Title: Ultrastructural and single-cell level characterization reveals metabolic versatility in a microbial eukaryote community from an ice-covered Antarctic lake

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Running Head: Metabolic strategies of Antarctic lake protists

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ABSTRACT

The McMurdo Dry Valleys (MCM) of Southern Victoria Land, Antarctica, harbor numerous ice-covered bodies of water that provide year-round liquid water oases for isolated food webs dominated by the microbial loop. Single-celled microbial eukaryotes (protists) occupy major trophic positions within this truncated food web ranging from primary producers (*e.g.*, chlorophytes, haptophytes, cryptophytes) to tertiary predators (*e.g.*, ciliates, dinoflagellates, choanoflagellates). To advance understanding of MCM protist ecology and their roles in nutrient and energy cycling, we investigated potential metabolic strategies and microbial interactions of key MCM protists isolated from a well described lake (Lake Bonney). Fluorescence-activated cell sorting (FACS) of enrichment cultures combined with single-amplified genomics/amplicon sequencing and fluorescence microscopy revealed that MCM protists possess diverse potential metabolic capabilities and interactions. Two metabolically distinct bacterial clades (*Flavobacteria* and *Methylobacteriaceae*) were independently associated with two key MDV lake microalgae (*Isochrysis* and *Chlamydomonas*, respectively). We also report on the discovery of two heterotrophic nanoflagellates belonging to the Stramenopila supergroup, one of which lives as a parasite of *Chlamydomonas*, a dominate primary producer in the shallow, nutrient-poor layers of the lake.

Single-celled eukaryotic cells called "protists" play critical roles in the cycling of organic matter in aquatic environments. In ice-covered lakes of Antarctica, protists occupy key roles in the aquatic food web, providing the majority of organic carbon to the rest of the food web (photosynthetic protists) and acting as the major consumers at the top of the food web (predatory

42 protists). In this report, we utilized a broad combination of techniques (microscopy, cell sorting,
43 genomics) to describe the trophic abilities as well as potential interactions between Antarctic
44 lake protists and other microbes. Our work reveals that Antarctic lake protists rely on metabolic
45 versatility for their energy and nutrient requirements in this unique and isolated environment.
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48 INTRODUCTION

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50 The microbial loop links the transfer of nutrients and carbon between the microorganisms of the
51 food web, and includes complex ecological interactions between microbial Eukaryotes, Bacteria,
52 Archaea and Viruses. Microbial activity is influenced by bottom-up (*e.g.*, availability of nutrient
53 and energy sources) and top-down (*e.g.*, predation, parasitism, viral lysis) controls, as well as
54 microbe-microbe interactions (*e.g.*, bacterial consortia). Interaction partnerships between
55 microorganisms lead to combinations of win, loss or neutral outcomes for each microbial partner.
56 For example, parasitism or predation are examples of win-loss outcomes (1), while mutualistic
57 interactions within biofilms and syntrophic interactions (cross-feeding of metabolic products
58 between two species) between an alga and a heterotrophic bacterium are examples of win-win
59 partnerships (2). Understanding microbial interactions and the influence of environmental factors
60 on microbial distribution patterns is a critical component of deciphering global nutrient fluxes
61 and biogeochemical cycles.

62

63 Single-celled eukaryotic microorganisms (*i.e.*, protists) are ubiquitous in every ecosystem on
64 earth and play critical ecological roles in food web dynamics and global carbon and nutrient
65 cycles (3). Diverse protist lineages possess multiple nutritional modes; playing key roles as
66 producers, decomposers, parasites, and predators. Phototrophic protists are important producers
67 that contribute to global primary production and incorporate a significant portion of inorganic
68 carbon into the food web (4). Predatory protists are major consumers of planktonic
69 phytoplankton and bacteria, providing control over the abundance of these organisms as well as

linking primary producers/consumers with higher trophic levels (*i.e.*, metazoans) (5). Mixotrophic protists (combined ability for photosynthesis and phagotrophic ingestion of food particles) are widespread in aquatic ecosystems (6, 7). Recent molecular surveys from marine and freshwater environments have revealed a high diversity of 18S rRNA sequences which are unrelated to existing cultured protists (8, 9). The application of high-throughput sequencing has begun to reveal protist biogeography (10-12) as well as seasonal variability (13, 14).

The McMurdo Dry Valleys (MCM) of Southern Victoria Land, Antarctica, is a polar desert: with average air temperatures of -20°C and precipitation rates of <10 cm per year (15, 16). Numerous marine-derived, perennially-ice covered lakes are the only source of year-round liquid water for life on the Antarctic continent. Permanent ice caps prevent wind mixing and significant nutrient inputs: MCM lake chemistry is vertically stratified in the water column and lakes exhibit oxygen-rich/ultra-oligotrophic surface waters which are separated by permanent chemoclines from ancient, anoxic/saline waters (17). Thus, the MCM lakes represent aquatic environments with strong selective pressures on microbial evolution including low temperatures, low annual levels of photosynthetically active radiation (PAR), and limited nutrient (N and P) availability (18). Salinity, oxygen and light appear to play important roles in the biogeography of Antarctic lake microorganisms (19). Each lake supports simple food webs which harbor little to no metazoans, with the exception of low numbers of copepods and rotifers in a few lakes (20). Protists play dominating roles in carbon and nutrient cycling in the MCM microbial food web. The majority of dissolved organic carbon is autochthonous (*i.e.,* derived from new photosynthetic activity within the water column). Protists represent the major producers of organic matter in the MCM lake food web (21, 22), while heterotrophic nanoflagellates and

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ciliates are the top predators of bacteria and smaller protists (23, 24). Despite the energetic cost of maintaining and regulating both photosynthetic and heterotrophic cellular apparatus, mixotrophy appears to be very prevalent in MCM aquatic food webs (23, 25). Mixotrophic metabolism is a survival strategy for MCM protists to exploit alternate sources of either nutrients (*i.e.*, under oligotrophic conditions) or energy (*i.e.,* in the absence of light) (26).

As part of the NSF Long Term Ecological Research (LTER) Program, several lakes within the MCM have been the focus of more than two decades of intensive study. Lake Bonney is one of the most well-studied MCM lakes, and the lake physical and chemical characteristics have been thoroughly described by Spigel and Priscu (27). Lake Bonney is divided into two basins (west and east lobes) which are separated by a narrow passage which allows mixing of the upper photic zones for a few weeks in the summer. The shallow waters of both lobes are generally freshwater; however, salinity levels increase steeply at the permanent chemocline (18 – 20 m depth for the east lobe). Below the chemocline, the deep anoxic zone is hypersaline (maximum salinity 125-150 PSU). Photosynthetically active radiation (PAR) is relatively low under the ice (<50 µmol m^{-2} s^{-1}) due to the attenuation of the ice cover and declines below detectable levels at depths >25 m (29). Recently, the diversity and spatial distribution of the planktonic microbial eukaryotic communities residing in Lake Bonney has been reported (28-31). Protist populations residing in the MCM lakes are vertically stratified and exhibit lake-specific differences in their distribution patterns. The water column of Lake Bonney is dominated by large chlorophytes (*Chlamydomonas*) in the shallow, nutrient-poor depths, while the dominant photosynthetic protists are nanoplankton (haptophytes, stramenopiles) which exhibit peak abundances within the permanent chemocline where PAR is extremely limited (28). While these studies have begun to

describe the biogeographic distribution of the protist communities residing in these ice-covered polar lakes, a full understanding of the trophic abilities of MCM protists and potential interactions with other MCM microorganisms is currently lacking.

