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Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries

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ABSTRACT: The Gotland Deep is a temporary anoxic basin in the central Baltic Sea that is frequently subjected to inflow events which result in oxygenation of the deep water. We examined the protistan community structure in this environment several months after such an inflow event by analyzing small subunit ribosomal RNA (18S rRNA) sequences from samples collected in the oxic–anoxic transition zone (200 m) and in the sulfidic waters below (220 m). While ciliates and fungal phylotypes in the clone libraries decreased (40 to 14% and 25 to 8%, respectively) from the transition zone to the deeper sulfidic waters, jakobid phylotype abundance increased from 7 to 32%. Heterotrophic choanoflagellates and stramenopiles accounted for 7 to 10% of the phylotypes at both depths. Other taxonomic groups (e.g. dinoflagellates, cercozoans, perkinsozoans, amoebae, centroheliozoans) were rare. Seven phylotypes were shared between the 2 libraries, with a higher number of phylotypes with broader taxonomic representation occurring in the sulfidic compared to the suboxic water sample. Only a small proportion (9%) of the 18S cDNA sequences in the Gotland Deep clone libraries were closely (>95% sequences similarity) related to previously described sequences.

KEY WORDS: Anoxic · Baltic Sea · Chemical stratification · Community structure · Gotland Deep · Molecular diversity · Protists · 18S rRNA

INTRODUCTION

In the past few years, anoxic environments have attracted increasing attention in protistan biodiversity research, as these habitats are promising for the discovery of unknown microbial eukaryotes (Epstein & López-García 2008). The exploration of protistan diversity in anoxic environments using phylogenetic analyses of small subunit ribosomal DNA (18S rDNA) fragments, amplified from environmental genomic DNA, started only a few years ago in anoxic coastal sediments (Dawson & Pace 2002) and hydrothermal deep-sea vent sediment (Edgcomb et al. 2002). Since then, several studies have analyzed 18S rDNA diversity in a variety of anoxic aquatic habitats like the Mid-Atlantic Ridge (López-García et al. 2003), the Cariaco Basin in the Caribbean Sea (Stoeck et al. 2003b), the sulfide-rich Zodletone spring (Luo et al. 2005), anoxic sediment around fumaroles (Takishita et al. 2007), the Framvaren Fjord in Norway (Behnke et al. 2006), the Mariager Fjord in Denmark (Zuendorf et al. 2006), Arctic sediment at Disko Island (Stoeck et al. 2007a) and the L'Atalante Basin in the Mediterranean (Alexander et al. 2009).

These studies revealed a tremendous phylotype richness exceeding all expectations based on previous microscopy- and cultivation-based studies. They detected not only representative sequences of all major phyloge-

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netic eukaryote lineages, but they reported sequences that point to the existence of a wealth of unknown organisms on high taxonomic ranks such as novel ciliate candidate classes (Stoeck et al. 2003b), and several rRNA sequences from oxygen-depleted environments seem to represent novel clades with sister relationships to Euglenozoa, Entamoeba, and Diplonemida (Stoeck et al. 2003b, Stoeck et al. 2006) as well as Euglenida in general (López-García et al. 2003, Behnke et al. 2006, Stoeck et al. 2006). Other sequences were deeply branching lineages unrelated to known eukaryotes as reported from hydrothermal vents and shallow water volcanic systems (Edgcomb et al. 2002, López-García et al. 2003). With few exceptions (Zuendorf et al. 2006), most of the environments previously studied represent permanently undisturbed anoxic habitats, some of them probably existing for tens of thousands of years (Edgcomb et al. 2002, Stoeck et al. 2003b).

In contrast, the Gotland Deep, located in the central Baltic Sea (Fig. 1), is a temporary anoxic basin that is subjected to frequent disturbance by inflow of oxygenated North Sea water entering the Baltic Sea through the Skagerrak and the Kattegat (Meier et al. 2006). The Baltic Sea is one of the largest brackish water basins in the world. Water exchange with the North Sea is naturally restricted by the islands and narrow channels around Denmark, resulting in stratified waters with heavier salt water on the bottom and less saline water (riverine and rainfall) on top, both separated by a stable halocline. Due to this minimal vertical mixing of the water column and high nutrient input, large areas of this shallow shelf sea become frequently devoid of oxygen. Hypoxic bottom waters may spread over the seafloor resulting in severe ecological and economic effects (Elmgren 2001).

At different time intervals, the oxygen-depleted bottom waters become re-oxygenated as a result of so-called major Baltic inflow events (Meier et al. 2006). During the past 2 decades, the frequency of major inflows has decreased and significant inflows occurred only in 1983, 1993 and 2003 (Meier et al. 2006). Between these inflow events, the stagnation periods result in decreasing oxygen and increasing hydrogen sulfide concentrations in the deep water of the Gotland basin. A sequence of smaller and larger inflow events that occurred in 2002 and 2003 resulted in marked changes of deep water salinity and oxygen conditions (Feistel et al. 2006). The major inflow event in 2003 led to high oxygen concentrations in the large basins of the central Baltic Sea. The entire water column of the Gotland Deep (Stn 271) was oxygenated for a period of about 1 yr, followed by a gradual increase of deep water H_2S concentrations and a rise of the oxic–anoxic interface (Fig. 2).

We sampled deep water of the Gotland Deep during the period of uprising of the oxic–anoxic interface. Here, we present a first 18S rRNA (cDNA) inventory of protistan diversity in this temporary anoxic environment and evaluate the influence of an oxygen concentration gradient in the water column on protistan diversity and community structure.

