

SHORT COMMUNICATION

Unveiling *in situ* interactions between marine protists and bacteria through single cell sequencing

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Heterotrophic protists are a highly diverse and biogeochemically significant component of marine ecosystems, yet little is known about their species-specific prey preferences and symbiotic interactions *in situ*. Here we demonstrate how these previously unresolved questions can be addressed by sequencing the eukaryote and bacterial SSU rRNA genes from individual, uncultured protist cells collected from their natural marine environment and sorted by flow cytometry. We detected *Pelagibacter ubique* in association with a MAST-4 protist, an actinobacterium in association with a chrysophyte and three bacteroidetes in association with diverse protist groups. The presence of identical phylotypes among the putative prey and the free bacterioplankton in the same sample provides evidence for predator–prey interactions. Our results also suggest a discovery of novel symbionts, distantly related to Rickettsiales and the candidate divisions ZB3 and TG2, associated with Cercozoa and Chrysophyta cells. This study demonstrates the power of single cell sequencing to untangle ecological interactions between uncultured protists and prokaryotes.

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Marine protists have a variety of fundamental ecological roles and comprise organisms with highly diverse evolutionary histories and lifestyles (Massana *et al.*, 2006; Worden *et al.*, 2006; Harada *et al.*, 2007; Worden and Not, 2008; Zubkov and Tarran, 2008; Not *et al.*, 2009; Heywood *et al.*, 2011). Phototrophic protists contribute significantly to phytoplankton biomass and primary production (Jardillier *et al.*, 2010), whereas heterotrophic protists have critical roles in microbial food webs by grazing on picoplankton, channeling organic carbon to higher trophic levels, controlling bacterial abundance and remineralizing nutrients (Azam *et al.*, 1983; Fuhrman and McManus, 1984; Pernthaler, 2005; Worden and Not, 2008; Massana *et al.*, 2009). Mixotrophy is also common among marine protists (Zubkov and Tarran, 2008). Furthermore, symbiotic relationships between prokaryotes and marine protists may be prevalent, but their studies so far have been limited to a few cultured model systems (Kneip *et al.*, 2008). Because of difficulties

in the cultivation of heterotrophic protists, little is known about the species-specific prey preferences of the predominant protists or the frequency and the nature of symbiotic relationships in their natural environment. Studies performed to date, using model prey and/or predator cells, suggest interactions between diverse microbial taxa (Fu *et al.*, 2003; Jezbera *et al.*, 2005; Massana *et al.*, 2009). However, to the best of our knowledge, identification of both the protistan predator and its natural prey or symbiont under *in situ* conditions has not been accomplished to date, because of methodological limitations. This represents a major gap in our knowledge of microbial ecology.

Here we present a new approach to disentangle the complexity of protist–prokaryote interactions by sequencing SSU rRNA genes of individual protists and the prokaryotes that are physically associated with them. We collected a coastal water sample from 1 m depth in Boothbay Harbor in the Gulf of Maine, USA (43°50′39.87″N 69°38′27.49″W) on 19 July 2009 and incubated it with Lysotracker Green as described in detail (Rose *et al.*, 2004). Lysotracker is a green fluorescing probe that stains food vacuoles in protists and has been previously used to detect heterotrophic protists from natural assemblages (Rose *et al.*, 2004; Heywood *et al.*, 2011). Individual cells displaying Lysotracker fluorescence were

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deposited into 384-well microplates by flow cytometric cell sorting, lysed using cold KOH and subjected to whole-genome multiple displacement amplification as described in detail in the Supporting Information. We refer to the multiple displacement amplification products from the sorted single cells as single amplified genomes (SAGs) that included amplified genomic DNA from prokaryotes associated with the sorted protists. The multiple displacement amplification products served as templates in real-time PCR, separately targeting the eukaryotic and the bacterial SSU rRNA genes (Supplementary Tables S1 and S2). The PCR amplicons were directly sequenced using Sanger technology. To compare the composition of bacteria associated with protists to the free bacterioplankton composition, we also generated SAGs of single bacterioplankton cells sorted from the same seawater sample (Supplementary Information).