The vast diversity of microbial eukaryotes has been largely inaccessible by conventional cultivation methods. Over the past decade the technology of single-cell genomics has been exploited to recover genomic information from single uncultivated cells from their natural habitats, and has revealed cell-specific interactions such as symbioses, predation and parasitism (32-34). In this study, single cells of protists originating from an enrichment culture from the zone of maximum primary production in the permanent chemocline of Lake Bonney (east lobe) were isolated on the basis of fluorescent properties which related to their potential nutritional mode (photosynthetic, heterotrophic, mixotrophic). We chose to utilize an enrichment cultivation approach to allow us to work with live organisms and avoid the logistical issues associated with preserving and shipping natural protist communities from Antarctica to our US laboratory. Our hypothesis is MCM lake protists possess wide metabolic versatility which drives nutritional mode-dependent microbial interactions. Specifically, we addressed these open questions regarding the ecology of the MCM lake microbial eukaryote communities: What is the range of trophic versatility in the MCM planktonic protist communities? Do MCM phototrophic and heterotrophic protists interact with specific microbial partners? In this study, whole genome amplification (WGA) was performed to produce single amplified genomes (SAGs) from a variety of the sorted eukaryote cells (with their microbial partners). Combining Illumina® sequencing with various microscopic methods, we were able to 1) identify nutritional mode and metabolic potential of several key MCM protists; 2) reveal a diversity of potential microbial

interactions, including predation, parasitism and endosymbiosis; 3) provide new information to understand microbial metabolic strategies for survival under permanent light limiting and nutrient poor conditions.

MATERIALS AND METHODS

Site description, sample collection and enrichment cultures

Samples were collected from the water column of the east lobe of Lake Bonney, an ice-covered meromictic lake located in the Taylor Valley, McMurdo Dry Valleys. Samples were collected through the ice hole located in the middle of the lake (77°42.825S, 162 ° 26.832E) during the austral summer on December 21, 2012. A sampling depth (13 m piezometric depth) was selected to reflect the depth of maximum phytoplankton biomass (28), which was verified by *in situ* chlorophyll fluorescence with a submersible FluoroProbe (BBE Moldaenke GmbH) (29). Lake water samples were collected with a 5 L Niskin bottle (General Oceanics), transferred to 1-L amber bottles, and stored at 4 ˚C in the dark until processing.

An enrichment cultivation approach was employed to allow for the transport of live cultures with higher biomass to our US laboratory (Miami University, Ohio). To ensure maximum recovery of protist diversity, a series of enrichment cultures were started in the presence of a number of autotrophic media types (35-37) and growth media concentration (10 – 100%). Lake water was inoculated into 25-mL sterile culture flasks and incubated in a photoincubator at 5 ˚C /25 µmol photons m^{-2} s^{-1} in McMurdo Station, Antarctica, for 2 weeks prior to shipment. Cultures were

regularly inspected under the microscope for growth and the presence of microbial eukaryotes. Cultures were then shipped to our US laboratory at Miami University at 4 ˚C in the dark. Upon arrival, enrichment cultures were transferred to a photoincubator set at the same temperature/light regime until cell sorting. Last, while cryopreservation in the presence of glycerol buffer works well for preserving bacterial communities(38, 39), preliminary experiments showed that this preservation approach failed to preserve internal structures of eukaryotic cells and caused lysis of some wall-less protists.

Single cell sorting

Prior to cell sorting, all enrichment cultures were visually inspected by light microscopy for growth and morphological diversity of the protist communities. Based on these microscopic observations, one enrichment culture (grown in 10% F medium) which exhibited relatively high protist morphological diversity was chosen to be shipped to Oak Ridge National Laboratory for single-cell sorting. Protist food vacuoles were stained using a pH-sensitive probe, LysoTracker Green DND-26 (Invitrogen, Carlsbad, CA, USA) (40). A 2mL aliquot of the culture was gently homogenized by several inversions and incubated in the dark on ice for 15 min in the presence of 75 nmol L^{-1} of LysoTracker. Microbial eukaryotic cells of various size, morphology and trophic ability (phototrophic, heterotrophic, mixotrophic) were identified and sorted with a Cytopeia INFLUX sorter (BD, Franklin Lakes, NJ, USA) in a Class 1000 clean room using a 488 nm argon laser for excitation. Green (528-538 nm) and red (670-730 nm) fluorescent emission indicated LysoTracker green fluorescence and chlorophyll autofluorescence, respectively. Targeted single eukaryotic cells were sorted into four 96-well plates which contained 3 µL UV-

sterilized TE buffer in each well (32). Individual cells were sorted from discrete sectors using the three criteria. Plate 1 (P1) contained cells showing high LysoTracker fluorescence only (*ie.*, heterotrophic/phagotrophic); Plate 2 (P2) contained cells exhibiting high red fluorescence (*ie.*, photosynthetic) and the absence of green fluorescence; and Plates 3 and 4 (P3, P4) contained smaller cells that exhibited intermediate levels of red and green fluorescence (*ie.*, mixotrophic; Fig. 1). Plates were stored at -80 ˚C until whole genome amplification.

DNA template preparation for PCR

For DNA preparation, single-sorted eukaryote cells were lysed using alkaline treatment by the addition of 3 µL of buffer that consisted of 0.13 M KOH, 3.3 mM EDTA pH 8.0 and 27.7 mM Dithiothreitol, heated to 95°C for 30 sec, and immediately placed on ice for 10 min, followed by addition of 3 µL neutralization buffer (0.13 M HCl, 0.42 M Tris pH 7.0, 0.18 M Tris 8.0). Genomic DNA from the cell lysates was amplified using multiple displacement amplification (MDA) by the addition of 11 µL of MDA master mix containing 90.9 µM random hexamers with two protective, phosporothioate bonds on the 3′ end (Integrated DNA Technologies, Coralville, IA, USA), 1.09 mM dNTPs (Roche Indianapolis, IN, USA), 1.8X phi29 DNA polymerase buffer (New England BioLabs, Ipswich, MA, USA), 4 mM DTT and ~100 U phi29 DNA polymerase enzyme (purified in house) (41). Whole genome amplification was performed for 10 hrs at 30°C followed by inactivation at 80°C for 20 min. MDA products were diluted 100-fold in sterile 1X TE buffer, then screened by PCR and sequencing.