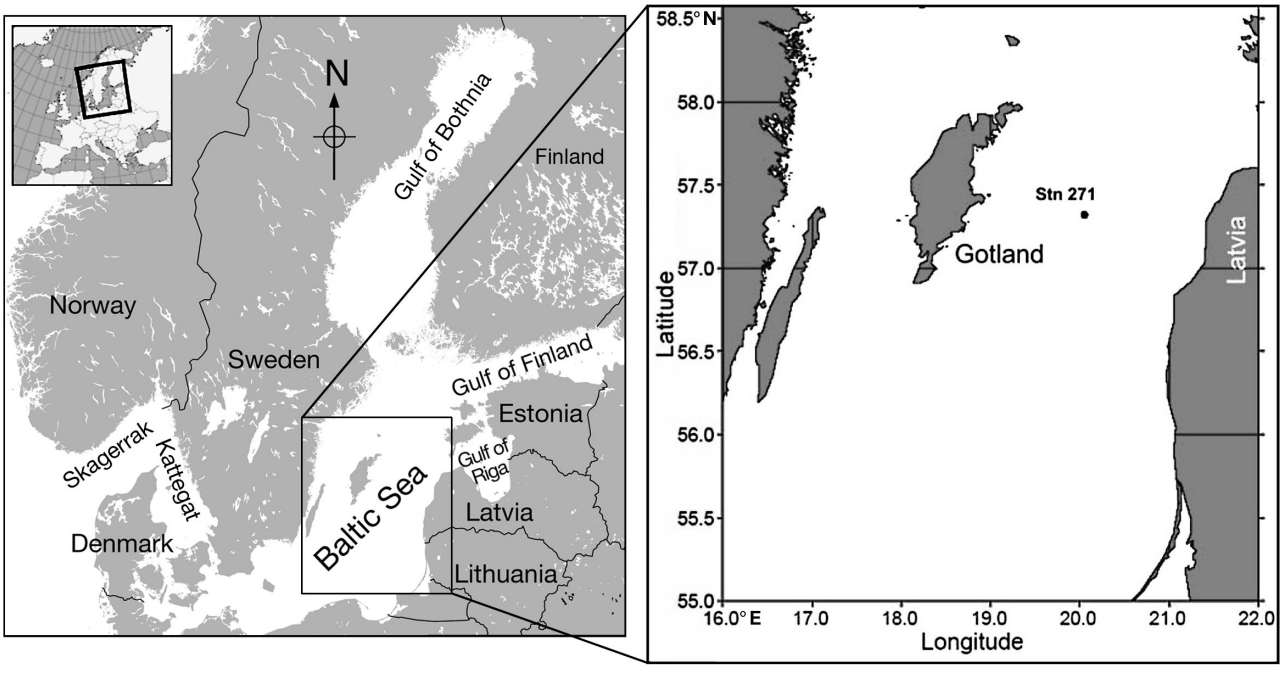

Fig. 1. Stn 271 in the central Baltic Sea (courtesy of Jan Donath, Leibniz-Institut für Ostseeforschung. Sources: http://commons.wikimedia.org/wiki/Image:Baltic_Sea_map.png#file; Labrenz et al. 2005)

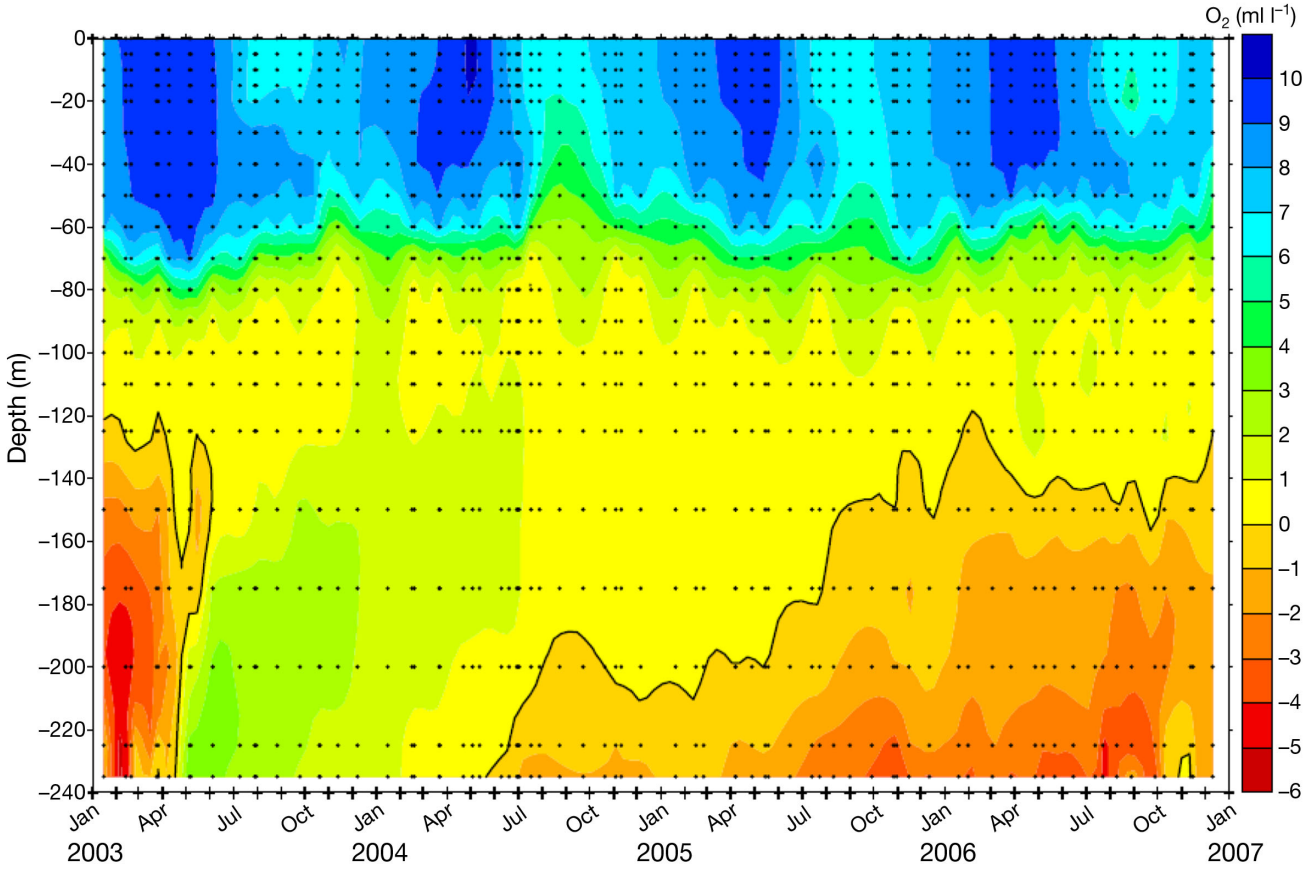

Fig. 2. Development of oxygen concentration in the eastern Gotland Basin (Stn 271) between January 2003 and January 2007 (source: www.io-warnemuende.de/documents/mebe70_2006-zustand-hc.pdf). The solid line optically separates the suboxic/anoxic zone from the oxygenated waters

MATERIALS AND METHODS

Sampling site and procedure. Samples were collected during a cruise on the RV 'Alkor' in the eastern Baltic Sea in May 2005. The sampling site was in the central Gotland Deep (Baltic Sea monitoring station, Stn 271; 57° 19.2′ N, 20° 03′ E) (Fig. 1), which is the second deepest basin (249 m) in the Baltic Sea. Using a CTD-system equipped with a rosette of twelve 5 l FreeFlow bottles (Hydrobios, Kiel-Holtenau), the chemocline (oxic–anoxic transition zone) was sampled at a depth of 200 m and the sulfidic deep water at a depth of 220 m.

Salinity, density, conductivity, temperature, NO_3^{2-}, bacterial abundance, NH_4^+, PO_4^{3-}, H_2S and Mn^{2+} were surveyed (Fig. 3). Temperature, conductivity and oxygen profiles were obtained with a CTD-system (SeaBird Electronics) or as described in Grasshoff et al. (1983).

Bacterial numbers were determined by flow cytometry according to Gasol et al. (2004). From seawater samples taken at each depth, 1 l was drawn onto 0.2 µm Durapore membrane filters (Millipore, Schwalbach) for harvesting microbial biomass. The filters were frozen immediately at –20°C until further processing in the laboratory.