Two optically distinct groups of small protists (<20 µm) displaying Lysotracker fluorescence were detected by flow cytometry and sorted separately (Figure 1). One of the groups displayed only Lysotracker (green) fluorescence, typical of heterotrophic protists (Heywood *et al.*, 2011), whereas another group showed both Lysotracker fluorescence and red autofluorescence from chlorophyll. The latter group may have been composed of mixotrophs and/or heterotrophs with ingested chlorophyll-containing prey. We operationally define them here as 'mixotrophic protists'. For each of these protist populations, 315 SAGs were generated and analyzed by PCR amplification and sequencing of their 18S and 16S rRNA genes (Supplementary Tables S1 and S2). We successfully sequenced the 18S rRNA gene from 47 and 38 SAGs from the putative heterotrophic and mixotrophic protists, respectively (Supplementary Table S2). Chloroplast

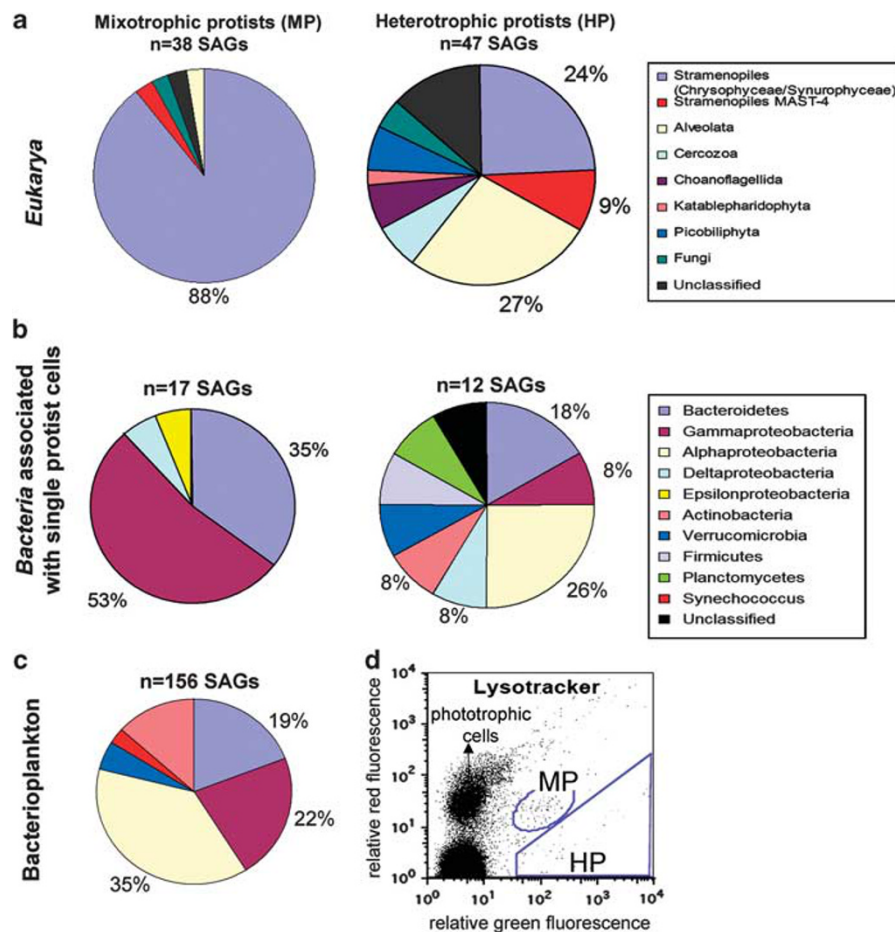


Figure 1 (a) Taxonomic composition of SAGs generated from the putative heterotrophic and mixotrophic protist populations and (b) the corresponding bacterial diversity of 16S rRNA genes found in protist cells. Key DNA tools (<http://keydnatools.com/>) and ARB software (Pruesse *et al.*, 2007) were used to assign phylogenetic position of SAGs. All sequences were screened for chimeras by Mallard 1.02 (Ashelford *et al.*, 2006) and Pintail 1.01 (Ashelford *et al.*, 2005). (c) Taxonomic composition of marine bacterioplankton SAGs generated from the same water sample used for the protist sorting. (d) Flow cytometric sort regions for the putative mixotrophic (MP) and heterotrophic protist (HP) populations. Putative heterotrophs showed high green fluorescence from Lysotracker-stained vacuoles, whereas putative mixotrophs displayed both green and red fluorescence from Lysotracker and chlorophyll. The large population in the lower left is noise and/or heterotrophic bacteria. Side scatter