

# Microbial eukaryote life in the new hypersaline deep-sea basin Thetis

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**Abstract** Only recently, a novel anoxic hypersaline (thalassic) basin in the eastern Mediterranean was discovered at a depth of 3,258 m. The halite-saturated brine of this polyextreme basin revealed one of the highest salt concentrations ever reported for such an environment (salinity of 348‰). Using a eukaryote-specific probe and fluorescence in situ hybridization, we counted  $0.6 \times 10^4$  protists per liter of anoxic brine. SSU rRNA sequence analyses, based on amplification of environmental cDNA identified fungi as the most diverse taxonomic group of eukaryotes in the brine, making deep-sea brines sources of unknown fungal diversity and hotspots for the discovery of novel metabolic pathways and for secondary metabolites. The second most diverse phylotypes are ciliates and stramenopiles (each 20%). The occurrence of closely related ciliate sequences exclusively in other Mediterranean brine basins suggests specific adaptations of the respective organisms to such habitats. Betadiversity-analyses confirm that microeukaryote communities in the brine and the interface are notably different. Several distinct morphotypes in

brine samples suggest that the rRNA sequences detected in Thetis brine can be linked to indigenous polyextremophile protists. This contradicts previous assumptions that such extremely high salt concentrations are anathema to eukaryotic life. The upper salinity limits for eukaryotic life remain unidentified.

**Keywords** Microbial eukaryotes · Brine basins · SSU rRNA · Protistan cell counts · Community analyses

## Introduction

Salinity is an ecological factor of considerable importance when exploring the limits known for life. High salinities impose cellular stress and create a series of challenges for organisms that have adapted to withstand a wide range of salinities (euryhaline species) or that even require high salt concentrations for survival (halophiles). Above all, euryhaline and halophilic organisms have to cope with tremendous osmotic pressure and low water activity, both of which are linked to each other. In high salt environments, non-adapted organisms lose intracellular cytoplasmic water, because water flows from higher to lower chemical potential until the gradient between the cell and the surrounding medium is abolished. In high salt environments cells may lose up to 80% of their total water content, leading to anhydrobiosis with typically fatal consequences due to irreversible phase changes, such as denaturation and breakage that occur in lipids, proteins and nucleic acids (Cox 1993). Therefore, salinity is a major barrier for unicellular eukaryotes. Inhabitants of high-salt environments have developed two principal mechanisms to guard against ambient environmental conditions. The first, described as the salt-in-cytoplasm strategy, raises the salt concentration

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in the cytoplasm and adapts the intracellular protein chemistry to these high salt concentrations. The organic-osmolyte strategy reduces the chemical potential of the cell water and keeps the cytoplasm largely free of NaCl in order to maintain an unchanged design of the cell's interior. Both strategies are described in detail in Kunte et al. (2002).

Our knowledge of the biology (including for example physiology, ecology, evolution and diversity) of halophiles is mostly based on data from studies of Archaea and Bacteria. But the taxonomic range of salt-loving organisms also spans the third domain of life, namely Eukarya, specifically microbial eukaryotes including protists and fungi. The first records of protists from hypersaline environments date back to the nineteenth century (Enz 1879; Butschinsky 1897; Florentin 1899), recognizing the diversity of unicellular eukaryotes able to live in high-salt environments like salterns, salt lakes and marine systems with salt concentrations higher than the typical marine salinity of ca. 3%. Since then, an admittedly low number of eukaryote (compared with bacterial or archaeal) studies in high-salt environments have been conducted. While few of them examined the ecological role of protists (e.g. trophic interactions) in high-salt systems (Pedrós-Alió et al. 2000; Park et al. 2003), most studied the diversity of specific taxon groups with a main focus on ciliates, which due to their large size, are relatively easy to identify morphologically (Wilbert 1995; Esteban and Finlay 2003). Other studies focused on individual organisms isolated from high-salt environments, including, for example, the phototrophic chlorophyte *Dunaliella salina* (Oren 2005), the halophilic heterotrophic flagellates *Halocafeteria seosinensis* (Park et al. 2006) and *Pleurostomum flabellatum* (Park et al. 2007) and the euryhaline ciliate *Fabrea salina* (Henneguy 1890). Such studies have significantly contributed to our knowledge of the ecology of high-salt systems and the biology of some of their inhabitants.

Studies targeting whole protistan communities in high-salt environments, however, are scarce (e.g. Post et al. 1983). A major reason for this might be ignorance of the ecological role protists play even in such extreme habitats (Pedrós-Alió 2004). A lack of such studies has prevented our complete (or at least as full as possible) understanding of protistan community structure and composition in any individual high-saline environmental system, making estimates of protistan diversity in high-saline environments impossible as well as comparisons of protistan community structures between different or similar high-saline habitats. Only recently, molecular based methods, i.e. the phylogenetic analyses of taxonomic marker genes (small subunit ribosomal RNA, SSU rRNA genes) amplified from genomic environmental DNA, were applied to provide initial descriptions of whole protistan communities in hypersaline systems. These studies targeted the hypersaline brine

basins L'Atalante (Alexander et al. 2009) and Bannock and Discovery (Edgcomb et al. 2009) at the bottom of the Eastern Mediterranean Sea and detected molecular signatures from a variety of different protists and fungi, suggesting that even in brines (highly saline water from which salts crystallize) eukaryotic life is not only possible but even diverse.

In this study, we targeted the whole protistan community of a newly discovered basin in the eastern Mediterranean Sea, namely Thetis. The thalassic homoiohaline Thetis brine is among the harshest environments on our planet, not only because it is one of the most saline systems ever reported to date, but also due to lack of oxygen and high pressure (water depth ca. 3,300 m) (La Cono et al. 2011). Therefore, the inhabitants of this environment are not "simple" halophiles, but polyextremophiles. In the current study we set out to gain the first insights into the taxonomic breadth of microbial eukaryotes in the Thetis brine. Therefore, we amplified and analyzed cDNA obtained from environmental samples. In contrast to DNA-based strategies, analyses of SSU rRNA genes obtained from environmental RNA identify active members of indigenous protistan communities (Stoeck et al. 2007; Alexander et al. 2009). We also compared the protistan community in the brine with the one in the overlaying interface, and also with previously surveyed deep hypersaline anoxic basins (DHABs) in the eastern Mediterranean Sea. This is the first study to count and visualize the number of protists in a DHAB brine environment in order to provide proof that the upper salinity limits for eukaryotic life have not yet been established, even for saturated brines.