Sanger sequencing

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209 A fragment of the 18S rRNA gene was amplified from each of the SAGs using universal
210 eukaryote primers [EK-82F (5' -GAAACTGCGAATGGCTC) and EK-1520R (5' -
211 CYGCAGGTTCACCTAC)] (42) to generate PCR products for sequencing. PCR was performed
212 in triplicate using 25 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min. Sequencing
213 reactions were performed using the BigDye Terminator v3.1 cycle sequencing kit (ABI, CA)
214 with M13R primer and the fragments were sequenced on an Applied Biosystems 3730×l DNA
215 Analyzer (ABI, CA) located in the Center for Bioinformatics and Functional Genomics (CBFG)
216 at Miami University.

217

218 *Illumina sequencing*

219

220 Following successful identification of the individual eukaryote SAGs, 79 samples were selected
221 for sequencing of the associated bacterial communities on an Illumina® MiSeq Platform in the
222 CBFG. 16S rRNA genes from the SAGs were amplified using the primer set (F515/R806) which
223 encoded sequence against the highly variable V4 region of 16s rRNA, barcodes and linkers. PCR
224 reactions and MiSeq sequencing reactions were strictly followed the protocol provided by the
225 Earth Microbiome Project (http://www.earthmicrobiome.org) (42-45). Samples were sequenced
226 according to the manufacturer's recommendations using a 300-cycle MiSeq Reagent Kit v2
227 (Illumina®) in a 2 X 150 bp paired-end run in the presence of 25 % PhiX DNA.

228

229 *Analysis of the sequences*

230

For 18S rRNA gene sequences derived from Sanger sequencing, representative and closest sequences were selected from GenBank and aligned using MUSCLE. A maximum-likelihood tree was generated using MEGA6 software. Bootstrapping was used to estimate node support of 1,000 replicate trees.

16S rRNA gene sequences generated on the MiSeq instrument were analyzed with QIIME (v1.8.0). OTUs were identified at 97% cutoff using Greengenes database (v13.8) (46). All OTUs with one sequence per sample were discarded. Samples were 120 times rarefied based on the number of sequences in the library with the lowest sequence number. Alpha diversity (number of OTUs, chao1 and Shannon index) was assessed (data not shown). Beta-diversity was integrated using weighted unifrac distance metric (47, 48). Principal coordinates analysis (PCoA) and ANOSIM similarity analysis was performed to identify co-existence of eukaryotic and prokaryotic organisms in the single-cell samples (49-52).

Confocal Laser Scanning Microscopy

Isochrysis sp. MDV and *Chlamydomonas* sp. ICE-MDV cultures were treated with LysoTracker Green DND-26 probe using the same procedure previously described for FACS. A Zeiss LSM-710 (Carl Zeiss Microscopy GmbH, Germany) confocal laser scanning microscope was used for images in Fig. 3. A 488 nm argon ion laser was used as elimination/excitation source. LysoTracker and chlorophyll fluorescence were detected within 520-540 nm and 650-750 nm respectively. Differential interference contrast (DIC) images were also generated using

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transmission light mode. Images were pseudocolored in Zeiss ZEN 2011 (Carl Zeiss Microscopy GmbH, Germany) software.

Scanning Electron Microscopy

Samples from the original enrichment culture were fixed with 1% paraformaldehyde and 1.25% glutaraldehyde, and a secondary fixation was applied in 1% osmium tetroxide. Specimens were dehydrated through an ethanol series, critical-point dried with CO_2, and sputter-coated with gold. Micrographs were generated with a Zeiss SUPRA-35 FEG SEM (Carl Zeiss Microscopy GmbH, Germany).

RESULTS AND DISCUSSION

Identities and trophic modes of sorted eukaryotes

To overcome the logistical constraints of transporting fresh samples from Antarctica to our US laboratory as well as low biomass issues associated with MCM planktonic communities, we used an enrichment cultivation-based approach to test FACS/ single cell sorting on McMurdo Dry Valley lake protist communities. A series of enrichment cultures were started in Antarctica using a range of growth media as described in the Experimental Procedures and incubated for 2 weeks prior to shipment to our US laboratory. Upon arrival in our US laboratory, we monitored algal diversity using a bbe FluoroProbe (see Table S2 in Supplemental Material) and visually inspected each culture using bright-field and fluorescence microscopy to qualitatively assess protist diversity. Inspection of one of the cultures (Enrichment MCM87) revealed large (>15 µm) biflagellate chlorophytes, several nanophytoplankton identified as haptophytes and cryptophytes (~ 5 µm), as well as dinoflagellates cells exhibiting variable pigmentation (owing to the presence or absence of phytoplankton prey) and several non-pigmented heterotrophic nanoflagellates (2-5 µm). Based on our recent work on natural protist diversity in Lake Bonney (28), we concluded that MCM87 harbored representatives of several key members of the Lake Bonney protist community, including chlorophytes, haptophytes, choanoflagellates, dinoflagellates, and nanoflagellates, all of which have been detected in environmental sequence libraries generated from the photic zone of the east lobe of Lake Bonney.

Based on LysoTracker and chlorophyll fluorescence signals combined with cell size information, we gated four eukaryote populations for single cell sorting: a group with high LysoTracker fluorescence (*i.e.,* presence of protist food vacuole; Fig. 1, Plate 1 – green group), a group with high red fluorescence (*i.e.,* presence of chlorophyll; Fig. 1, Plate 2 – red group) and two groups with smaller cells that exhibited intermediate levels of red and green fluorescence (Fig. 1, Plates 3 and 4 – blue groups). Targeted single cells were deposited into four 96-well plates according to distinct gates that separated various types of organisms based on the size and fluorescent characteristics mentioned above. Following cell lysis and whole genome amplification using phi29 DNA polymerase, 65% (250 out of 384 samples) of the SAGs were successfully amplified using 18S rRNA primers based on electrophoretic separation of the PCR products on a 1% agarose gel. All SAGs which exhibited a band of the correct size (~1400 bp) were Sanger-sequenced. We recovered 79 high quality partial 18S rRNA sequences (~600bp) for phylogenetic analysis (GenBank accession numbers: KU196097- KU196166). Representative sequences were aligned with SILVA and GenBank databases using SINA and BLAST. Phylogenetic analysis revealed that the SAGs library harbored a total of 16 OTUs (based on a cut-off of 97% similarity) related to the Stramenopila supergroup and several other major phyla, including Chlorophyta, Choanoflagellida, Dinoflagellata, Cryptophyta and Haptophyta. The majority of SAG 18S rRNA sequences were identical to uncultivated eukaryote clones recovered in an earlier study on natural protist populations residing in Lake Bonney (28).

All SAGs recovered from the photosynthetic group (*i.e.,* high autofluorescence, low LysoTracker fluorescence; Fig. 1; SAG Plate 2; n=13) were related to a large biflagellate Chlorophyte, *Chlamydomonas,* which was closely related (99% identity) to an Antarctic marine species,

Chlamydomonas sp. Antarctic 2E9, and two Antarctic ice algal strains, *Chlamydomonas* sp. ICE-W and ICE-L (53). The *Chlamydomonas* SAGs were also closely related to sequences from clone libraries generated from the depth of maximum productivity (13 m depth) in the east lobe of Lake Bonney (28) (Fig. 2). Recent studies on phylogenetic and functional genes indicated that this organism is the dominant chlorophyte in the east and west lobes of Lake Bonney (28, 30). Last, our FACS results which suggest that this organism is photoauotrophic were supported by earlier physiological studies on *Chlamydomonas* ICE sp. (53) as well as a highly studied Lake Bonney chlorophyte, *Chlamydomonas* sp. UWO241 (22, 54).