was used to limit the sort to cells that were < ~20 µm in diameter (data not shown). Genbank accession numbers are as follows: 18S rRNA gene (JF488764–JF488851), 16S rRNA bacterial gene (JF488634–JF488667), 16S rRNA mitochondrial gene (JF488737–JF488763) and 16S rRNA chloroplast gene (JF488668–JF488736).

SSU rRNA genes were detected in 66 SAGs of the putative mixotrophs (21% of total SAGs), further supporting a mixotrophic lifestyle (Supplementary Table S2). No chloroplast sequences were recovered from the putative heterotrophs, confirming the specificity of our analytical techniques. Mitochondrial 16S rRNA gene sequences were recovered from five putative heterotrophs and 22 putative mixotrophs. The putative mixotrophs were composed mainly of chrysophytes (88% of SAGs), whereas the putative heterotrophs were highly diverse (Figure 1), consistent with our previous study on heterotrophic protists in the Gulf of Maine (Heywood *et al.*, 2011).

Bacterial 16S rRNA gene sequences were recovered from 12 and 17 putative heterotrophic and mixotrophic protists, respectively. In most cases

they were similar to those recovered from free bacterioplankton SAGs, with the predominance of *Gammaproteobacteria*, *Bacteroidetes* and *Alphaproteobacteria* (Figure 1). Among the represented bacterial classes, only the frequency of *Gammaproteobacteria* was significantly higher in mixotrophic protist SAGs as compared with free bacterioplankton SAGs (α selectivity index for predator food preference = 0.8 (Cheson, 1983; Jezbera *et al.*, 2006); Z-test for two proportions, $P=0.02$). This suggests that *Gammaproteobacteria* were more likely than other bacterial classes to be grazed by (or attached to) protists in the studied marine sample.

We recovered both eukaryote and bacterial SSU rRNA gene sequences from seven SAGs, in this way obtaining direct evidence for their *in situ* ecological interaction (Figure 2). In five of these cases,