## Materials and methods

### Sampling site and sampling

For details about the sampling site we refer to the Thetis description by La Cono et al. (2011). In short, the seawater-brine interface of basin Thetis is located at a depth of 3,258 m below sea level. Temperature measured at the interface was 13.97°C, increasing to 15.06°C in the bottom water (3,415 m). At 3,258 m, salinity increases sharply to reach the highest salt concentration of 348 g L<sup>-1</sup> just 2 m deeper and remains that high throughout the brine to the seafloor. Molecular oxygen is undetectable from the middle of the interface starting at 3,259 m depth. Likewise, the redox potential changes from +213 mV in the seawater just above the interface to <−340 mV in the brine, indicating highly reducing conditions. The chemical composition of the brine seems to consist of typical late-stage evaporates with high Br<sup>-</sup> concentrations (6 mmol L<sup>-1</sup>), as well as >80% halite, 12% bischofite, 7% carnallite and 2%

magnesium sulfide (La Cono et al. 2011). Thetis brine is nine times more saline and contains nine times more bromide compared with seawater, which indicates that the brine in basin Thetis like the Bannock brine (Vengosh and Starinsky 1993) originates from ancient interstitial seawater rather than from de novo dissolution of halite (La Cono et al. 2011). Sulfide concentration in the brine is  $2.12 \text{ mmol L}^{-1}$ . Samples were taken in June 2010 aboard *RV Urania* at  $34^{\circ}40'11.34''\text{N}$  and  $22^{\circ}8'43.68''\text{E}$  in the framework of the MAMBA program using 12-L Niskin bottles on a rosette equipped with SBE-911plus conductivity–temperature–depth (CTD) sensors (Sea-Bird Electronics, Inc., Bellevue, WA, USA). For nucleic acid extraction from each sampled horizon (interface at 3,259 m and brine at 3,415 m), ca. 12 L of sample water was collected on Durapore membranes (47 mm, 0.65  $\mu\text{m}$ , Millipore, Germany) under gentle pressure ( $<50 \text{ ml min}^{-1}$ ), using a peristaltic pump (Ecoline ISM 1079, Ismatec, Germany) under  $\text{N}_2$ -atmosphere in case of the lower halocline. Measures were taken to avoid exposure of the samples to atmospheric conditions during filtration as described previously (Alexander et al. 2009). Until further processing of the samples, filters were stored at  $-20^{\circ}\text{C}$  in RNeasy (Ambion, Germany).

#### cDNA library construction

This method has been described in detail previously (Alexander et al. 2009). In short, total RNA was extracted using Qiagen's AllPrep DNA/RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions following a chemo-mechanical cell disruption by bead-beating (45 s, 30 Hz). Residual DNA was removed by DNase I (Qiagen) digestion. The concentration of extracted and purified RNA was determined spectrophotometrically using a Nanodrop ND-1000 UV–vis spectrometer (Nanodrop Technologies, Wilmington, DE, USA). The integrity of the RNA was checked with an RNA 6000 picoassay with an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). To minimize extraction bias we extracted total RNA from three individual filters per depth. Total RNA was then transcribed into cDNA using the Two-Step Omniscript Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. After transcription of each individual sample, the transcribed products were pooled and subjected to SSU cDNA amplification. The HotStart PCR protocol (50  $\mu\text{L}$ -reactions with Qiagen HotStart Taq Polymerase) for eukaryote SSU cDNA amplification employed the primers 528F (Medlin et al. 1988) and 1179R (Lane 1991) resulting in ca. 650 bp fragments. The PCR program consisted of an initial 5-min incubation at  $95^{\circ}\text{C}$ , followed by 35 identical amplification cycles (denaturing at  $95^{\circ}\text{C}$  for 45 s, annealing at  $52^{\circ}\text{C}$  for 45 s, extension at

$72^{\circ}\text{C}$  for 90 s) and a final extension at  $72^{\circ}\text{C}$  for 5 min. To minimize PCR-bias, we ran five individual reactions per depth. As negative controls with each PCR setup we ran extracted RNA prior to transcription (each individual sample was checked for DNA contaminations after RNA extraction) and PCR reactions with PCR-water but without the addition of template cDNA. Prior to clone library construction we pooled all reactions of the same depth during the PCR product purification process (MiniElute PCR purification kit, Qiagen, Germany). Purified PCR products were cloned using pGEM-T-Vector Systems kit (Promega GmbH, Mannheim, Germany) and plasmids were extracted from overnight cultures by LGC Genomics (Berlin, Germany). From the pooled and then amplified samples, we constructed one clone library for Thetis interface and one library for Thetis brine.

#### Sequencing and phylotype grouping

We sequenced 384 clones from Thetis interface and 192 clones from Thetis brine (LGC Genomics) using M13F as the sequencing primer and an Applied Biosystems (ABI) 3730 XL DNA Stretch Sequencer, with the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. Initially, we had the intention to sequence two 96-well plates for each sampling site; however, because one individual ciliate amplicon dominated the Thetis interface clone library, we had to increase our sequencing effort for that sample to obtain adequate taxonomic coverage (see “Results and discussion” section).

After checking sequences for chimeras using KeyDNA Tools (Guillou et al. 2008, results available from the authors upon request) and removal of low-quality sequence reads (too short sequences or sequences with biased nucleotides), sequences were grouped into operational taxonomic units (OTUs) using the software package JAguc (<http://www.wagak.informatik.uni-kl.de/JAguc>) based on a 98.0% sequence similarity cutoff. For justification of this OTU definition, we refer to Caron et al. (2009) and Nebel et al. (2010). One representative sequence from each OTU (the longest read in an OTU) was subjected to an automated gapped BLAST analysis (Altschul et al. 1997) against GenBank's nucleotide database release 178 using the JAguc software package. This program automatically identified non-target sequences (e.g. metazoa sequences, bacteria or archaea), which then were excluded from phylogenetic and statistical analyses. Furthermore, JAguc identified and retrieved the sequences of closest relatives deposited in the 178 release of the GenBank nucleotide database for subsequent phylogenetic analyses. From these sequences we manually selected the closest described species match for each OTU representative as well as sequences from environmental studies (maximum of five

sequences if available, each from a different environmental study or habitat) for phylogenies. Clone library coverage (sampling saturation) was assessed using the sampcurve tool (<http://www.wagak.informatik.uni-kl.de/JAguc>). The protistan and fungal sequences of this study (one representative of each OTU) have been deposited in the GenBank database under accession numbers JF308229–JF308284.

### Phylogenetic analyses

Environmental SSU cDNA sequences along with their closest GenBank matches (see above) were initially aligned against a backbone alignment of eukaryote SSU rRNA gene sequences representing the taxonomic breadth of major eukaryote lineages using the program MUSCLE (Edgar 2004). Alignments were refined using Gblocks (Castresana 2000), followed by visual inspection and manual refinement. An evolutionary distance tree (neighbor joining, NJ) was constructed from this refined alignment. Information about the phylogenetic placement of each environmental Thetis sequence together with BLAST information was used to select the sequences for alignments of defined taxonomic groups, namely four individual analyses in total, including alveolates, stramenopiles, opisthokonts (fungi and choanoflagellates) and “others” (haptophytes, chlorophytes and jakobids). The four data sets were aligned and refined as described above (information about alignments are available in the legends to the respective evolutionary distance tree Figs. 2, 3, 4, 5) before being subjected to phylogenetic analyses. Distance and maximum likelihood analyses were conducted for final phylogenies. Neighbor joining evolutionary distance (BioNJ) analyses were carried out in the Seaview program package version 4.2, (Galtier et al. 1996). Maximum-likelihood bootstrapping analyses were carried out with 100 replicates using RAxML with the setting as described in (Stamatakis et al. 2008). ML analyses were conducted online on the CIPRES Portal V 2.0 (<http://www.phylo.org>). Pairwise sequence similarities were calculated with the module pairalign as implemented in the JAguc software package.

### Community analyses

Estimates of OTU richness, partitioning of diversity shared among the two communities (beta-diversity) as well as indices of alpha-diversity were calculated with EstimateS v. 8.2.0 (Colwell 2009).