SAG sequences related to a nanoflagellate haptophyte (*Isochrysis galbana*; 97% identity) represented a significant proportion of samples (80 %) recovered from SAG Plate 4 (n=17; Fig. 2), which represented one of two plates with intermediate levels of green and red fluorescence (Fig. 1, SAG Plate 4). 18S rRNA sequences from these SAGs were very similar to sequences recovered from clone libraries generated from 13 m-deep waters from east and west lobe Lake Bonney (28), and recent reports suggest that haptophytes communities related to this organisms are key primary producers in this lake (29, 30, 55). Communities of *Isochrysis* dominate the chemoclines of the east and west lobes of Lake Bonney (29) Several recent studies using quantitative PCR to monitor abundance of photosynthetic functional genes (*rbcL, psbA*) indicates that in contrast with chlorophyte populations, abundance (DNA) and activity (RNA) of haptophyte populations is not correlated with light availability (29, 55). These data suggest that the dry valley *Isochrysis* may rely on alternative metabolic strategies, such as mixotrophy.

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Stramenopiles represent a poorly understood supergroup of microbial eukaryotes that exhibit broad trophic ability (*e.g.*, phototrophy, mixotrophy, predation, parasitism). The marine stramenopiles (MASTs) lineage is a diverse group of microbial eukaryotes and represent a large proportion of heterotrophic nanoflagellates (HNFs) in marine environments (56). HNFs are small microbial eukaryotes (2 to 20 µm) which graze on bacteria and picophytoplankton and are recognized as the dominant consumers of picoplanktonic biomass in marine environments (7, 57). Our 18S rRNA gene sequence library contained several SAGs that were related to stramenopiles (Fig. 2). The most abundant stramenopile SAGs were closely related to *Pteridomonas danica* (SAG Plate 4; 99% identification, n=22) and *Pirsonia verrucosa* (SAG Plate 3; 94-100% identification, n=22). Sequences related to both stramenopiles were recovered in clone libraries from the west and east lobes of Lake Bonney (28) (Fig. 2). Recent work has reported that stramenopiles make up a significant proportion of the protist population (up to 60%) in both lobes of Lake Bonney (29, 31). *Pirsonia* is a phycovorous nanoflagellate which feeds on marine centric diatoms (58). *Pteridomonas* is a ubiquitous marine heterotrophic nanoflagellate (5). In addition, two OTUs within the Stramenopile supergroup (SAG clones P3E2 and P4C7) exhibited very low similarity (<81%) to the closest related identified sequence in the database (*Thraustochytrium* sp.). Both OTUs were, however, closely related to the sequences generated in Lake Bonney clone libraries from a previous study (28).

Several other protists groups were represented in the SAG libraries at low abundance. A single SAG was recovered for two choanoflagellates and one dinoflagellate. One SAG related to a psychrophilic marine cryptophyte *Geminigera cryophila* was also recovered. *G. cryophila* is abundant in the shallow layers of Lake Bonney (28, 29). Dinoflagellates have been detected in

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355 relatively high abundance in the west lobe of Lake Bonney, while sequences related to
356 choanoflagellates were less abundant (28, 31). All low abundance SAGs were recovered from
357 the two mixotrophic groups (SAG plates 3 and 4; Fig. 1) and were closely related to sequences
358 recovered from clone libraries generated from east or west lobe Lake Bonney, with the exception
359 of one choanoflagellate sequence (SAG P4B8; Fig. 2).
360
361 *Isolation and description of two key photosynthetic protists*
362
363 Recent work on the natural communities of Lake Bonney suggested that a chlorophyte related to
364 *Chlamydomonas* sp. ICE-L and a haptophyte related to *Isochrysis galbana* occupy important yet
365 distinct roles in the Antarctic lake food web (28-31). Our FACS analyses indicated that the
366 chlorophyte is a pure photoautotrophic species while the haptophyte may possess the ability to
367 combine photosynthetic metabolism with heterotrophic activity, such as digestion of captured
368 bacterial prey or particulate carbon. To confirm our hypotheses regarding the trophic capability
369 of these key MCM protists, we purified isolates of both organisms from enrichment cultures. A
370 culture of the *Chlamydomonas* sp. (hereafter named *Chlamydomonas* sp. ICE-MDV) was
371 recovered by plating of an enrichment culture on Bold's Basal Medium (59) with 1.5% agar. The
372 *Isochrysis* strain (hereafter named *Isochrysis* sp. MDV) was isolated by dilution to extinction of
373 an enrichment culture in 0.5X seawater supplemented with F/2 medium. The identity of both
374 strains was confirmed by 18S rRNA gene sequencing (data not shown). Confocal microscopy
375 showed that *Chlamydomonas* sp. ICE-MDV is a large (15 - 20 µm) biflagellate cell exhibiting
376 high autofluorescence (Fig. 3a, d and e). As LysoTracker probe is fluorescent acidotrophic;
377 therefore, we wondered if the acidic compartment (lumen) of chloroplasts (60) might cause false

positive fluorescence signal under flow cytometry. We did not detect green fluorescence in the presence of LysoTracker in *Chlamydomonas* sp. ICE-MDV cells, despite the presence of a large chloroplast (Fig. 3d and e). This also confirms that it is unlikely that any of the SAGs sorted in the mixed SAG plates (Plates 2 and 3; Fig. 1) were a product of spurious chloroplast staining. *Isochrysis* sp. MDV is a small (2 - 5 µm) brown-pigmented biflagellate alga. It possesses bi-lobed chloroplasts which exhibited high autofluorescence (Fig. 3i and j). In contrast with the chlorophyte strain, when cells of *Isochrysis* sp. MDV were treated with LysoTracker probe, green fluorescence was localized to a single vacuole (Fig. 3g). The presence of green fluorescence was associated only with LysoTracker-stained cells, as neither strain exhibited green fluorescence in the absence of LysoTracker (see Figs. S1 and S2 in supplemental material). We have also confirmed in growth experiments that *Isochrysis* sp. MDV can grow either in the dark (heterotrophic) or the light (phototrophic), but grows optimally in the presence of a variety of organic carbon sources and low irradiance (mixotrophic). In contrast, *Chlamydomonas* ICE-MDV cannot grow in the dark in the presence of organic carbon, but exhibits ability to grow under a broader range of light intensities (see Table S1 in supplemental material for description of basic growth physiology). Therefore, the chlorophyll and LysoTracker staining fluorescence information from confocal microscopy combined with growth physiology confirmed results from the FACS analyses that *Chlamydomonas* sp. ICE-MDV is a pure photosynthetic protist while *Isochrysis* sp. MDV is likely capable of mixotrophic metabolism. These results fit well with data from functional gene analyses that Lake Bonney chlorophyte populations that occupy the under-ice layers of the water column are strongly correlated with light availability on spatial and seasonal scales (55). By contrast, haptophyte populations dominating planktonic communities in

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the permanent chemocline are not influenced by light availability but can supplement light-dependent photosynthesis with ingestion of bacterial prey or particulate carbon.