Total RNA extraction. Total RNA was extracted from the frozen filters by isolation of total nucleic acids using a phenol extraction protocol (Weinbauer et al. 2002). Subsequently, RNA extracts were purified by incubation of total nucleic acids with DNAse I (Roche Diagnostics) for 60 min at 37°C. The concentration and purity of the extracted and purified total RNA was determined spectrophotometrically using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). To exclude any residual environmental DNA in the extracted RNA samples, PCR was performed as described in the next section, using the RNA extracts as the template. In all cases, amplification was negative (results not shown).

cDNA library construction. To retrieve 18S cDNA, 7.25 µl (ca. 500 ng) of template total RNA were reverse transcribed at 42°C using the iScript Select cDNA Synthesis Kit (BioRad) according to the manufac-

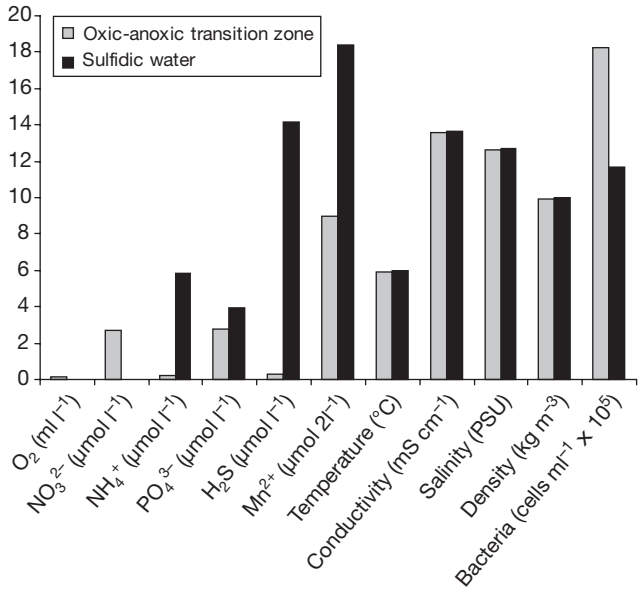

Fig. 3. Environmental parameters and bacterial abundance in the suboxic and the anoxic layer. The gradient between the 2 layers is shown for O_2, NO_3^{2-}, NH_4^+, PO_4^{3-}, H_2S and Mn^{2+}, and for conductivity, temperature, salinity, density and the bacterial cell numbers. Note the constant and the changing parameters between the 2 environments

turer's instructions. For reverse transcription (RT), the eukaryote-specific primer Euk B (Euk1791R; 5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′; Medlin et al. 1988) was applied. The transcribed double-stranded DNA (cDNA) was PCR-amplified using the eukaryote-specific forward primer Euk 528F (5′-CGG TAA TTC CAG CTC C-3′; Edgcomb et al. 2002) and the universal reverse primer U1517R (5′-ACG GCT ACC TTG TTA CGA CTT-3′; Shopsin et al. 1999), resulting in ca. 1 kb fragments. The PCR protocol for eukaryote 18S cDNA has been described previously (Stoeck et al. 2007b). Four individual reactions per depth were run to minimize PCR bias. Before construction of the clone libraries, reactions from the same depth were pooled prior to purification of the PCR products (MinElute PCR Purification Kit, Qiagen). Purified PCR products were cloned using a pGEM-T Vectors System (Promega) and a One Shot® aTOP10 Chemically Competent *E. coli* (Invitrogen). Plasmids were extracted from overnight cultures using a DirectPrep 96 Miniprep Kit (Qiagen) as described earlier (Stoeck et al. 2007b).

Sequencing and phylotype grouping. Nearly 300 clones per depth were partially sequenced using the specific M13F-sequencing primer (M13uni (-21), 5′-TGT AAA ACG ACG GCC AGT-3′; MWG Biotech AG) and a 3730 DNA Stretch Sequencer with the XL Upgrade and the Prism BigDye terminator v. 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Partial sequences were grouped into operational taxonomic units (phylotypes) using the program DOTUR (Distance-based OTU and Richness) (Schloss & Handelsman 2005; www.plantpath.wisc.edu/fac/joh/dotur.html) based on a 99% sequence similarity cut-off value. Afterwards, one representative clone was chosen for each phylotype, defined on a 99% sequence similarity level, for a full bidirectional sequencing reaction. The final DOTUR analysis included all full-length sequences together with the partial sequences from the M13F-sequencing reaction. Final phylotypes were defined on the 99% sequence similarity level and performed sequence quality assessments, PHRED and PHRAP analyses, and assembled the sequences with the CodonCode Aligner software v.1.2.4 (CodonCode Corporation). Low quality sequence reads and non-target prokaryote and metazoan sequences were excluded from phylogenetic analyses.

The 18S rRNA sequences determined in the present study were deposited in GenBank database under accession numbers FJ153624 to FJ153746.

Phylogenetic analyses. Environmental 18S cDNA sequences were compared to GenBank using gapped BLAST analysis (Altschul et al. 1997) to roughly determine their phylogenetic affiliation. Environmental sequence data and their next GenBank matches were imported into an ARB database (www.arb-home.de) and aligned using the FastAligner utility tool of the ARB software package (Ludwig et al. 2004). Alignments were refined manually using phylogenetically conserved secondary structures and sequences were added to the ARB tree using QuickAdd Parsimony. Potential chimeric sequences were identified using secondary structure predictions, the Chimera_Check command v. 2.7 provided by the Ribosomal database project II (RDP II; http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU), and partial treeing analyses (Robison-Cox et al. 1995). After analyzing the approximate positions of the 18S cDNA sequences, partial alignments comprising sequences of defined taxonomic groups were exported from ARB and used to construct detailed phylogenetic subtrees. Minimum evolutionary distance analyses were carried out under maximum likelihood criteria, with all characters equally weighted and unordered, using PAUP* v. 4.0b8 (Swofford 2002). Using Modeltest (Posada & Crandall 1998), we chose the evolutionary model that best fit our alignment data sets from 56 possible models. The trees were constructed under maximum-likelihood criteria using a GTR + I + G DNA substitution model with individually variable-site gamma distribution shape parameter (G) and proportion of invariable sites (I); base frequencies and a rate matrix for the substitution model were set as suggested by Modeltest (Posada & Crandall 1998). One thousand boot-

strap replicates were performed using heuristic search to support the analyses. Bayesian analyses were carried out with MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001), supported by posterior probabilities using 4 chains/ two runs and running 10 million generations for each alignment. Every 1000th generation tree was sampled. The first 25% of all sampled trees were considered 'burn-in' trees and were discarded prior to tree reconstruction. We collected 1502 trees (2 runs, 100 000 generations, 1001 sampled trees per run, burnin = 250). Posterior probability was calculated with a 50% majority rule consensus of the remaining trees. Modeltest v. 2 (Posada & Crandall 1998), using hLTR, recommended the GTR + I + G evolutionary model.

Comparison of the communities. The Jaccard index was calculated with the program package SPADE (Species Prediction And Diversity Estimation) (Chao & Shen 2003; http://chao.stat.nthu.edu.tw/softwareCE.html) as a measure of similarity between the 2 communities based on abundance ($J_{abundance}$) with adjustment for the effect of unseen shared phylotypes in order to reduce bias due to undersampling ($J_{adjusted}$).

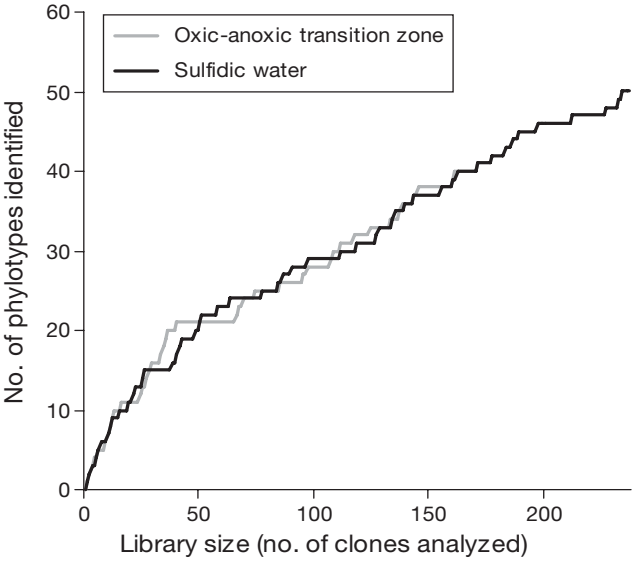

Fig. 4. Sampling saturation profiles. Phylotype accumulation curves for the oxic–anoxic transition zone at 200 m and the sulfidic water layer at 220 m of the Gotland Deep. The number of phylotypes is plotted as a function of numbers of clones sampled. Clone samples were randomly resampled to completion without replacement to quantify coverage of phylotype diversity. Phylotypes are defined to encompass clones that shared 99% sequence similarity based on a pairwise comparison of homologous 18S cDNA sequence regions

RESULTS

We sampled 2 water layers in the Gotland Deep of the central Baltic Sea: the oxic–anoxic transition zone at 200 m depth and sulfidic water 20 m below (at 220 m). Salinity, density, conductivity and temperature showed only little variation between these 2 depths. In contrast, NO_3^{2-} concentration and bacterial abundance decreased significantly while a variety of electron acceptors and donors (NH_4^+, PO_4^{3-}, H_2S and Mn^{2+}) increased considerably (Fig. 3).