### Protistan cell numbers

The number of protists in Thetis interface and brine samples was assessed using hybridization of the 5'-end

fluorescence-labeled (CY3) oligonucleotide probe EUK1209 (5'-GGGCATCACAGACCT-3', Giovannoni et al. 1988) to formaldehyde-fixed (3.5% final) aliquots of a sample (varying from 25 to 80 ml) drawn onto Isopore membranes (0.2 µm, 25 mm, Millipore). The procedure followed the protocol as described in the standard fluorescence in situ hybridization (FISH) protocol described by (Pernthaler et al. 2001) for planktonic protists. The stringency for the eukaryote-specific probe was adjusted with 30% formamide and hybridization time was 1.5 h. As a counterstain we used DAPI to visualize the nucleus of target cells. We used a Zeiss Axiophot II epifluorescence microscope to inspect the stained samples with DAPI- and CY3-specific filter sets. From three replicate filters, we analyzed one-eighth of a filter for protistan abundance. We did not observe the probe binding to prokaryotes. We also could not confirm that the probe hybridizes with fungi.

## Results and discussion

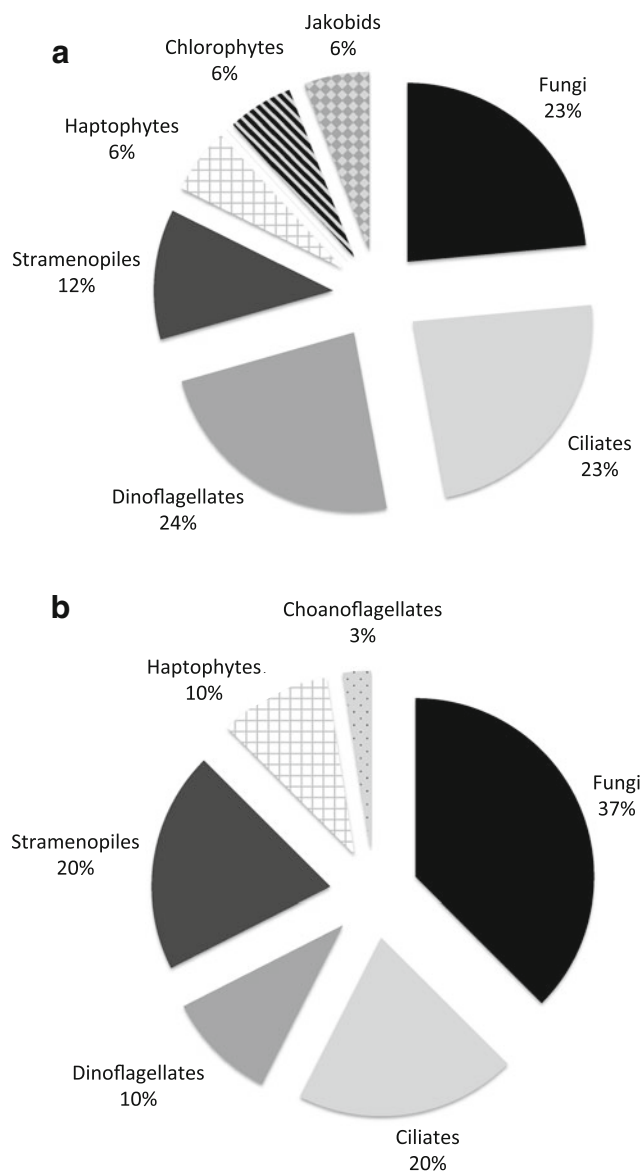
From the 384 Thetis interface clones and the 192 Thetis brine clones, 340 and 122 sequences, respectively, were identified as high-quality reads assigned to protists and fungi. The remaining clones were either of lower quality (incomplete sequences or biased nucleotides) or assigned to non-targets (metazoa). The 340 interface clones grouped into 17 distinct OTUs and the 122 brine clones into 39 OTUs at 98.0% similarity. In the interface clone library, 276 sequence reads clustered in the most abundant OTU and 42 sequences in the second most abundant OTU (both OTUs are taxonomically closely related to the ciliate *Pleuronema coronatum*), together accounting for 93% of the target sequences in the interface. This explains the relatively low number of OTUs in the interface compared with the brine, despite a higher sequencing effort. Taxonomically, the OTUs retrieved from the Thetis basin are distributed throughout a number of different major eukaryote evolutionary lineages.

### Phylogenetic assignment of Thetis sequences

#### Fungi

In the brine, fungi are the dominant microeukaryotes accounting for 37% of the total number of OTUs (Fig. 1). In the past few years, fungi were identified as one of the most successful eukaryotic taxon groups regarding adaptation to extreme environments including anaerobic habitats (Jebaraj et al. 2010), the deep subsurface (Edgcomb et al. 2002, 2011), high pressure and hydrothermal systems (Bass et al. 2007; Burgaud et al. 2009; Le Calvez et al. 2009), acidic environments (Voronin 2010), or extremely





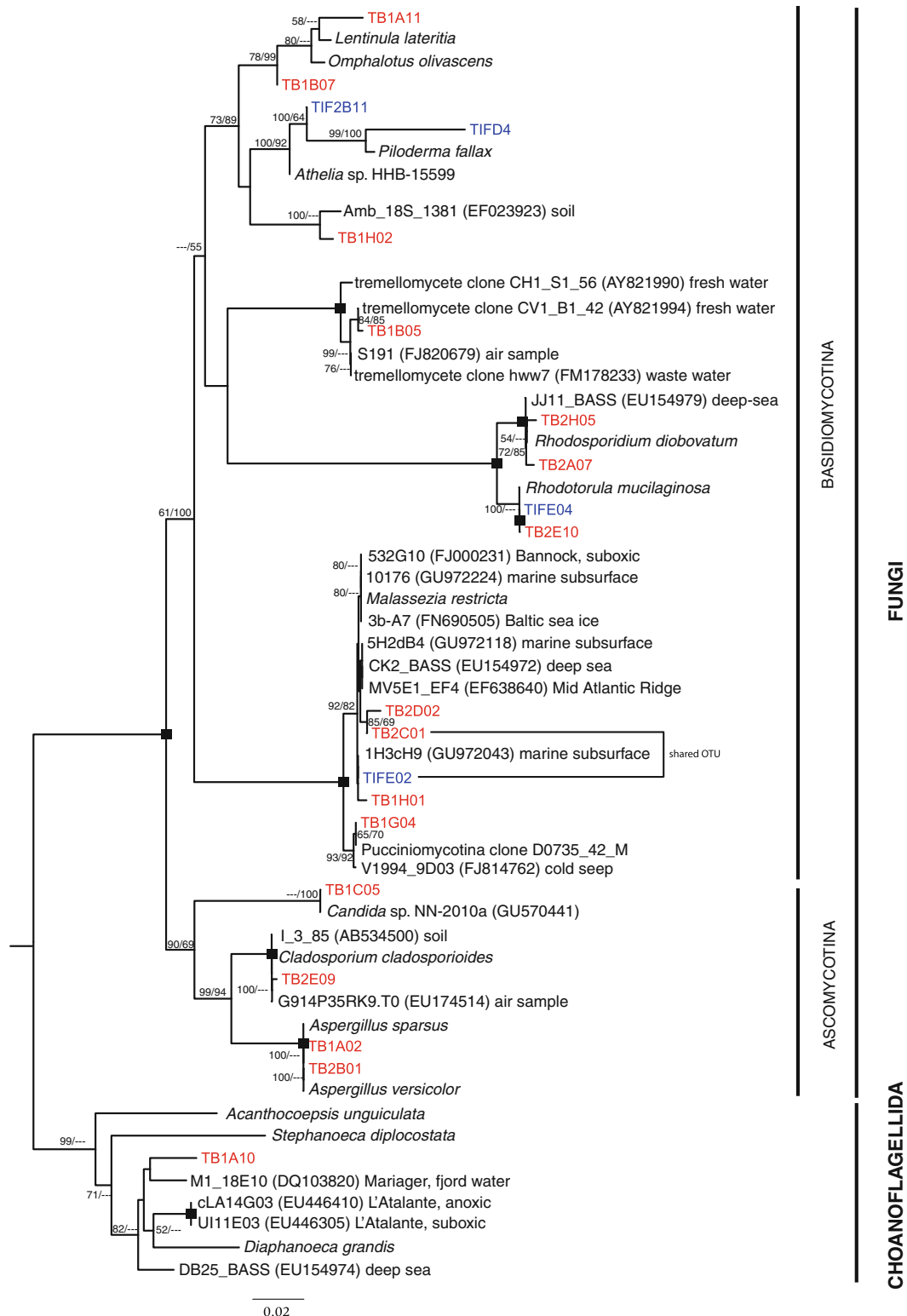
**Fig. 1** Relative taxonomic composition of analyzed clone libraries from Thetis interface (a) and brine (b). The relative proportions reflect the number of different OTUs within each taxonomic category ( $n = 17$  in interface and  $n = 39$  in brine). OTUs unite sequences sharing at least 98% sequence similarity

