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Diversity of Heterotrophic Protists from Extremely Hypersaline Habitats

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Heterotrophic protists (protozoa) are a diverse but understudied component of the biota of extremely hypersaline environments, with few data on molecular diversity within halophile 'species', and almost nothing known of their biogeographic distribution. We have garnered SSU rRNA gene sequences for several clades of halophilic protozoa from enrichments from waters of >12.5% salinity from Australia, North America, and Europe (6 geographic sites, 25 distinct samples). The small stramenopile *Halocafeteria* was found at all sites, but phylogenies did not show clear geographic clustering. The ciliate *Trimyema* was recorded from 6 non-European samples. Phylogenies confirmed a monophyletic halophilic *Trimyema* group that included possible south-eastern Australian, Western Australian and North American clusters. Several halophilic Heterolobosea were detected, demonstrating that *Pleurostomum* contains at least three relatively distinct clades, and increasing known continental ranges for *Tulamoeba peronaphora* and *Euplaesiobystra hypersalinica*. The unclassified flagellate *Palustrimonas*, found in one Australian sample, proves to be a novel deep-branching alveolate. These results are consistent with a global distribution of halophilic protozoa groups (~morphospecies), but the *Trimyema* case suggests that is worth testing whether larger forms exhibit biogeographic phylogenetic substructure. The molecular detection/characterization of halophilic protozoa is still far from complete at the clade level, let alone the 'species level'.

Key words: Diversity; halophiles; microbial eukaryotes; molecular phylogeny; protozoa.

Introduction

Hypersaline habitats near or above 150 ‰ salinity are a type of extreme environment that is widely though sparsely distributed across the Earth, and that harbors a diverse biota adapted to high salinity waters (Javor 1989; Oren 2002). Extremely halophilic or halotolerant microorganisms capable of growth under these conditions are found across all domains of life (Kushner 1978). Halophilic bacteria are spread over 50 genera including *Salinibacter* and *Halomonas*, while halophilic archaea are distributed into 20 genera, mostly in the clade *Haloarchaea* (Oren 2002). The diversity of microbial eukaryotes is more poorly understood. The best known halophiles are photosynthetic algae, especially *Dunaliella* spp., and several forms of fungi (Javor 1989; Plemenitaš and Gunde-Cimerman 2005), but there is also a considerable

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if little-studied diversity of extremely halophilic or halotolerant protozoa (Cho 2005; Hauer and Rogerson 2005; Park et al. 2009; Ruinen 1938). For example, >25 apparently distinct morphospecies of protozoa have been observed even at ~300 ‰ salinity, albeit mostly in microscopy surveys of natural samples or crude enrichments (representing ~50 distinct sampling sites in total; see Supplementary Material table 1 in Park et al. 2009). Meanwhile, Galotti et al. (2014) reported 10 species of ciliate from a ~150 ‰ salinity dilution of brine from a single salt pan (and many more species from lower salinity dilutions from several salt pans). Most free-living protozoa are phagotrophs that directly consume prokaryotes and other microbes (Laybourn-Parry 1992), so the potential or actual presence of protozoa in the most extreme categories of hypersaline habitats has important consequences for understanding the microbial ecology of these systems, especially if protozoa are abundant, as has been observed at least occasionally (Elloumi et al. 2009; Park et al. 2003).

Over several years, a number of obligately halophilic or highly halotolerant protozoa have been cultured from hypersaline environments and characterized using rRNA gene sequencing and (usually) electron microscopy examinations (Cho et al. 2008; Foissner et al. 2014; Kirby et al. 2015; Park et al. 2006, 2007, 2009; Park and Simpson 2011). They form at least 8 different genera, and belong to several distinct groups, including Stramenopiles (*Halocafeteria*), several groups of ciliates (*Trimyema, Fabrea*, and *Platynematum*), and several clades within Heterolobosea (*Euplaesiobystra*, *Pharyngomonas*, and the *Pleurostomum*/*Tulamoeba* clade). The obligately halophilic protozoa cultured to date are genetically distinct from freshwater or marine protozoa, and often considered separate genera (Cho et al. 2008; Park et al. 2006, 2007, 2009; Park and Simpson 2011). Also, culture-independent sequencing-based studies of Mediterranean deep hypersaline anoxic basins (DHABs), the extremely hypersaline Lake Tyrrell in south-eastern Australia, and extremely hypersaline ponds in Spain and Chile have uncovered a number of 18S rRNA gene phylotypes representing protozoa that have not been detected in non-hypersaline samples (Alexander et al. 2009; Edgcomb et al. 2009; Heidelberg et al. 2013; Stock et al. 2012; Triadó-Margarit and Casamayor 2013). However, there is little overlap between the halophilic protozoa identified to date using culturing and the probable halophiles detected in sequence surveys, indicating that our knowledge of the diversity of halophilic protozoa remains poor. Furthermore, most of these recent studies of halophilic protozoa have used material from a single geographic region, or rarely two regions. As a result, there are few data on the sequence variation within the known groups of obligately halophilic protozoa, and almost nothing known about their global distribution, whether considering the level of groups (~genera), of morphospecies, or of sequence-defined phylotypes. Since extremely hypersaline habitats are very small and sparse relative to marine, freshwater or soil systems, it is unclear whether generalisations about protist biogeography based on marine, freshwater or soil systems would necessarily apply.

In this study we investigated halophilic protozoa enriched from six sampling sites across western North America (California), south-eastern Australia (specifically Whyalla, in eastern South Australia), Western Australia, and central Europe (Poland), using 18S rRNA gene sequencing and light microscopy. At >125‰ salinity, all organisms identified on phenotype-based and molecular criteria, except one, were closely related to *Halocafeteria*, *Trimyema*, *Pleurostomum*, *Tulamoeba* or *Euplaesiobystra*. Our data support the broad geographic spread of these halophilic groups (i.e. genera and/or morphospecies). A clear biogeographic signal was absent from internal phylogenies of the small flagellate *Halocafeteria*, but phylogenies of halophilic *Trimyema*, a ciliate which is several times larger, are consistent with biogeographic structuring, although our sampling is too limited to rigorously test this. We also obtained gene sequence data for the unclassified flagellate *Palustrimonas*, which proves to be a deep-branching alveolate and to represent a previously undocumented lineage of probable halophiles.

Results

Light Microscopy

Halocafeteria group

Cells were observed in cultures derived from 14 extremely hypersaline subsamples (260-322‰ or salt) from south-eastern Australia (Whyalla), Western Australia (Hutt Lagoon and Shark Bay), Europe (Wieliczka salt mine, Poland), and North America (Salton Sea periphery and Chula Vista solar saltern; Table 1). *Halocafeteria* cells were infrequently observed below 150‰ salinity (Table 1; unpublished observations). Cells were ovoid, 4-6 μm long and 2-4 μm wide, with two sub-equal flagella, each 1.5-2 times cell-length (Fig. 1 A-N). Cells usually either swam erratically with both