Comparison of the communities and taxonomic affiliation of the cDNA sequences

From the nearly 600 sequences (ca. 300 per library) analyzed in total, 171 protistan target clones, grouped into 41 phylotypes, remained for the transition zone sample after short sequence reads and suspected chimeric sequences were excluded from the analyses. In the sulfidic sample, the remaining 237 protistan target clones grouped into 50 phylotypes (see Appendix 1). Neither of the clone libraries reached saturation (Fig. 4). Only few phylotypes are shared between the 2 libraries (n = 7, Fig. 5). For the 200 m clone library, 34 phylotypes were unique, and 43 phylotypes occurred exclusively in the 220 m clone library. Consequently, it was not unexpected that the Jaccard index showed a relative high dissimilarity in the community structure when adjust-

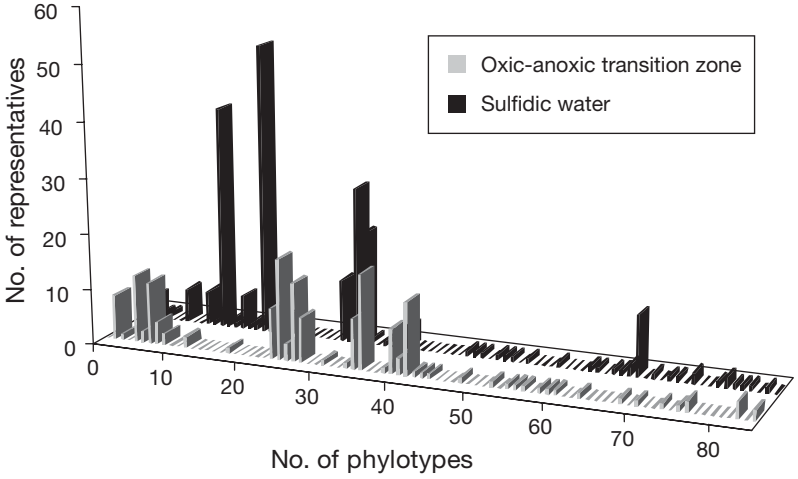

Fig. 5. Representative distribution of differing phylotypes in the oxic–anoxic transition zone and the sulfidic water layer. The number of phylotypes is plotted as a function of numbers of representatives in the 2 independent clone libraries of the 2 layers (200 and 220 m). The order of phylotypes along the *x*-axis reflects the output from the DOTUR software. Taxonomic assignment of each phylotype together with a representative sequence from the Gotland Deep libraries is given in Appendix 1

ing for the number of unseen phylotypes ($J_{adjusted}$ = 0.25 [phylotypes defined on 99% sequence similarity], 0.26 [phylotypes defined on 98% sequence similarity] and 0.31 [phylotypes defined on 95% sequence similarity], with a value of 1 indicating identical community structures).

The major taxa accounting for the significant differences between the 2 samples become obvious from Fig. 6. The transition-zone community (Fig. 6A) was dominated by ciliates (16 phylotypes, 40%). In contrast, ciliates accounted for only a small proportion of the anoxic library (14%), while jakobid flagellate phylotypes (32%) dominated this assemblage (Fig. 6B). Representative phylotypes of some major taxonomic groups were unique for the sulfidic library (Lobose amoebae, centrohelids, chloroplastids, and cryptophytes), while dinoflagellate phylotypes were exclusively present in the transition-zone sample. Furthermore, fungal phylotypes accounted for 25% of the transition-zone library, but for only 8% in the sulfidic sample. Only a few phylotypes were shared between the 2 libraries. These are: 1 ancyromonade phylotype, 1 fungal phylotype, 2 choanoflagellate phylotypes, and 3 jakobid phylotypes (see also Table A1). Some phylogenetic groups showed similar proportions in both libraries (Perkinsidae, Stramenopila, Cercozoa, Ancyromonadidae and Choanoflagellata), but these groups were usually represented by different phylotypes. Comparing the abundance of phylotypes with the abundance of clones at both depths we noted that, in the oxic–anoxic transition zone, the proportions were comparable to those obtained from the phylotype composition (Fig. 6C), whereas the taxonomic affiliation of clones from the sulfidic waters represented a larger tax-