cold habitats (D'Elia et al. 2009) and even nuclear reactors (Zhadanova et al. 2000). Fungi are among the best-studied groups of eukaryotes in high-salt environments. Since their first discovery in solar salterns only 10 years ago (Gunde-Cimerman et al. 2000), fungi have been detected and isolated in high-salt environments around the globe (reviewed in Gunde-Cimerman et al. 2009). Their abundance and relative ease of cultivation make fungi excellent model organisms when it comes to studying the adaptation of eukaryotes to high osmotic pressure: for example, fungi use glycerol, erythritol, arabitol or mannitol as osmotic solutes, thereby maintaining low cytoplasmic salt concentrations.

The most prominent examples of halophilic fungal model organisms are *Debaryomyces hansenii*, *Hortaea werneckii* and *Wallemia ichthyophaga*. These fungi belong to different and also distantly related evolutionary lineages and have developed different strategies to cope with ion concentration toxicity and loss of water. Detailed elucidation of the pathways involved in fungal halotolerance may prove to be very useful for improving salt resistance in crops (Vaupotič et al. 2007). Furthermore, halophilic fungi are very promising for new drug discoveries, some of which are showing a cytotoxic effect against certain cancer cell lines (Ma et al. 2010). Therefore, brines of DHABs are not only a source of unknown fungal diversity, but also potential hotspots for the discovery of novel metabolic pathways of potential economical importance and for secondary metabolites for medical treatment.

We note that at present, there is no useful guideline as to how to translate 18S rRNA gene sequence similarities in fungi into taxonomic units. The highly conserved nature of the 18S rRNA genes in fungi limits the use of this gene for taxonomic resolution to family level (Horton and Bruns 2001). Therefore, a high 18S rRNA gene sequence similarity between two fungi hardly allows the inference of a similar autecology and life style of these two fungi. Some of the Thetis brine fungi are closely related to the described species (Fig. 2), including, for example, the red yeast *Rhodotorula mucilaginosa*, which is widely distributed and common in deep-sea environments (Nagahama et al. 2001) and exhibits a halotolerance (Lahav et al. 2002). Other OTUs are related to the ascomycetes *Cladosporium* and *Aspergillus*, both of which have been isolated previously from solar salterns (Cantrell et al. 2006). Some OTUs, however, are only distantly related to described and sequenced fungi and may represent novel taxa on high taxonomic levels, such as the basidiomycete TB1H02, which is closely related to another environmental sequence, detected in a soil habitat (Amb\_18S\_1381). Interestingly, only two further Thetis brine sequences are related to previously published environmental sequences, TB2D02 and TB2C01. It is noteworthy that these related environmental sequences originate exclusively from extreme marine habitats, including hypersaline environments (Bannock Basin and fluid inclusions in sea ice), hydrothermal Mid-Atlantic ridge site or the marine subsurface (Fig. 2). This clade nicely demonstrates the capability of closely related fungal phylotypes to adapt successfully to a number of different extreme conditions. Due to a historical focus on their diversity and metabolism, the ecological role of fungi in hypersaline systems remains largely unknown.

Based on our results, it could be concluded that fungi are among the dominant taxa found in the Thetis interface; however, they account for a much smaller proportion there



**Fig. 2** Minimum evolution (NJ distance) phylogenetic tree of eukaryotic small subunit rRNA genes showing the position of fungal and choanoflagellate OTUs<sub>98%</sub> from Thetis interface (TIF-clones, blue) and brine (TB-clones, red). For details about tree construction see “Materials and methods”. The tree is based on 612 unambiguously aligned positions. Two stramenopile sequences were chosen as

outgroup taxa (not shown). Numbers at respective nodes show support values. The first numbers are distance bootstrap values over 50% from an analysis of 1,000 replicates. The second numbers maximum likelihood bootstrap values over 50% from an analysis of 100 replicates. Black squares at nodes indicate full support from both analyses