Community composition of organisms co-sorted with Lake Bonney eukaryotes

To gain further insight into the trophic modes as well as potential microbial partners of the MCM microbial eukaryotes, we selected a number of eukaryote SAGs of varying trophic potential for 16S rRNA amplicon community sequencing. From the population of successfully amplified and sequenced SAGs, we sequenced a fragment of the 16S rRNA gene from 79 eukaryote SAGs using the Illumina® MiSeq platform (NCBI BioProject ID PRJNA304193). We recovered both bacterial 16S and plastid SSU rRNA genes from these sequencing libraries. The plastid sequences were filtered from the 16S rRNA gene libraries of all SAG OTUs known to be photosynthetic, including *Chlamydomonas* sp. ICE-MDV, *Isochrysis* sp. MDV, and *Geminigera*. To assess both the diversity and co-occurrence patterns of organisms that co-sorted with the individual eukaryote SAGs, we generated a heat map of 16S rRNA genes associated with each individual SAG sample (see Fig. S3 in supplemental material). The distribution of recovered 16S rRNA gene sequences was highly variable and dependent upon both the trophic mode and identity of the microbial eukaryote partner. Bacterial sequences associated with the photosynthetic *Chlamydomonas* sp. ICE-MDV and the mixotrophic *Isochrysis* sp. MDV were generally diverse, with a predominance of the phyla *Actinobacteria* and *Bacteriodetes*. In contrast, 16S rRNA gene sequences recovered from the two stramenopile SAGs related to *Pteridomonas* and *Pirsonia,* which dominated the heterotrophic (Plate 1) and one of the

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422 mixotrophic (Plate 4) populations, respectively, were enriched with plastid sequences (see Fig.
423 S3 in supplemental material).
424
425 Principal Coordinates Analysis (PCoA) based on UniFrac distance metrics of 16S rRNA OTUs
426 from the four most abundant SAGs (*Isochrysis* sp. MDV, *Chlamydomonas* sp. ICE-MDV,
427 *Pteridomonas* and *Pirsonia*) exhibited clustering by protist trophic mode (Fig. 4). Similarity
428 analysis using ANOSIM method indicated that bacterial OTUs associated with individual
429 eukaryote SAGs were significantly different to each other ($p<0.001$, $R^2=0.40$). Bacterial OTUs
430 associated with *Isochrysis* sp. MDV and *Chlamydomonas* sp. ICE-MDV were clustered
431 separately from each other, but were more closely related compared with either of the
432 straemophile SAGs. 16S rRNA gene libraries generated from SAGs of the heterotrophic
433 nanoflagellates (*Pteridomonas* and *Pirsonia*) were more related to each other than to either of the
434 photosynthetic protists or the environmental samples (Fig. 4).
435
436 *Potential interactions between Dry Valley protists and bacteria*
437
438 Complex ecological interactions exist between protists and other microorganisms, including
439 predation of bacteria by heterotrophic protists and syntrophic interactions between
440 photosynthetic algae and heterotrophic bacteria (2, 61). Traditionally, heterotrophic protists were
441 identified via culture-dependent and microscopic methods (62-65). Recent studies have shown
442 that a single-cell genomic approach can provide direct evidence of specific predator-prey
443 interactions (66, 67). We recovered 22 samples that had high LysoTracker probe-associated
444 fluorescence and were closely related to the stramenopile *Pteridomonas danica* (Figs. 1 and 2).

445 *Pteridomonas* is a ubiquitous heterotrophic nanoflagellate in marine systems, and is an important
446 linkage between bacteria and higher trophic levels in the food web (68-70). Interestingly, we
447 observed that more than 90% of 16S rRNA gene sequences from *Pteridomonas* SAGs were
448 closely related to a stramenopile chloroplast sequence that was neither reported in environmental
449 sequence libraries (28, 31) nor detected in other SAG 16S rRNA libraries in this study. A
450 previous study reported that *Pteridomonas danica* harbors non-pigmented non-photosynthetic
451 plastids or vestigial chloroplasts (71); therefore, it is likely that the chloroplast sequences we
452 recovered in 16S rRNA gene sequence libraries generated from *Pteridomonas* SAGs represent
453 plastid sequences from vestigial chloroplasts. This interpretation was supported by a lack of
454 pigmentation under light microscopy in *Pteridomonas* cells (data not shown). We removed the
455 stramenopile plastid sequences from the 16S rRNA gene libraries generated from *Pteridomonas*
456 SAGs and investigated the remaining 16S rRNA gene sequences in the *Pteridomonas* libraries
457 (Fig. 5a). The most abundant sequences were related to a haptophyte plastid rRNA gene (32 ±
458 23% in total OTUs) and a firmicute, *Streptococcus* (13±8% in total OTUs) (Figure 6a). HNFs
459 represent a diverse ecological group cable of various trophic preferences (*e.g.,* bacteriovorous,
460 phycovorous or omnivorous). *P. danica* feeds on both bacteria and picophytoplankton and is
461 therefore an omnivore (72); however, we did not find any reports of this genus predating on
462 haptophytes. Picocyanobacteria are largely absent from the water column of the MCM lakes (29,
463 30); therefore, we suggest that in addition to heterotrophic bacteria, the haptophyte population
464 which dominates the photic zone of Lake Bonney could be the natural prey for the MCM
465 *Pteridomonas*.
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467 A number of feeding strategies have been reported for heterotrophic flagellates such as filtration
468 of food particles with an array of radial pseudopodia or tentacles or direct interception of prey by
469 flagella. In addition, flagellates are often attached to particles such as marine snow when feeding
470 (73, 74). Examination of the Lake Bonney *Pteridomonas* by scanning electron microscopy
471 revealed the presence of a single long flagellum surrounded by a radial array of tentacles (Fig. 5
472 b-d) which are characteristic morphological features of *Pteridomonas* species (68). In addition,
473 we observed particles attached to the tentacles and cells were often attached to a surface of
474 particle by a stalk (Fig. 5b). To our knowledge, our images are some of the first published SEMs
475 of this genus. Thus, the Lake Bonney *Pteridomonas* appears to rely on a filter feeding
476 mechanism and may be particle-attached within the water column. This would be an
477 advantageous strategy within the permanent chemocline where potential prey would be abundant
478 and particulate organic carbon from the upper photic zone may accumulate. In support of this
479 suggestion, Roberts et al. (24) reported that HNF dominated heterotrophic protozoa in Lake
480 Bonney and peaked within the permanent chemocline of the west lobe of Lake Bonney.
481
482 A second highly abundant SAG (n=22) within the Stramenopile supergroup exhibited a relatively
483 high proportion of chloroplast sequences within the 16S rRNA gene sequence libraries (Figs. 6
484 and S3 in supplemental material). These SAGs were recovered from Plate 4, which exhibited
485 relatively high green fluorescence and intermediate levels of autofluorescence, and were closely
486 related to a parasitoid nanoflagellate, *Pirsonia* sp. (Figs. 1 and 2). Parasitoid protists comprise a
487 diverse taxonomic group which are thought play major roles in controlling their prey abundance;
488 however, their role in microbial food webs is poorly understood (75). Plastid sequences
489 recovered from the *Pirsonia* SAGs were all related to *Chlamydomonas* chloroplast sequences