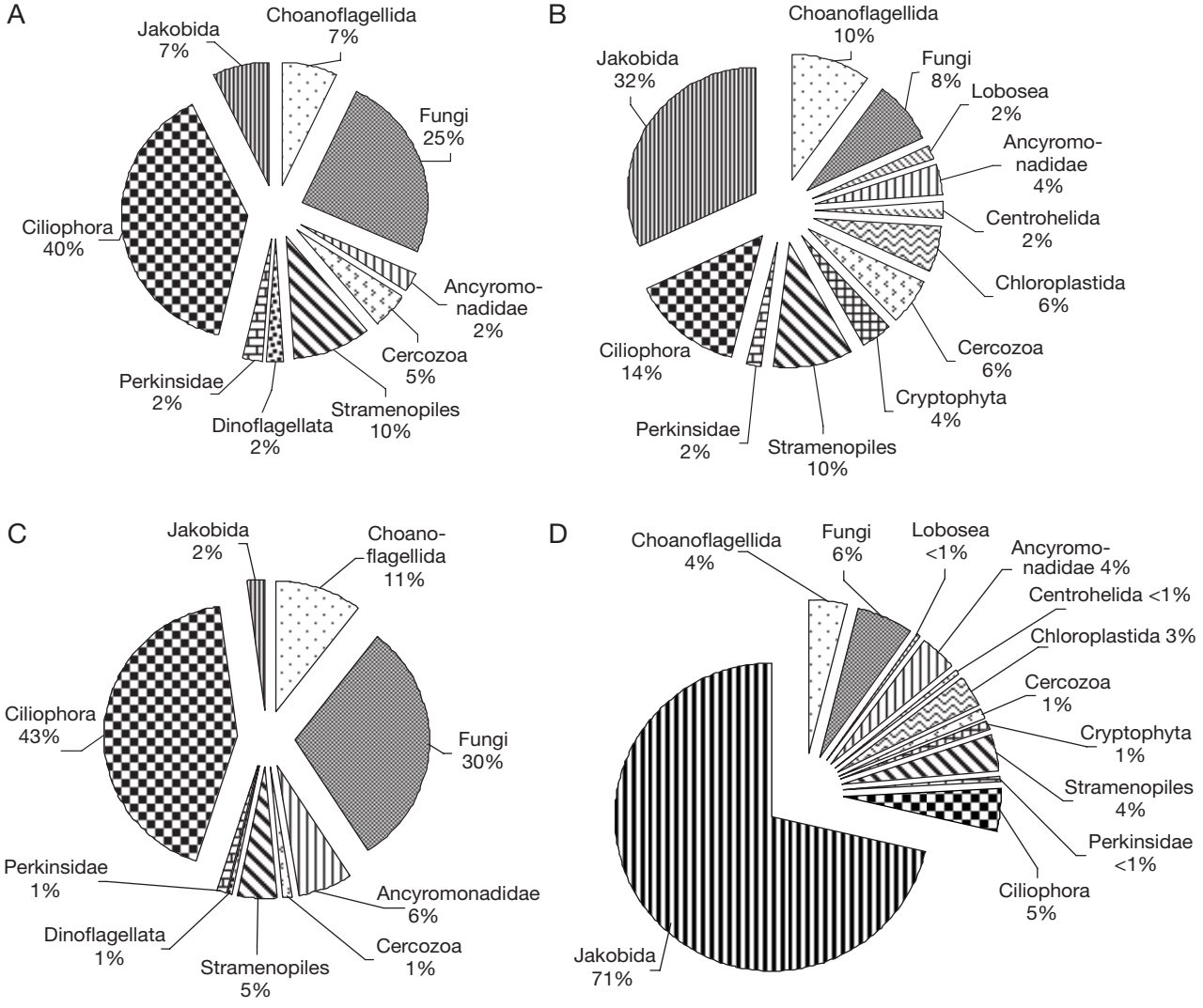

Fig. 6. Taxonomic affiliation of phylotypes and clones. Relative abundance of phylotypes (A) in the oxic–anoxic transition zone (200 m) and (B) in the sulfidic water layer (220 m) of the Gotland Deep ($n_{transition\ zone}$ = 41; $n_{sulfidic}$ = 50) identified using a 99% sequence similarity cut-off level. Abundance of clones among the different taxonomic groups (C) at 200 m and (D) at 220 m depth

onomic breadth (Fig. 6D). The major difference was due to the jakobid sequences, which did not account for just 32% as in the phylotype composition, but for 71% of all clones from the sulfidic layer at 220 m depth.

Phylogenetic analyses

Of the 18S cDNA sequences we retrieved from the Gotland Deep, none were identical to any deposited sequences in public databases. The majority of phylotypes (31 in the 200 m and 43 in the 220 m library) had <95% sequence similarity to any previously reported sequences. The most divergent sequence that we retrieved from the Gotland Deep (GoC3_E04) had only 78.7% sequence similarity to a previously deposited 18S rDNA sequence (*Endogene pisiformis*; Fungi, incertae sedis). We performed partial treeing analyses for the major taxonomic protistan groups discovered in the Gotland Deep to identify detailed phylogenies of the Gotland clones.

Alveolata

The transition-zone community comprised 18 different alveolate phylotypes and the sulfidic community only 8 (Fig. 7). Most alveolate sequences fell within the ciliates. We found representatives from 7 out of 11 described riboclasses (Lynn 2003), indicating a high taxonomic ciliate diversity in the Gotland basin. No ciliate phylotypes occurred simultaneously in both libraries. The classes Plagiopylea, Nassophorea, Spirotrichea and Litostomatea were exclusively detected in the oxic–anoxic transition zone, while Armophorea were unique for the sulfidic community. We recovered representatives from the Prostomatea and the Oligohymenophorea in both libraries. Many ciliate sequences from the Gotland libraries showed similarities to environmental sequences from permanently anoxic marine sites like the Framvaren Fjord in Norway, the Cariaco deep-sea basin off Venezuela, anoxic sediments from the Guaymas deep-sea basin in the Gulf of California, anoxic coastal waters in Japan, but also anoxic samples from a freshwater lake in France. For example, 3 Gotland clones from the library constructed out of the sulfidic water samples (GoC1_D05, GoC1_E02, and GoC3_G07) branched together with a similar sequence from the permanently anoxic Cariaco Basin (H60) in a highly supported cluster (100/100), with the next related sequence of a described organism (*Cyclidium porcatum*) being at least 19% divergent. Two further Gotland sequences (GoC1_C05 from 220 m and GoC6_B10 from 200 m) formed an uncultured sequence cluster together with an environmental 18S clone (C1_E031) from the anoxic sediment in the Guaymas Basin in the Gulf of California. Interestingly, this cluster could not be assigned to any of the 11 described ciliate riboclasses. However, its intermediate branching position between the classes Nassophorea and Plagiopylea was not supported. Many of the closest-related sequences from described organisms belonged to ciliates with a lifestyle adapted to anoxia (e.g. *Trimyema*, Fenchel & Finlay 1990; *Metopus*, Biagini et al. 1997), or hypoxia (*Pleuronema*, Zubkov et al. 1992; *Prorodon*, Fenchel & Finlay 1990; *Strombidium*, Fenchel & Bernard 1993; *Euplotes*, Fenchel & Finlay 1995).

Two alveolate phylotypes branched within the phylum Perkinsozoa. One (GoC6_F02, oxic-anoxic transition zone) was related to environmental sequences from an anoxic Mid-Atlantic Ridge sample and a freshwater sample (Lake George, NY, USA). Both perkinsid Gotland sequences were ca. 18% divergent from each other and also from the closest related sequence of a described organism (*Perkinsus mediterraneus*). Only 1 phylotype branched within the dinoflagellates, namely GoC6_C10 from the oxic–anoxic transition zone. This sequence was 10% divergent to the next known dinoflagellate sequence, *Gymnodinium beii*.

Stramenopila

Stramenopile phylotypes (Fig. 8) were present in both communities, but none were shared between both libraries. Three (GoC6_D07 and GoC4_D05 from the transiton zone, GoC3_B08 from the sulfidic water) were closely related to the heterotrophic chrysophyte flagellate *Spumella*. Clone GoC1_E06 branched together with a sequence from the anoxic Framvaren Fjord (Behnke et al. 2006) as a relative of the chrysophyte *Paraphysomonas*. Three further sequences (GoC1_F05 and GoC3_C12 from the sulfidic water and GoC4_C09 from the transition zone) fell into the Eustigmatales as close relatives of *Nannochloropsis*, which is capable of mixotrophy (Xu et al. 2004). One clone from the sulfidic community (GoC2_C01) fell within the Bacillariophyta. The next related sequence was an uncultured clone from the Framvaren Fjord (FV23_1F6) and the next described relative was *Chaetoceros* (98% sequence similarity). The sequence GoC5_C06 branched within the labyrinthulomycetes, as a relative of Tagiri-16 from an anoxic fumarole sediment in Japan (92% sequence similarity).