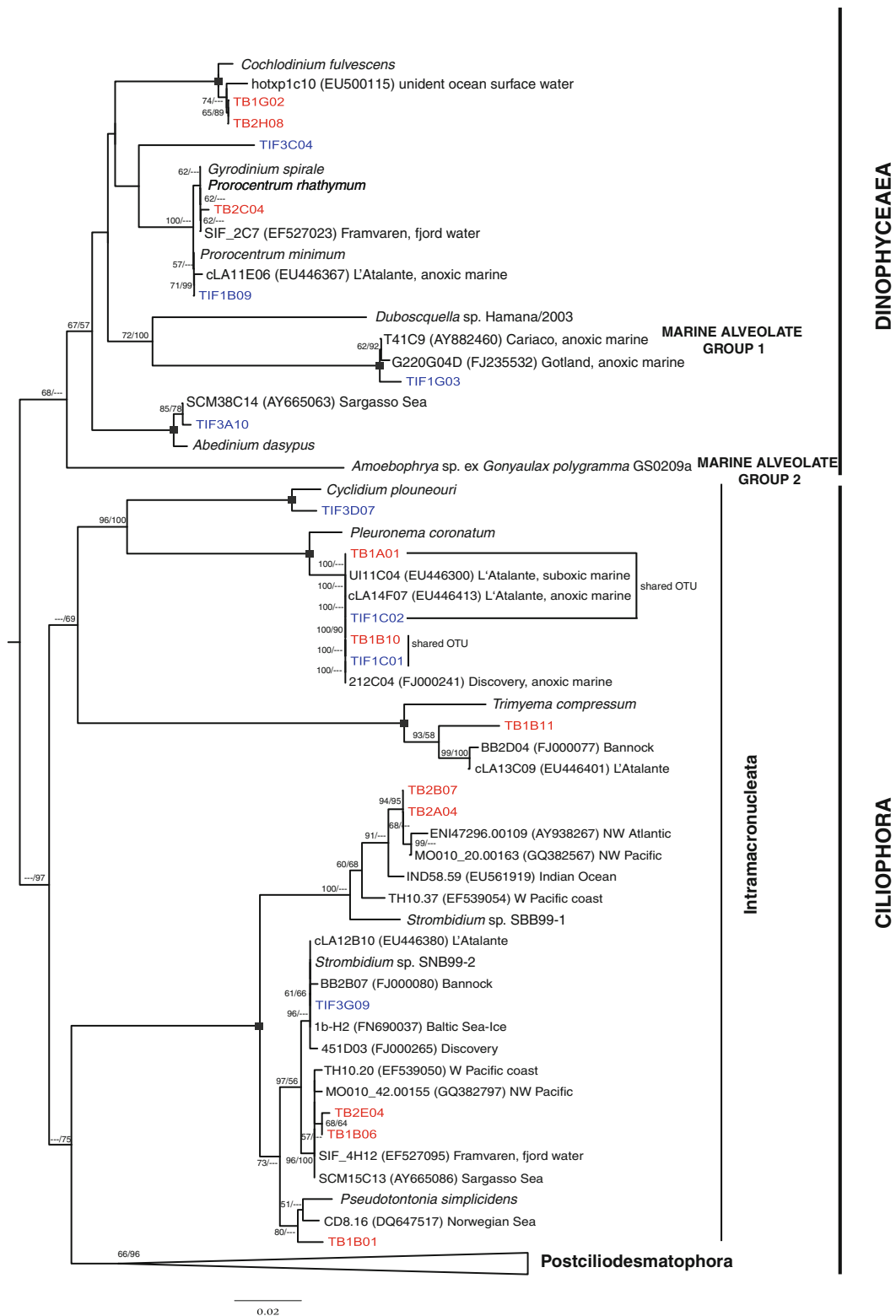
(23%) compared with the brine (Fig. 1). Only one phylo-type is shared between the interface and the brine sample (TIFE02 and TB2C01, belonging to the versatile extremophile basidiomycete clade, Fig. 2). This raises the question whether most fungi in the brine are halophilic rather than halotolerant, which would support the existence of discrete fungal communities in the interface and the brine.

### Alveolates

Pronounced differences in the protistan communities of Thetis brine and interface are also observed. On a higher taxonomic level, ciliates account for a substantial protistan component in both habitats (23% in the interface and 20% in the brine, Fig. 1). But within ciliates, only two phylotypes are shared between the two habitats, both of which are closely related to the relatively small scuticociliate *Pleuronema coronatum* (Fig. 3). The relative abundance of the respective species, however, seems to be much higher in the interface (276 individual sequences belonging to OTU TIF1C02 and 42 to OTU TIF1C01, together accounting for 93% of the target sequences in the interface) compared with the brine (9 sequences belonging to TB1B10 and 8 sequences to TB1A01). This indicates that despite being halotolerant, growth conditions are much better for *Pleuronema* in the mixo- to metahaline interface. Interestingly, very similar sequences (Fig. 3) have been found previously in the interfaces of the Discovery and L'Atalante basins in the Eastern Mediterranean deep-sea (Alexander et al. 2009; Edgcomb et al. 2009). Halotolerance combined with a facultative anaerobe metabolism (Fenchel and Bernard 1996) might enable a more ubiquitous dispersal of this species, at least among the geographically isolated DHABs in the eastern Mediterranean Sea. Another brine sequence (TB1B11) is to some extent (92.7% similarity) related to the strict anaerobe *Trimyema compressum*. Similar sequences have been discovered previously in the hypersaline Bannock and L'Atalante basins (Alexander et al. 2009; Edgcomb et al. 2009). At least phylogenetically, the halotolerant or halophilic forms of the respective species are different from the marine forms, suggesting a potential allopatric speciation through long-term separation of populations by an environmental gradient (salinity). Only very few records are available of *Trimyema* isolates from high-salt environments (Ruinen 1938a; Tucolesco 1962; Esteban and Finlay 2003), but none of them exhibited such high-salt concentrations as Thetis brine. This might be due either to the rare occurrence of *Trimyema* species in such habitats (restricted dispersal of hypersaline ecotypes) or to cell death of *Trimyema* upon exposure to oxygen.

Unfortunately, no molecular sequences are available from these previously described species for comparison with the sequences obtained from Thetis, Bannock and L'Atalante. Also *Strombidium* species are reported from some high-salt habitats (Wilbert 1995; Elloumi et al. 2006). Four brine OTUs and one interface OTU cluster within two *Strombidium* clades, together with sequences from non-extreme environments and from other anoxic and/or hypersaline habitats, demonstrating the diverse array of environmental conditions to which ecotypes within a ciliate genus can adapt to or develop a tolerance to. An additional brine sequence (TB1B01) branches as a distant relative to *Pseudotontonia*, a genus with only two described species (one of which is sequenced) that occurs in marine plankton samples (Skovgaard and Legrand 2005) but has not been reported previously from high-salt systems. Another interface sequence is related to the scuticociliate *Cyclidium*, a genus containing species that have adopted an anaerobic life style (Clarke et al. 1993) and that occur in high-salt environments (Finlay et al. 2006).

Another group of alveolate protists, the dinoflagellates, accounts for 24% of the sequences in the interface and for 10% in the brine (Fig. 1). None of the OTUs have been detected in both the brine and the interface. All but one brine sequence has *Cochlodinium* as the closest described relative (Fig. 3). To our knowledge, halotolerance for the globally distributed *Cochlodinium* has not been previously described. However, considering the recent discovery of a novel *Cochlodinium* species (Iwataki et al. 2007), it is reasonable to assume that not all species within this genus have been described to date and that halophilic or halotolerant species exist. TB2C04 is a *Prorocentrum*-like clone that is similar to an environmental sequence from a sulfidic anoxic marine fjord (Framvaren). Also in the interface we found a *Prorocentrum*-like clone, which is distantly related to the brine clone and more closely related to an environmental sequence from the L'Atalante interface. Another very interesting interface clone (TIF3C04) cannot be taxonomically assigned and there is no support for its phylogenetic placement as ancestral to the *Prorocentrales* and *Gymnodiniales*. We did not retrieve any dinoflagellate sequences branching within the marine alveolate group 2, and we only retrieved one sequence (TIF1G03) belonging to the marine alveolate group 1. This is very unusual because such sequences are found frequently and in high diversity in marine samples (Caron and Gast 2008). We explain this as a lack of potential hosts for these endosymbiotic protists in Thetis. We note that we cannot exclude technical artifacts like undersampling and possible PCR or transcription bias as factors contributing to the results discussed here.