(Fig. 6a); however, unlike *Pteridomonas*, *Pirsonia* is not known to harbor a vestigial plastid. Therefore, the presence of plastid sequences within the 16S rRNA gene sequencing libraries suggested that *Pirsonia* potentially interacts with *Chlamydomonas* in a predator-prey relationship. However, microscopic examination indicated that the average size of *Pirsonia* was ~5 µm which was noticeable smaller than *Chlamydomonas* (~15 µm; Fig. 6b, c). Past studies have observed *Pirsonia* species are host specific for marine centric diatoms. The feeding mechanism involved attachment to a host diatom cell, formation of a trophosome inside the host cell and transfer of digested material into the parasite cell (58, 76). We observed an extracellular organelle on the Dry Valley *Pirsonia* which likely functions as part of this feeding mechanism (Fig. 6b, arrow). While Diatoms are abundant in the ephemeral streams and microbial mats around the Dry Valleys (77-79), they are rare in MCM lake water columns (28, 29, 31). To our knowledge there are no reports of *Pirsonia* interacting with chlorophytes; however, we observed numerous *Pirsonia* cells attached to *Chlamydomonas* cells (Fig. 6 d and e), which is a critical step for infection of host cells.

Phytoplankton-bacteria interactions are well known in aquatic environments and are largely driven by the dependence of heterotrophic bacteria on the production of alga-derived organic substrates in the form of extracellular phytoplankton products or decaying algal biomass. There is clear evidence that phytoplankton community distribution and dynamics influences specific bacterial assemblages (80-82). Algal-bacteria interactions involve either free-living, non-particle attached communities of heterotrophic bacteria or cell-to-cell contact between specific bacteria and algal hosts: bacteria groups associated with either interaction appear to be significantly different from each other (83, 84). Bacterioplankton can exhibit differential abilities for

incorporation of specific algal-derived substrates (85). The Dry Valley lakes are essential closed systems for most of the year, and thus heterotrophic bacteria are heavily reliant on newly fixed carbon from phytoplankton production. Recent studies based on environmental sequencing of phytoplankton and bacterial communities have shown that spatial distribution of planktonic communities strongly varies within and between lakes (28, 29, 31); however, interactions between MCM phytoplankton and heterotrophic bacteria have not been resolved. In this present study, SAGs of either the chlorophyte *Chlamydomonas* sp. ICE-MDV or the haptophyte *Isochrysis* sp. MDV were associated with a number of bacterial OTUs (Figs. 5 and S3 in supplemental material). In general, the evidence for strong algal-bacteria interactions was lacking. However, we did note two potential bacterial associations with the Lake Bonney phototrophic SAGs. First, a significant proportion of *Chlamydomonas* ICE-MDV single cells were associated with OTUs related to the Methylobacteriaceae. Members of this group are obligate methylotrophs that can oxidize a number of methylated compounds including methanol and methylamines. These organisms are relatively abundance in marine environments, particularly during phytoplankton blooms (86, 87). Many marine microalgae produce a number of single-carbon compounds as osmolytes (88); however, less is known about algal-methylotroph interactions in freshwater environments (89). While the presence of methylotrophy in the MCM lakes has not been reported, it seems probable that algal production of osmolytes would be a likely adaptation to survive harsh conditions such as low temperatures combined with high salinity.

In contrast with the *Chlamydomonas* sp. ICE-MDV SAGs, *Isochrysis* sp. MDV SAGs were not generally associated with methylotrophic bacterial sequences. Instead, the majority of haptophyte

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single cells were associated with a number of bacterial sequences from Flavobacteriaceae (see Fig. S3 in supplemental material). In the marine environment, *Flavobacteria* represent a dominant Bacteriodetes which breakdown complex organic matter and biopolymers by either direct attachment to algal cells or algal-derived detritus (90, 91). Haptophyte blooms in the Southern ocean are associated with peaks in abundance in *Flavobacteria* (92), and sequences related to *Flavobacteria* are highly abundant in the bacterioplankton communities residing in the chemocline of Lake Bonney (31). Our work supports a putative interaction between Lake Bonney haptophytes and *Flavobacteria*.

The relatively high number of bacterial OTUs associated with the phototrophic SAGs could also represent evidence of low algal-bacterial coupling in the MCM lakes. Phytoplankton exudate quantity and composition are influenced by environmental factors including nutrient limitation, light availability and temperature (91, 93). The conditions of the MCM lakes (*e.g.*, limited light and nutrients, low temperatures) may have selected for algal species which excrete a low fraction of algal-derived compounds, and thus bacterioplankton rely more heavily upon exudate release during algal lysis or death. In support of this latter hypothesis, we have observed heavy recruitment and colonization of bacteria on dead and dying algal cells in our enrichment cultures; an interaction which would have been excluded in the FACS sorting. There is also evidence that natural bacterioplankton communities obtain a significant fraction of organic carbon from ancient, relict pools of organic matter (94).

CONCLUSIONS

Microbial eukaryotes represent the majority of diversity across the eukaryotic tree of life and are capable of complex metabolic modes and interactions. While important in marine ecosystems, their influence reaches profound levels in aquatic systems where the microbial loop dominates. In our current study, we discovered that the MCM protists possess a diverse array of metabolic capabilities (pure photosynthetic, to mixotrophy, heterotrophy and parasitism) which likely contributes to the strong vertical layering of key protists communities in the water column of Lake Bonney. Metabolic versatility of MCM protists underpins specific microbe-microbe interactions in some cases (*e.g.*, *Pteridomonas*-chlorophyte), while for other protists (*e.g.*, haptophytes), interactions with heterotrophic bacteria do not appear to be an important survival strategy.

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851

852 **Figure Legends:**

853 **Figure 1.** Sample cytogram showing flow cytometric sort regions for microbial eukaryotes from
854 an enrichment culture (MCM87) generated from the Antarctic Dry Valley Lake Bonney. Prior to
855 sorting the sample was stained with LysoTracker and samples were gated on green fluorescence
856 (LysoTracker-stained food vacuoles) vs. red fluorescence (chlorophyll autofluorescence). Ovals
857 indicate groups that were selected for single cell sorting. Colors represent high chlorophyll
858 autofluorescence (red), high LysoTracker fluorescence (green) or intermediate of either (blue).
859 P1 – P4, sorted 96-well plates 1 to 4.