Rhizaria

Three sequences from the sulfidic depth and 2 sequences from the transition zone branched within the

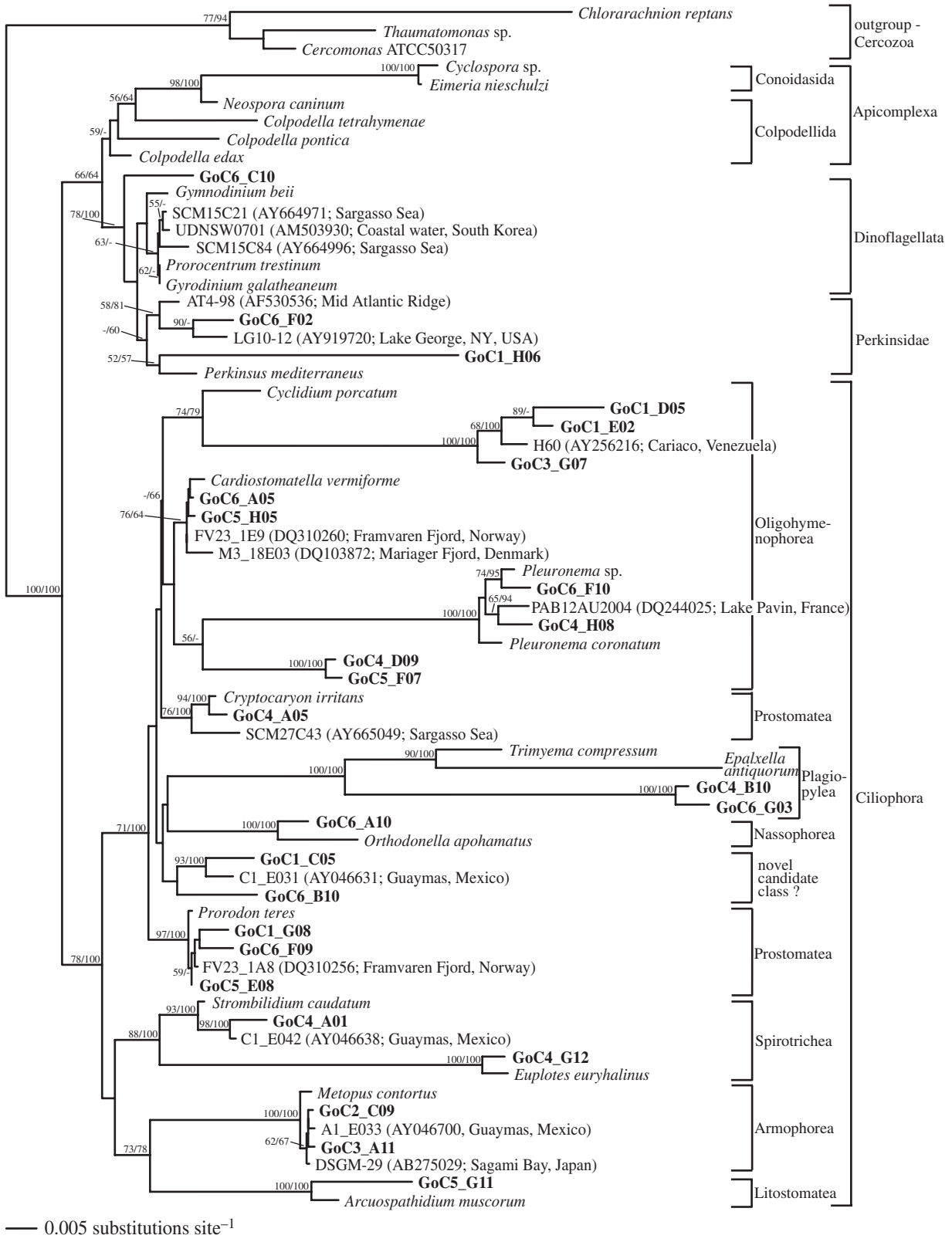

Fig. 7. Alveolata phylogeny. Minimum evolution phylogenetic tree of eukaryotic 18S rRNA showing the position of alveolate phylotypes from the oxic–anoxic transition zone (bold GoC4/5/6-clones) and the sulfidic water layer (bold GoC1/2/3-clones) of the Gotland Deep GTR + I + G: (G) = 0.4939, (I) = 0 based on 750 unambiguously aligned positions. Numbers at respective nodes show support values (distance bootstrap values/posterior probabilities)

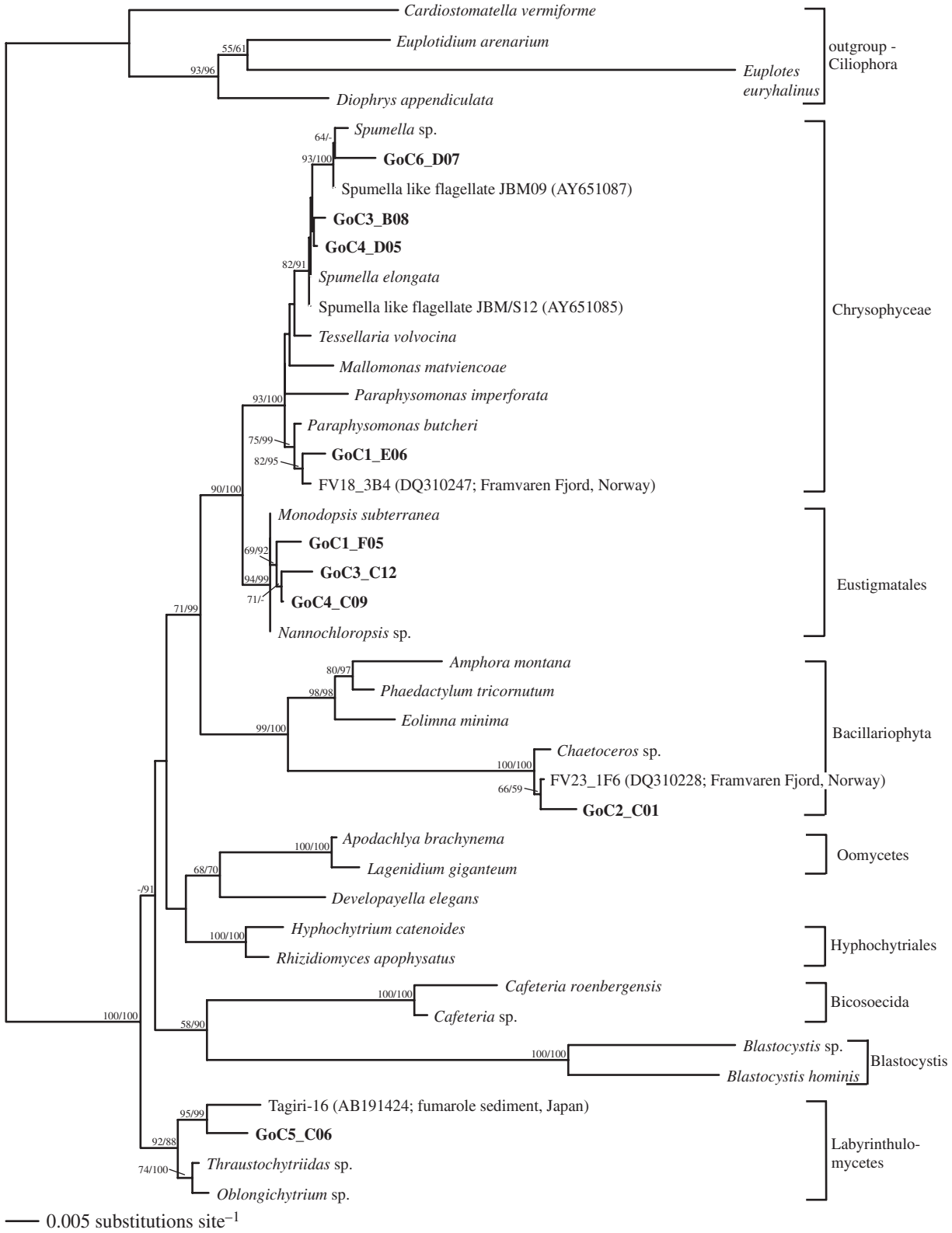

Fig. 8. Stramenopiles phylogeny. Minimum evolution phylogenetic tree of eukaryotic 18S rRNA showing the position of stramenopile phylotypes from the oxic–anoxic transition zone (bold GoC4/5/6-clones) and the sulfidic water layer (bold GoC1/2/3-clones) of the Gotland Deep. GTR + I + G: (G) = 0.8144, (I) = 0.4646 based on 850 unambiguously aligned positions. Numbers at respective nodes show support values (distance bootstrap values/posterior probabilities)