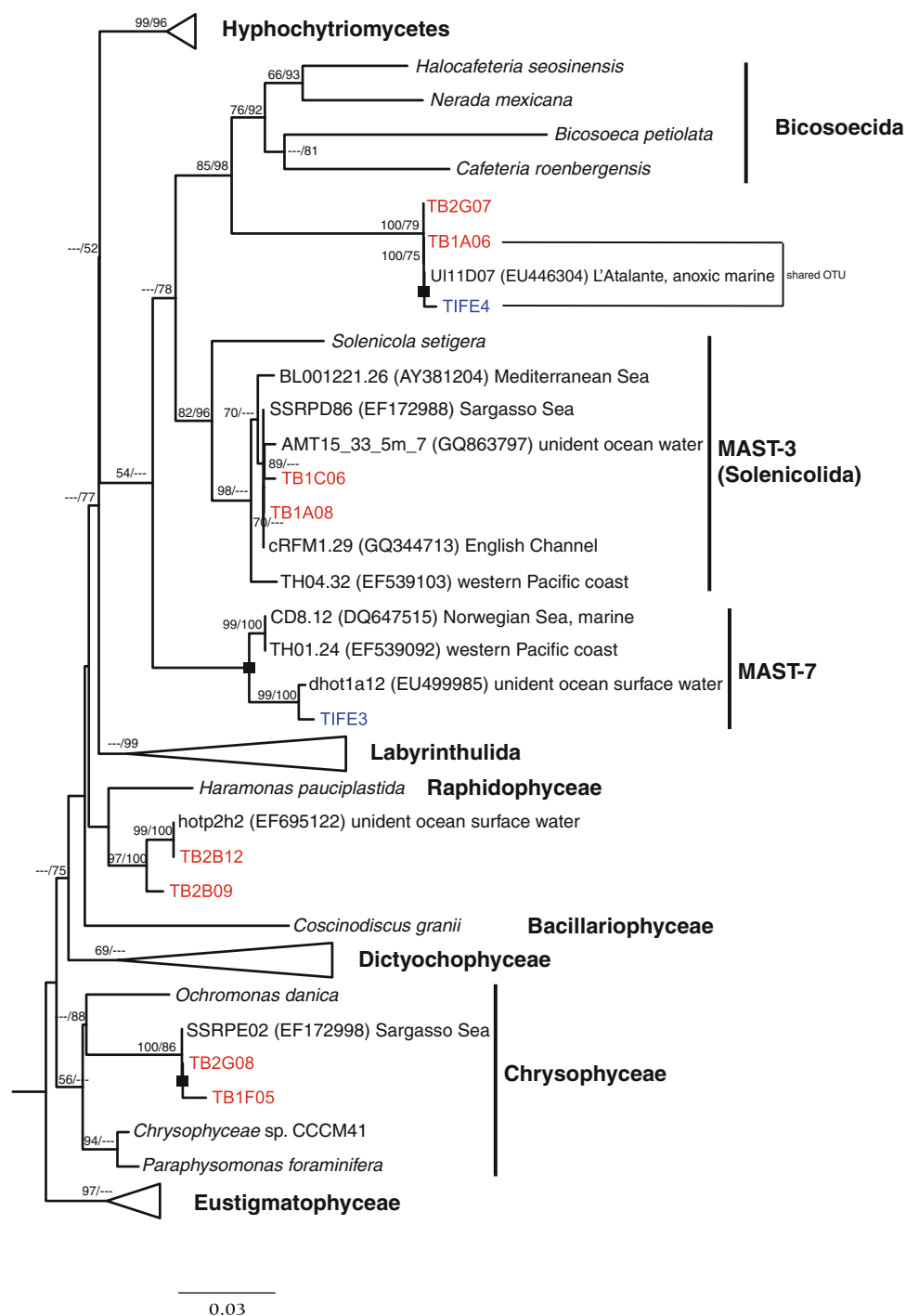


**Fig. 3** Minimum evolution (NJ distance) phylogenetic tree of eukaryotic small subunit rRNA genes showing the position of alveolate OTUs<sub>98%</sub> from Thetis interface (TIF-clones, blue) and brine (TB-clones, red). For details about tree construction, see “Materials and methods”. As for fungi and choanoflagellates (Fig. 2), the tree is based on 612 unambiguously aligned positions and two

stramenopile sequences were chosen as outgroup taxa (not shown). Numbers at respective nodes show support values. The first numbers are distance bootstrap values over 50% from an analysis of 1,000 replicates. The second numbers maximum likelihood bootstrap values over 50% from an analysis of 100 replicates. Black squares at nodes indicate full support from both analyses



**Fig. 4** Minimum evolution (NJ distance) phylogenetic tree of eukaryotic small subunit rRNA genes showing the position of stramenopile OTUs<sub>98%</sub> from Thetis interface (TIF-clones, blue) and brine (TB-clones, red). For details about tree construction see “Materials and methods”. The tree is based on 615 unambiguously aligned positions. Two ciliate sequences were chosen as outgroup taxa (not shown). Numbers at respective nodes show support values. The first numbers are distance bootstrap values over 50% from an analysis of 1,000 replicates. The second numbers maximum likelihood bootstrap values over 50% from an analysis of 100 replicates. Black squares at nodes indicate full support from both analyses



### Stramenopiles

Stramenopiles are heterokont flagellates from which a number of taxa have been recorded in and isolated from high-salt environments (Ruinen 1938b; Post et al. 1983; Patterson and Simpson 1996; Park et al. 2006), and which are often morphologically and phylogenetically distinct from freshwater and typical marine stramenopiles (Park and Simpson 2010). Therefore, it is not surprising that in

the brine samples, stramenopiles are among the dominant protistan taxa accounting for 20% of all OTUs (Fig. 1). Only two stramenopile OTUs were found in the Thetis interface sample. All stramenopile Thetis brine clones are distantly related to described species (Fig. 4), showing that a vast number of stramenopiles are still awaiting discovery even on relatively high taxonomic levels. The shared OTU (TB1A06/TIFE4) from both clone libraries and the brine OTU (TB2G07) together with a sequence from the

hypersaline L'Atalante basin branch as distant relatives of *Halocafeteria seosinensis* (sequence similarity between Thetis brine clones and *Halocafeteria* is 84.4% for TB1A06 and 84.3% for TB2G07), a bicosoecid flagellate that can tolerate up to 36% salinity and that has been designated as a “borderline halophile” (Park et al. 2006). Two brine clones (TB2B12 and TB2B09) are distantly related to the raphidophycean species *Haramonas pauciplastida*. Whether these sequences belong to species from the same genus or are truly raphidophyceans (unsupported relationship, Fig. 4) is unknown. TB2G08 and TB1F05, together with an environmental sequence from the Sargasso Sea, form a novel uncultured clade of unknown taxonomic identity within the chrysophytes. Not much is known about hypersaline chrysophytes, but because they can develop high abundances in brine channels of sea ice (Stoecker et al. 1997), it is reasonable to assume that also this taxonomic group has successfully adapted to hypersaline environments. Two additional brine clones (TB1C06 and TB1A08) branch within an environmental sequence clade together with sequences from a variety of non-extreme habitats, with *Solenicola setigera* being the closest described relative (90.8 and 91.2% sequence similarity). *S. setigera* is an unclassified colonial heterotroph that is a representative of the recently described Marine STra-menopile clade 3 (MAST 3) (Gomez et al. 2011). Members of this clade are globally distributed and abundant in oceanic waters and include sequences retrieved from anoxic and sulfidic environments (Gomez et al. 2011). We report here the first representative from a hypersaline environment in the MAST 3 clade, indicating the wide physiological capabilities of MAST 3 members. Likewise, we report here the first halotolerant or halophilic representative of the uncultured MAST 7 clade retrieved from Thetis interface (TIFE3). In a previous study in the DHAB Bannock (Edgcomb et al. 2009), we found a phylotype that branches within the MAST 9 clade. Consequently, we now identify three MAST clades that include members from high-salt environments, expanding the known salinity regime of these clades. While marine-freshwater transitions between heterotrophic stramenopiles have been remarkably rare (Park and Simpson 2010), hypersaline habitats seem to be colonized more frequently from marine environments. Interestingly, most heterotrophic stramenopile flagellates reported earlier are active at waters with 15–17% salinity or below (Ruinen 1938b; Post et al. 1983; Patterson and Simpson 1996; Pedrós-Alió 2004; Park and Simpson 2010). The traditional view is that above 25% salinity, heterotrophic flagellates (and also ciliates) disappear (Pedrós-Alió 2004). Rarely reported exceptions of heterotrophic flagellated protists are *Halocafeteria* (Park et al. 2006) and the heterolobosean flagellate *Pleurostomum flabellatum* (Park et al. 2007). Our molecular survey