860

861 **Figure 2.** Maximum-likelihood tree (1000 bootstrap) showing identity of Lake Bonney microbial
862 eukaryote single amplified genomes (EUK-SAGS) recovered from enrichment culture MCM87.
863 SILVA and GenBank data bases were used to assign phylogenetic position of EUK-SAGS.
864 Colors of the SAG sequences are correlated with Figure 1 sorting parameters. * indicates
865 sequences from the natural eukaryote populations in Lake Bonney generated in a previous study
866 (28). Genbank accession numbers for EUK-SAG 18S rRNA gene sequences are: KU196097-
867 KU196166.

868

869 **Figure 3.** Confocal microscopic images of *Chlamydomonas* sp. ICE-MDV (a - e) and *Isochrysis*
870 sp. MDV (f - j) isolates stained with LysoTracker Green and DAPI. a and f, DIC images; b and g,
871 LysoTracker fluorescence; c and h, DAPI fluorescence; d and i, chlorophyll autofluorescence; e
872 and j, overlays of a – d and f – i respectively. Bars indicate 5 µm.

873

874 **Figure 4**. Principle Coordinates Analysis (PCoA) of weighted UniFrac distances of 16S rRNA
875 genes associated with EUK-SAGs of *Isochrysis* sp. MDV (crosses), *Chlamydomonas* sp. ICE-
876 MDV (diamonds), *Pteridomonas* (squares) and *Pirsonia* (circles). Each data point represents one
877 SAG sample. Ovals represent the 50% similarities within each sample group.
878
879 **Figure 5**. SEM micrographs and the diversity of microbial partners associated with the
880 heterotrophic nanoflagellate *Pteridomonas* (n=22). a. Diversity of 16S rRNA gene OTUs
881 recovered from *Pteridomonas* EUK-SAGs. b-d, SEM images of *Pteridomonas* sp. cells from the
882 enrichment culture. Bars: 1µm.
883
884 **Figure 6**. Evidence of parasite-host interactions between Lake Bonney microbial eukaryotes. a,
885 Diversity of 16S rRNA gene OTUs recovered from *Pirsonia* EUK-SAGs (n=22). b, c, SEM
886 images showing the size comparison of *Pirsonia* vs. *Chlamydomonas* sp. ICE-MDV,
887 respectively. The arrow indicated the extrusive organelle of *Pirsonia*. d, e, SEM images showed
888 *Pirsonia* cells attached to *Chlamydomonas* sp. ICE-MDV. Bars: 5 µm.
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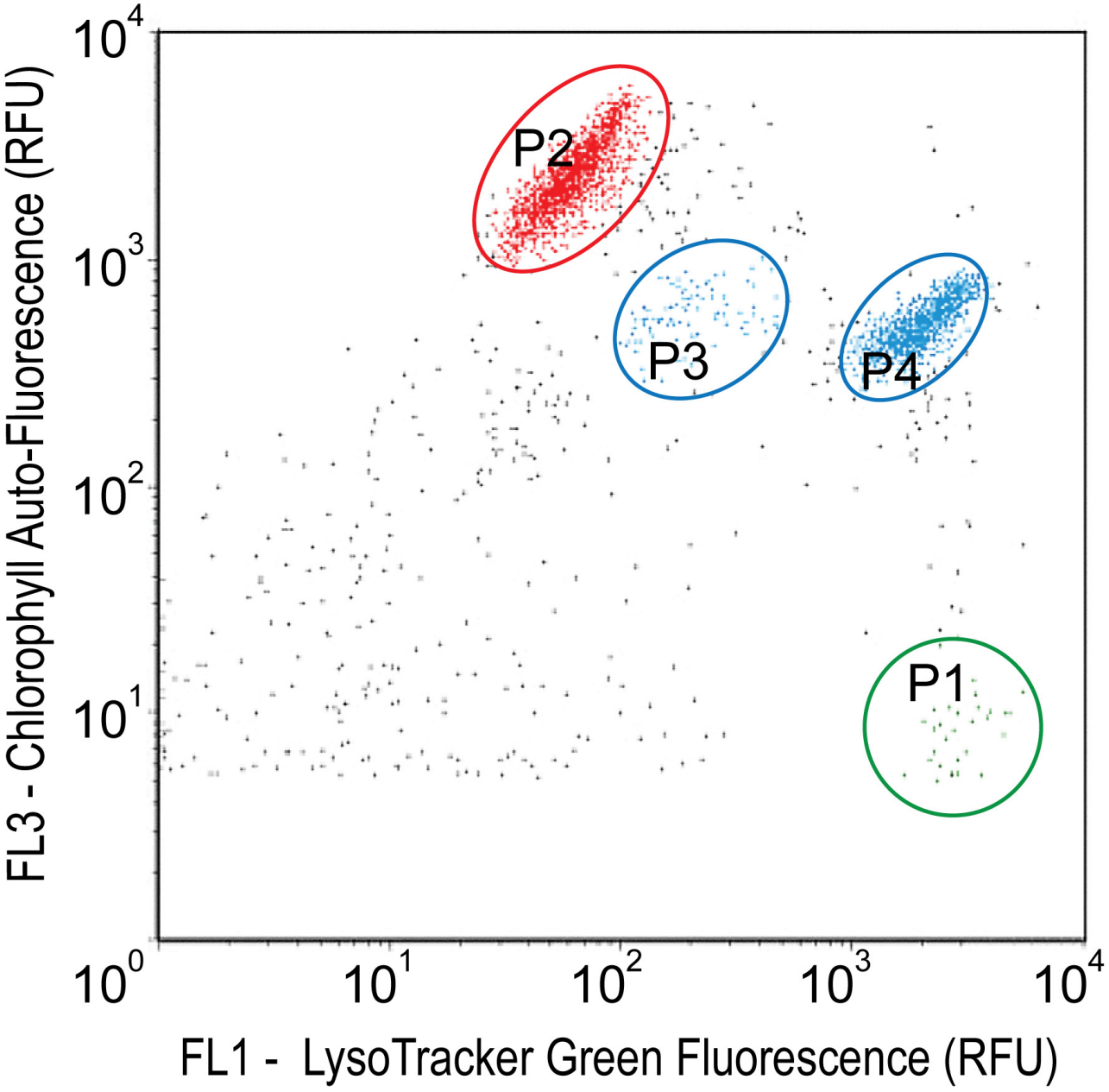
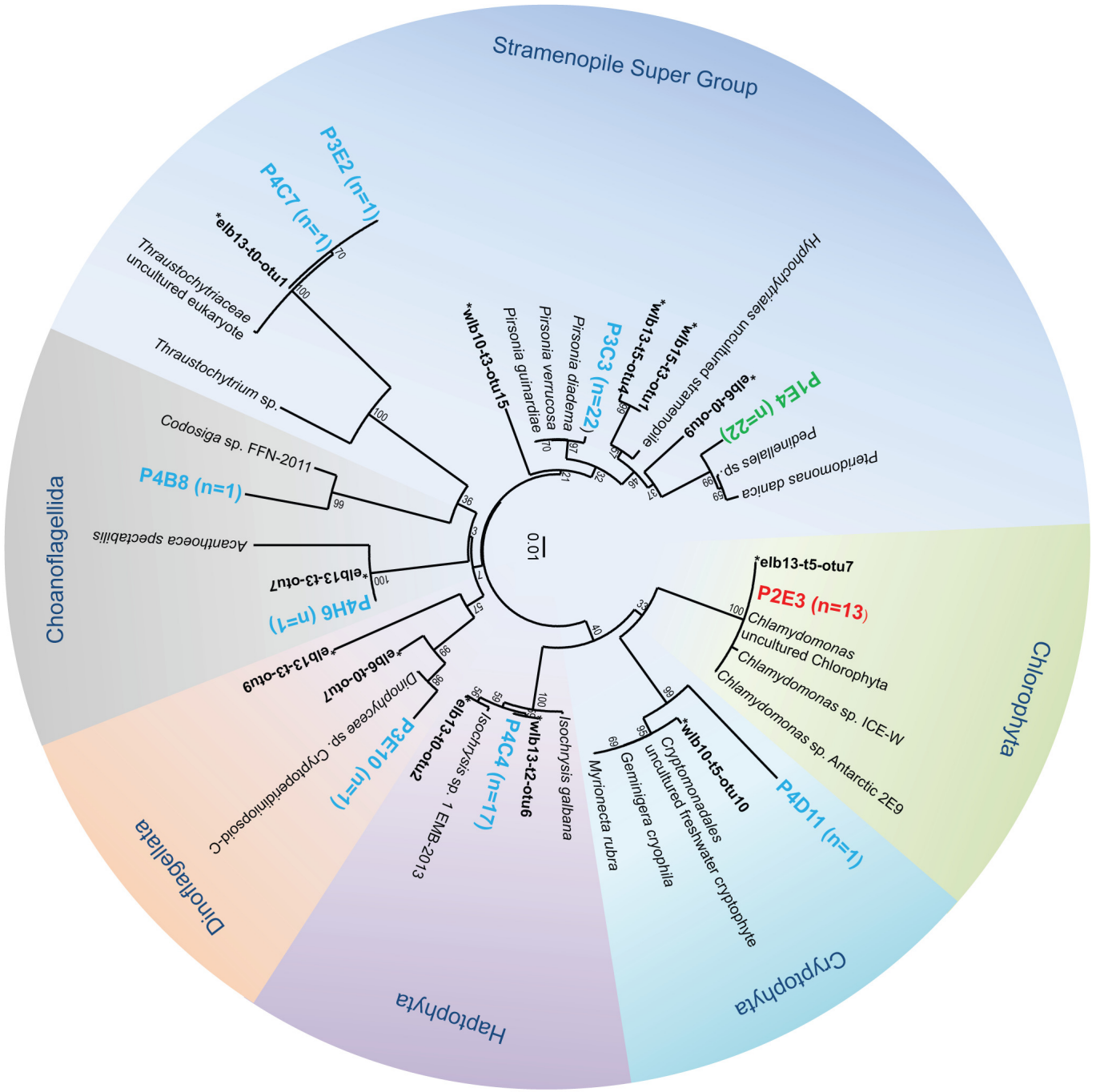
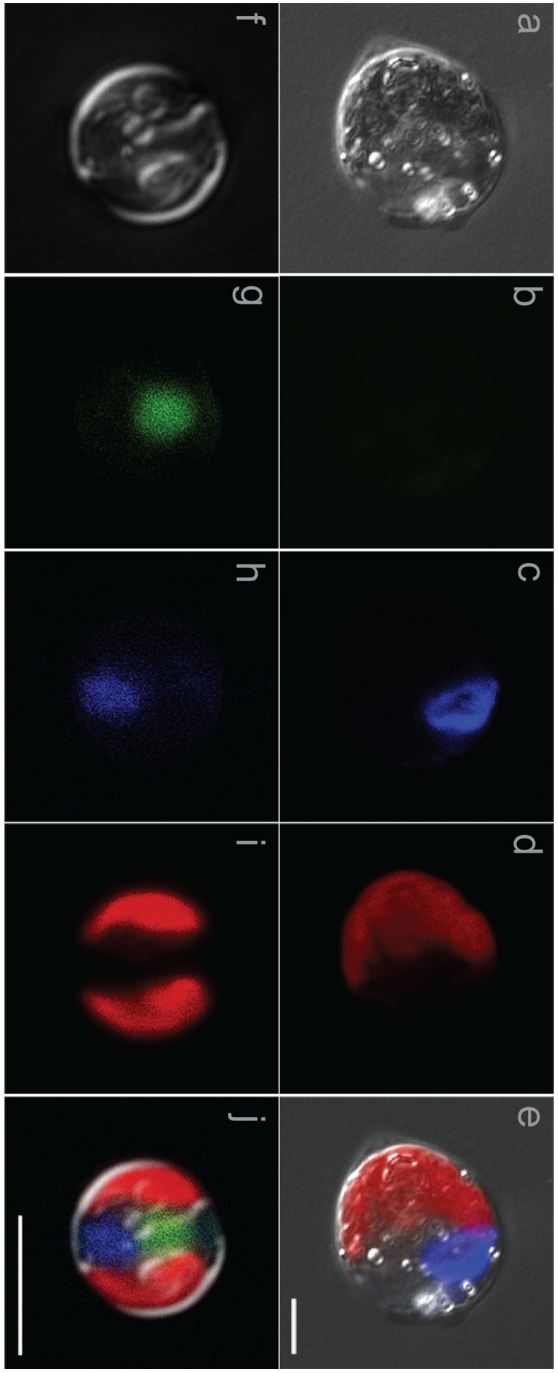