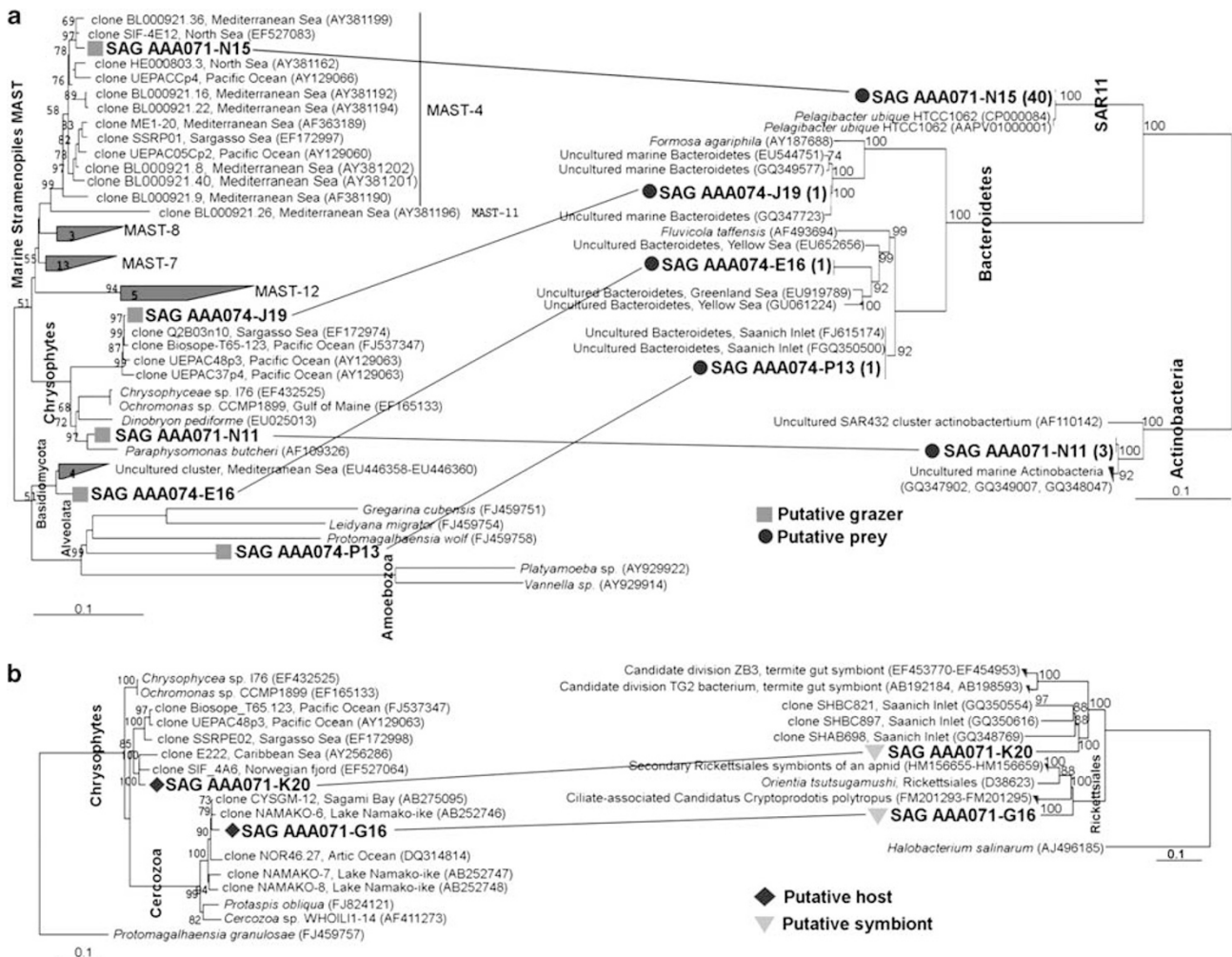


Figure 2 Best maximum likelihood trees (1000 bootstrap) showing the taxonomic identities for both the protist (left) and the associated bacteria (right) obtained by single cell sequencing. Bootstrap values ≥ 50 are displayed. Phylogenetic analysis was performed with RAxML version 7.0.3 (Stamatakis, 2006) implemented in ARB package using the reference ARB database 102 (<http://www.arb-silva.de>). The core tree was calculated with the closest reference sequences and then partial eukaryotic and bacterial sequences from SAGs (736–949 nucleotide positions) were added using the ARB parsimony tool. SAG names ‘AAA071’ indicate SAGs generated from the putative heterotrophic protist population, whereas ‘AAA074’ were from the putative mixotrophic protist population. (a) Phylogenetic tree showing the identity of both the protist grazer and its putative bacterial prey. In parentheses is the number of SAGs with the identical 16S rRNA gene detected in the bacterioplankton SAG library generated from the same water sample used for protist sorting. (b) Phylogenetic tree showing the taxonomic identities of two single cell protists harboring putative bacterial symbionts.