suggests that the diversity of heterotrophic flagellates in hypersaline systems may be larger than previously thought.

#### Other taxa

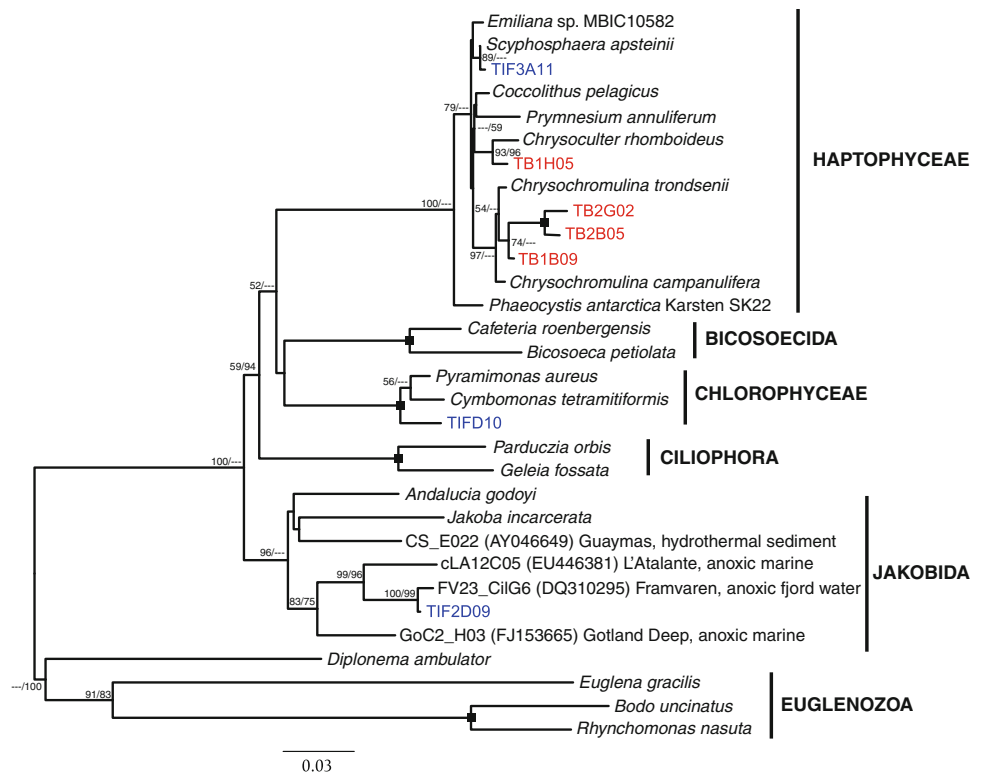
Other taxonomic groups that we detected in individual OTUs include an uncharacterized choanoflagellate sequence in the brine (Fig. 2), a chlorophyte sequence and one jakobid sequence (TIF2D09) in the interface (Fig. 5), the latter branching in an environmental sequence clade together with sequences from other anoxic marine sites and the hypersaline L'Atalante basin. The SSU rRNA gene similarity of TIF2D09 to its closest described relative (*Andalucia godoyi*) is only 68.1%. We detected five haptophyte OTUs, four in the brine and one in the interface (Fig. 5). Studies of the relevance and ecology of haptophytes in our oceans are just being revealed (Liu et al. 2009) and we are not aware of any records of haptophyte isolates from hypersaline systems. This may be due to the difficulties of culturing and morphologically identifying haptophytes in general and also to the fact that there are few molecular surveys from hypersaline systems. A previous study recorded haptophyte SSU rRNA gene sequences from the hypersaline L'Atalante basin (Alexander et al. 2009), indicating that haptophytes might be common members of high-salt environments.

In general the higher taxon level community structure in Thetis is similar to other (terrestrial) saline habitats such as salt lakes or solar salterns, but on a lower level we find clear differences. For example, very common halophiles like the ciliate *Fabrea salina* or some fungi like *Hortaea werneckii* and *Wallemia ichthyophaga* could not be detected in this or in any previous study in DHAB brines. We assume that the polyextreme conditions in the DHABs most likely select for even more specialized communities that include different phylogenetic lineages specifically adapted to DHAB environmental conditions. It will be interesting to reveal the detailed diversity patterns within some of the DHAB communities in large-scale studies using massively parallel tag sequencing.

#### Are the organisms behind the sequences alive?

The question emerges, whether the environmental sequences we detected do indeed derive from indigenous extremophiles. Alternatively, they might only represent preserved nucleic acids from allochthonous sources or contamination. This idea has been discussed in detail previously, and can be largely excluded, due to the amplification of cDNA instead of DNA (Alexander et al. 2009). In short, here we analyzed SSU rRNA gene sequences amplified from cDNA that was obtained from environmental RNA. Traditionally, clone libraries are constructed

**Fig. 5** Minimum evolution (NJ distance) phylogenetic tree of eukaryotic small subunit rRNA genes showing the position of jakobid, chlorophyte and haptophyte OTUs<sub>98%</sub> from Thetis interface (TIF-clones, blue) and brine (TB-clones, red). For details about tree construction, see “Materials and methods”. The tree is based on 615 unambiguously aligned positions. Three euglenozoan sequences were chosen as outgroup taxa. Numbers at respective nodes show support values. The first numbers are distance bootstrap values over 50% from an analysis of 1,000 replicates. The second numbers maximum likelihood bootstrap values over 50% from an analysis of 100 replicates. Black squares at nodes indicate full support from both analyses



from DNA-amplified genes (Epstein and López-García 2007). However, a major shortcoming of this molecular SSU rRNA gene approach is that environmental DNA is not necessarily a marker of viability (Mills et al. 2005). This is particularly important when cataloging SSU rRNA gene sequences from systems with steep chemical gradients (MacGregor et al. 2001) and also from the deep-sea where numerous non-indigenous organisms and debris from the water column may accumulate. Because it is very difficult most of the time to infer the ecology and lifestyle of an organism based on a SSU rRNA gene sequence, we are not able to affirm the indigenous nature of each organism behind each individual sequence. For this purpose, the design and application of specific probes to preserved samples from discrete depths would be crucial (Massana et al. 2002). However, we did at least verify the existence of life in Thetis brine using FISH. Figure 6 depicts some distinct morphotypes, which we detected in the Thetis basin and which leave no doubt about the existence of protistan life in this harsh environment. Using universal eukaryote probe, we cannot directly link the specific genetic signatures found in Thetis to specific morphotypes labeled with the general FISH probe. Therefore, we will have to design, test and apply clade- or taxon-specific probes in the future, which will be helpful for assessing the quantitative contribution of specific taxa in these samples. This will be subject of a follow-up study.