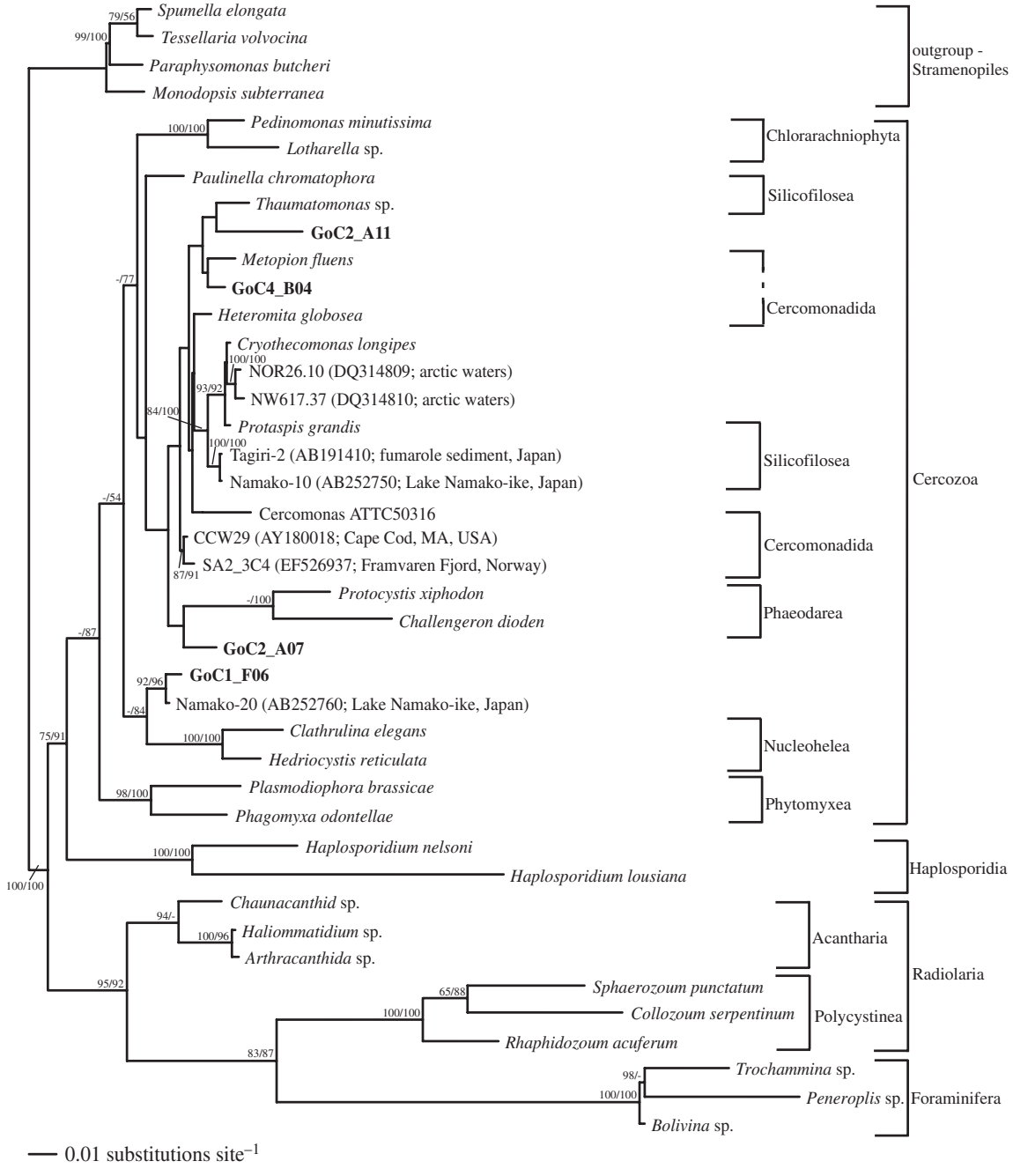

Fig. 9. Rhizaria phylogeny. Minimum evolution phylogenetic tree of eukaryotic 18S rRNA showing the position of Rhizaria phylotypes from the oxic–anoxic transition zone (bold GoC4/5/6-clones) and the sulfidic water layer (bold GoC1/2/3-clones) of the Gotland Deep. GTR + I + G: (G) = 0.5999, (I) = 0.1483 based on 815 unambiguously aligned positions. Numbers at respective nodes show support values (distance bootstrap values/posterior probabilities)

Cercozoa (Fig. 9). Two cercomonad sequences, GoC2_A11 (220 m) and GoC4_B04 (200 m), were related to *Thaumatomonas* (88.6% sequence similarity) and *Metopion* (90.7% sequence similarity). The pheodarean clone GoC2_A07 (sulfidic) was highly divergent to the closest known described taxa *Protocystis* (87.3% sequence similarity) and *Challengeron* (85.2% sequence similarity). Clone GoC1_F06 was a close relative of the environmental clone Namako-20, retrieved from a Japanese saline anoxic lake sediment. The branching position of these clones was uncertain, but posterior probabilities (84) suggested a sister relation to the Nucleohelea.