identical 16S rRNA sequences of the protist-associated bacteria were detected in the SAG library of randomly sorted bacterioplankton (Supplementary Table S2). Interestingly, we detected a *Pelagibacter ubique* sequence in one MAST-4 SAG (AAA071-N15), which are among the most abundant groups of marine bacterioplankton and heterotrophic protists, respectively (Massana *et al.*, 2004; Worden and Not, 2008; Not *et al.*, 2009). In addition, *Bacteroidetes* sequences were recovered from SAGs representing a widespread, uncultured marine Chrysophyte cluster (SAG AAA074-J19), an alveolate belonging to the Apicomplexa (AAA074-P13) and one protist related to an uncultured, widespread Basidiomycota cluster (AAA074-E16), previously found in oxygen-depleted regions of the Mediterranean and Arabian Seas. Finally, one protist SAG (AAA071-N11), phylogenetically related to the known heterotroph *Paraphysomonas butcheri*, contained a 16S rRNA gene related to *Actinobacteria* clones found in other coastal areas, and the *Actinobacteria* SAR432 cluster. Although *Bacteroidetes* are often found associated with (Jasti *et al.*, 2005) or attached to (Sapp *et al.*, 2007) phytoplankton, we are not aware of such associations being reported between bacteria and heterotrophic protists. Furthermore, *Pelagibacter* and SAR432 are known free-living taxonomic groups and their finding in/on protists most likely is a result of protist grazing activity. Thus, we interpret these results as a likely indication of bacteria being recently ingested by the sorted protists. Further studies will be required to unequivocally determine whether these associations represent predator–prey interactions or bacterial attachment on the protist cell surfaces.

Protist SAGs AAA071-K20 (Chrysophyta) and AAA071-G16 (Cercozoa) contained bacterial 16S rRNA genes that were not related to known marine bacterioplankton. In fact, the closest entries in Genbank displayed <89% of the SSU rRNA gene similarity and belonged to Rickettsiales and the candidate divisions ZB3 and TG2. The latter group is formed by endosymbionts of aphids and termites (Figure 2b), whereas rickettsia are often intracellular parasites or pathogens of different hosts, including protists (Ferrantini *et al.*, 2009). This suggests the discovery of novel symbionts/parasites in the uncultured, widely distributed marine picoeukaryote groups, although further verification is needed to confirm these findings.

The use of single cell sequencing to assess the composition of natural heterotrophic protist assemblages was recently proven a reliable approach (Heywood *et al.*, 2011). Because of the stringent cell sorting conditions applied here, random co-sorting of multiple cells is unlikely, as demonstrated in prior studies using our methodology (Stepanuskas and Sieracki, 2007; Woyke *et al.*, 2009; Heywood *et al.*, 2011; Fleming *et al.*, 2011; Hess *et al.*, 2011). The limitations of the method applied here include

primer-target mismatches in the PCR screens of SAGs, the presence of PCR template secondary structures (Potvin and Lovejoy, 2009), incomplete cell lysis and uneven amplification of genomic DNA regions during multiple displacement amplification (Zhang *et al.*, 2006; Woyke *et al.*, 2009). All these factors may contribute to the relatively low observed frequency of 18S and 16S rRNA gene co-recovery from the same SAG. Other plausible causes include rapid degradation of prey DNA in food vacuoles, and the competition of bacterial and organelle 16S rRNA genes in PCR reactions. It is also important to recognize that some protists may have had multiple prokaryote taxa associated with them, which would have been detected only by a deep sequencing of the 16S rRNA genes or genomic DNA of individual SAGs.

In this pilot study, we demonstrate how single cell sequencing enables the analysis of ecological interactions between uncultured protists and prokaryotes, opening novel opportunities to examine microbial grazing and symbiotic relationships *in situ*. The PCR-based SAG sequencing used here should be viewed as the first step, enabling the formulation of specific hypotheses, which can be tested by complementary studies, such as protist SAG whole genome sequencing, fluorescence *in situ* hybridization of detected groups of interest and manipulative experiments.

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Supplementary Information accompanies the paper on The ISME Journal website (<http://www.nature.com/ismej>)