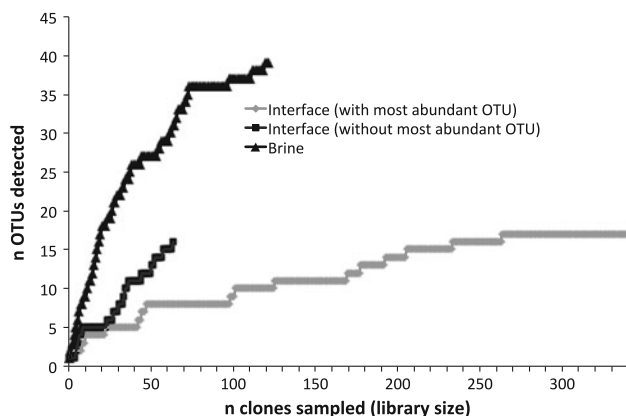
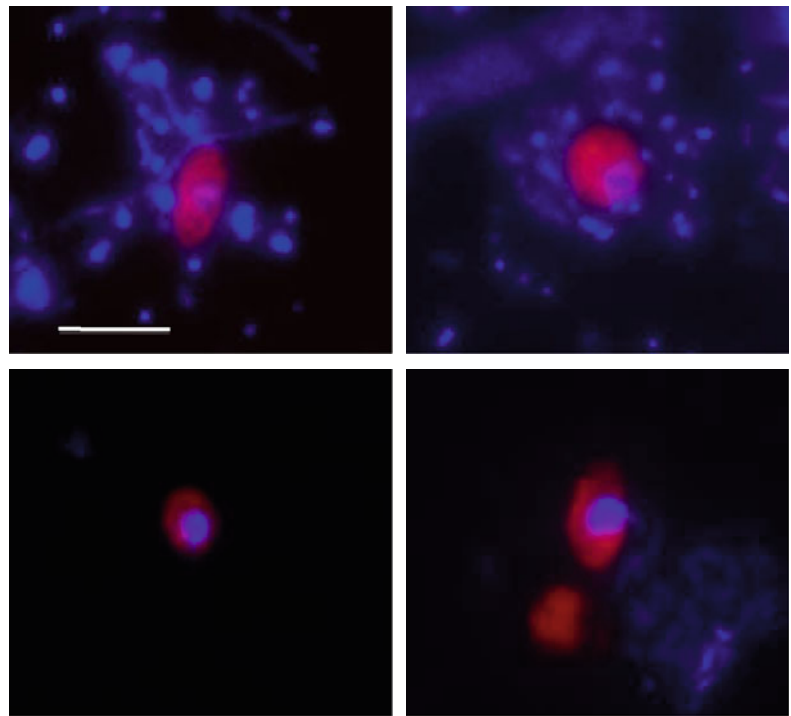
#### Comparing the Thetis interface with brine eukaryote communities

Even though we clearly find a higher numerical abundance of protists in the interface than in the brine (interface contained  $1.10 \times 10^4 \pm 0.23$  cells  $L^{-1}$ , and brine  $0.55 \times 10^4 \pm 0.16$  cells  $L^{-1}$ , respectively), a solid detailed comparison between the microbial eukaryote communities of Thetis interface and brine is not trivial. This is mainly because both communities have not been sampled to saturation. Even though the sampling saturation profile of the interface suggests nearly saturated clone library coverage (Fig. 7), this impression is misleading. When removing the most abundant OTU with 276 individual sequence reads from the sampling saturation analyses, it becomes obvious that the remaining 16 interface OTUs detected represent only a very small fraction of the total community. Likewise, the brine community with 39 distinct OTUs is heavily undersampled. Statistical tools for community comparisons have been developed in order to account for missing species or phylotypes in a sampled community (Lozupone and Knight 2008), but the smaller the fraction of the taxon coverage, the less accurate even these strategies are (Chao and Shen 2003; Chao et al. 2005). Another issue that biases such estimates is that they are abundance-based, which means that the abundance of individual sequences within each OTU is considered as taxon abundance. However, this

**Fig. 6** Fluorescence in situ hybridization (FISH) of formaldehyde-fixed and filtered samples from Thetis interface (upper two pictures) and brine (lower two pictures) using a universal eukaryotic probe labeled with the red fluorochrome CY3 and counterstained for nuclei with the blue fluorescent dye DAPI. The pictures are overlays from two individual pictures taken with a cooled black and white CCD camera colored post record. The figures depict selected different morphotypes, which demonstrate the existence of eukaryotic life in the harsh Thetis environment. Scale bar is 5  $\mu\text{m}$  in size

Thetis  
Interface

Thetis  
Brine



**Fig. 7** Sampling saturation profile (OTU<sub>98%</sub> accumulation curve) for interface and brine samples of the Thetis deep-sea basin. The number of OTUs is plotted as a function of numbers of clones sampled. Clone samples were randomly resampled to completion without replacement to quantify coverage of OTU diversity. For the interface, two curves are plotted, one of which includes the most abundant OTU accounting for 82% of the total number of target sequences analyzed for the interface, and one of which does not include this most abundant OTU. This demonstrates the bias and potential of misinterpretation of the collector's curve when one individual OTU is heavily overrepresented

is not true for microbial eukaryotes, because of highly variable SSU rRNA gene copy numbers and also genome copy numbers within different eukaryote taxa (Prokopowich et al. 2003; Zhu et al. 2005). Therefore, the OTUs with the most abundant genes in a clone library are not necessarily the most abundant taxa in nature. The numbers we calculated for OTU richness using EstimateS should therefore

not be considered as absolute, but rather as relative in a comparison between the brine and interface communities. The non-parametric richness estimator Chao1 predicts 31–66 OTUs in the interface and 55–81 OTUs in the brine. How significant this difference is, is unknown because in both cases the data (lower and upper diversity estimate values) vary greatly and there is no statistic that assess the accuracy of the predictions. Therefore, we are hesitant to claim that the OTU richness in the brine is higher than in the interface.

What we can conclude, however, with higher confidence, is that community membership in the brine differs notably from that in the interface. Only four OTUs are shared between the two communities. Increased sampling will most likely increase the number of shared OTUs, but stochastically also the number of OTUs unique to each community will increase in the same order of magnitude. Statistical analysis that accounts for the number of unseen OTUs (Chao's Jaccard abundance-based similarity index, Chao et al. 2005) is only 0.3, confirming the conspicuous differences between brine and interface samples (note: 1 indicates identical communities). We assume that hyper-saline conditions in the brine select for other taxa than the less saline waters of the interface.

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