Stramenopile Super Group
Choanoflagellida
Dinoflagellata
Haptophyta
Cryptophyta
Chlorophyta
0.01
P3E2 (n=1)
P4C7 (n=1)
*elb13-t0-otu1
Thraustochytriaceae uncultured eukaryote
Thraustochytrium sp.
Codosiga sp. FFN-2011
P4B8 (n=1)
Acanthoeca spectabilis
*elb13-t3-otu7
P4H6 (n=1)
*elb13-t3-otu9
*elbb-t0-otu7
Dinophyceae sp. Cryptoperidiniopsoid-C
P3E10 (n=1)
*elb13-t0-otu2
Isochrysis sp. 1 EMB-2013
P4C4 (n=17)
*wlb13-t2-otu6
Isochrysis galbana
*wlb10-t3-otu15
Pirsonia guinardiae
Pirsonia verrucosa
Pirsonia diadema
P3C3 (n=22)
*wlb13-t5-otu4
*wlb15-t3-otu1
Hyphochytriales uncultured stramenopile
*elbb-t0-otu9
P1E4 (n=22)
Pedinellales sp.
Pteridomonas danica
*elb13-t5-otu7
P2E3 (n=13)
Chlamydomonas uncultured Chlorophyta
Chlamydomonas sp. ICE-W
Chlamydomonas sp. Antarctic 2E9
*wlb10-t5-otu10
Cryptomonadales uncultured freshwater cryptophyte
Geminigera cryophila
Myrionecta rubra
P4D11 (n=1)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