morphology and motile behavior of *Cyranomonas* are distinct. *Cyranomonas* has only been described by light microscopy observations of natural samples. Because no culture of *Cyranomonas* has been available, little is known about the taxonomic position of the genus. Cultivation of *Cyranomonas* may help to clarify its uncertain phylogenetic affinities.

In this paper, we report on the first successful isolation and cultivation of two *Cyranomonas* strains from marine sediments off Halifax (Canada) and Garorim Bay (Korea). The two *Cyranomonas* isolates, KM009 (Canadian strain) and KM010 (Korean strain), are very similar to *Cyranomonas australis* under the light microscope, and are shown to belong to the subphylum Filosa within the Cercozoa by 18S rRNA gene phylogenies. However, *Cyranomonas* does not belong to a well-characterized subclades within Filosa, and it form a 'novel clade' together with some previously reported environmental sequences.

Material and Methods

Isolation, cultivation, and light microscopy

Samples were collected from intertidal marine sediments in Horseshoe Island Park in Halifax, Nova Scotia, Canada (N44°38′23″, E63°36′46″) on 31 May 2012, and from Garorim Bay, Korea (N36°55′32″, E126°20′21″) on 17 July 2015. The salinities were about 30 psu. The samples were processed as described elsewhere (Lee and Patterson, 2000). Briefly, sandy surface sediments were collected, placed in trays in layers 1 cm deep, and allowed to settle for several hours before excess water was drained off. The material was covered with a sheet of lens tissue, upon which 22 mm × 22 mm No. 1 coverslips were placed. After 12 h, the coverslips were removed and flagellates were observed with a Zeiss Axiocam HR digital camera and its associated software (Axiovision 4.6). A single cell corresponding to the morphospecies *Cyranomonas australis* Lee 2002 was isolated by micropipetting on a coverslip, inoculated into a well plate containing sterile seawater supplemented at 1% v/v with Lysogeny Broth (LB), and maintained at 21 °C. The resulting cultures, KM009 and KM010, also contained natural prokaryotes. For routine maintenance, subculturing was performed every month. The isolate KM009 isolated from marine sediment off eastern Canada was maintained in the laboratory for ~2 years, but is now dead; KM010 isolated from marine sediment off Korea remains extant. Light micrographs were collected from cultures about one week old using differential interference contrast optics. Culture of *Cyranomonas australis* strain KM010 is kept with the Korean Culture Collection of Protists at Kyungnam University (Korea).

Scanning electron microscopy

A culture sample about one week old was fixed with 4% (w/v) OsO_4 for 30 min at room temperature, and then one drop of 4% (w/v) OsO_4 was added to the sample. Cells were transferred onto a 1 μm polycarbonate membrane filter (Corning Separations Division, Acton, MA, USA), washed with sterile distilled water, dehydrated with a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95% [2 times], 100% [3 times]), and critical-point-dried with CO_2. Filters were mounted on stubs, sputter-coated with gold, and viewed under a Hitachi S4700 scanning electron microscope.

Molecular sequencing and phylogenetic analysis

A 10-mL sample of about one-week-old culture was centrifuged at 5,900 *g* for 5 min, and then the DNA was extracted using a Qiagen Blood & Cell Culture Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. 18S ribosomal DNA (18S rDNA) sequences were obtained by PCR amplification using a combination of the eukaryote primers EukA (5′-AACCTGGTTGATCCTGCCAGT-3′) and EukB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al., 1988). For the PCR amplification, a reaction volume of about 20 μL was used that included 1.5 μL of 10 μM stocks of the primers EukA and EukB, 2 μL of 0.25 mM dNTP-mix, 0.8 μL of 50 mM $MgCl_2$, 0.2 μL total of 5U/μL *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), and 1–3 μL of DNA template. The cycling conditions started with a denaturing step at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 1 min of annealing at 55 °C, and extension at 72 °C for 2 min (10 min at 72 °C for the final cycle only). A PCR product corresponding to the expected size was gel-isolated and directly purified, and then sequenced using various eukaryotic internal sequencing primers. These 18S rRNA gene sequences of the two isolates have been deposited in GenBank under the accession numbers KX618913 (strain KM009) and KX618914 (strain KM010).