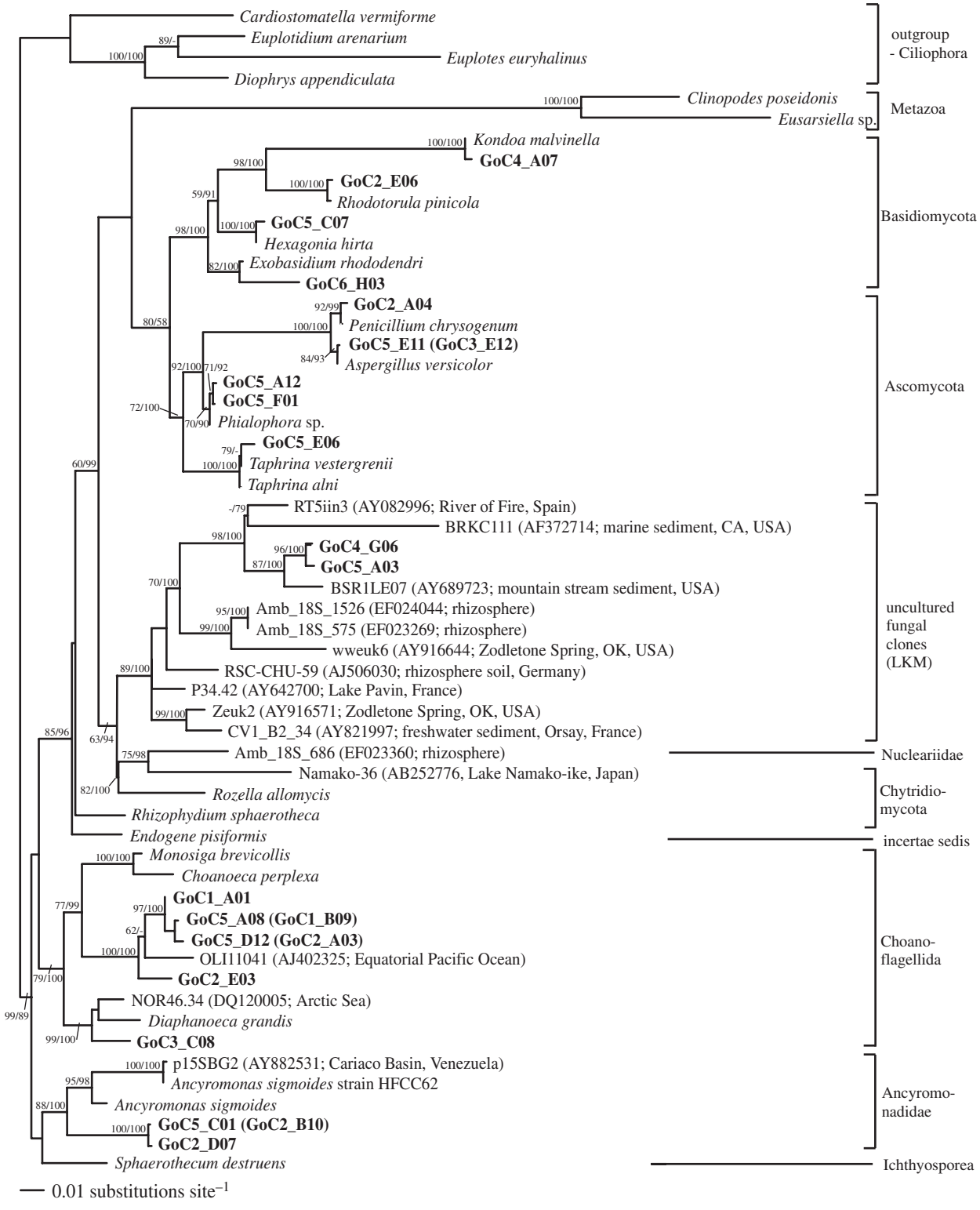

Fig. 10. Opisthokonts phylogeny. Minimum evolution phylogenetic tree of eukaryotic 18S rRNA showing the position of opisthokont phylotypes from the oxic–anoxic transition zone (bold GoC4/5/6-clones) and the sulfidic water layer (bold GoC1/2/3-clones) of the Gotland Deep. GTR + I + G: (G) = 0.4585, (I) = 0.2800 based on 915 unambiguously aligned positions. Numbers at respective nodes show support values (distance bootstrap values/posterior probabilities). In case a phylotype was found in both clone libraries, one representative sequence of this phylotype from the oxic–anoxic transition zone library is displayed as an individual branch and a representative sequence from the sulfidic water library is given in parentheses

Ophistokonts

Fungal sequences (Fig. 10) were retrieved from both layers; however, the vast majority occurred in the transition zone (n = 9). Four phylotypes (GoC4_A07, GoC2_E06, GoC5_C07, and GoC6_H03) branched within the Basidiomycota, and 5 phylotypes could be assigned to the Ascomycota, all of which were relatively closely related to described taxa. Two phylotypes from the transition zone (GoC4_G06 and GoC5_A03) were highly interesting as they branched within a clade of as yet uncultured fungi, including sequences retrieved from an acidic river (Amaral Zettler et al. 2002), anoxic marine sediment (Dawson & Pace 2002) and anoxic freshwater (J. Baeseman et al. unpubl. data). We identified 5 choanoflagellate phylotypes, 4 of which, together with a clone from the equatorial Pacific (OLI11041), branched as an environmental sequence clade without a cultured representative. Their closest related described organism was *Monosiga brevicolis* with a maximum sequence similarity of only 92.7%. The choanoflagellate sequence GoC3_C08 from the sulfidic layer was affiliated with *Diaphanoeca* (92.8% sequence similarity). Three further sequences from the Gotland Deep (GoC2_B10, GoC2_D07, GoC5_C01) were highly divergent to their next known described relatives, i.e. *Ancyromonas* and *Sphaerothecum* (89% sequence similarity in both cases).

Gotland 18S cDNA sequences branching within the jakobids

We assigned 19 phylotypes from the Gotland Deep to the jakobids (Fig. 11). Three of these occurred both in the transition zone and in the sulfidic waters. None were unique to the transition zone. The Gotland sequences were highly divergent from each other (up to 19% differences in sequence primary structure between GoC1_A03 and GoC3_D05). This does not become obvious from the distances in the phylogenetic tree (Fig. 11), as most of this divergence was in regions 1–187 and 660–722 of the sequences. However, most of these start (5′) and end (3′) regions were removed from the alignments in order to account for a comparison of homologue regions of all sequences in the respective phylogenetic analyses. They branched within a large clade that includes exclusively environmental sequences retrieved from anoxic marine sites like the Framvaren Fjord in Norway (Behnke et al. 2006), the Mariager Fjord in Denmark (Zuendorf et al. 2006), the Sippewisset salt marsh in the USA (Stoeck & Epstein 2003), the L'Atalante deep-sea basin in the Mediterranean (Alexander et al. 2009), and the sediment of the Mexican Guaymas deep-sea basin (Edgcomb et al. 2002). The closest related jakobid species was *Andalucia incarcerata* (= *Jakoba incarcerata*), with a maximum sequence similarity of 76.7%.

DISCUSSION

The goals of the present study were to complete a first inventory of molecular protistan diversity in the temporary anoxic Gotland Deep in the Baltic Sea, which is subjected to frequent disturbance by inflowing oxygenated seawater, and an assessment of the influence of physicochemical stratification in the Baltic Sea on protistan community structure.

We constructed and analyzed cDNA clone libraries from 18S rRNA from samples that we retrieved from the oxic–anoxic transition zone (200 m) and from below the chemocline (220 m) of the Gotland Deep. This is a standard technique in bacterial ecology and diversity research to identify active indigenous organisms and key players in biogeochemical processes in a specific environment (MacGregor et al. 2001) that was only recently adapted (Stoeck et al. 2007b) and successfully applied (Alexander et al. 2009) for eukaryote plankton communities.

Ciliates, jakobids and fungi comprised major fractions of the Gotland cDNA libraries. The high abundance of ciliate phylotypes is a typical feature of clone libraries constructed from anoxic aquatic environments (Epstein & López-García 2008). A dominance of ciliate morphotypes in such environments has previously been documented using microscopy-based methods (Fenchel et al. 1990). The successful establishment of ciliates in oxygen-depleted habitats is probably a result of a combination of their evolutionary history and feeding modes. Most ciliates are predators and grazers (Bernard & Rassoulzadegan 1990) that find abundant prey along stratification gradients in deeper waters, where bacteria develop high-density populations as they benefit from the availability of organic carbon, nutrients and particulate materials sinking down through the water column and reduced compounds like sulfide, ammonia and methane diffusing upwards from the anoxic sediment (Taylor et al. 2006). This was also observed for bacterial communities in the Gotland Deep (Höfle & Brettar 1995). These bacterial assemblages support a secondary food web along oxygen-depleted stratification gradients (Taylor et al. 2006).

Several times in their evolutionary history, ciliates have acquired various adaptations to anoxia like hydrogenosomes, detoxification of hydrogen sulfide and symbiotic relationships with bacteria (Fenchel & Finlay 1995), which makes them very strong competitors in this secondary food web. Indeed, several Gotland sequences

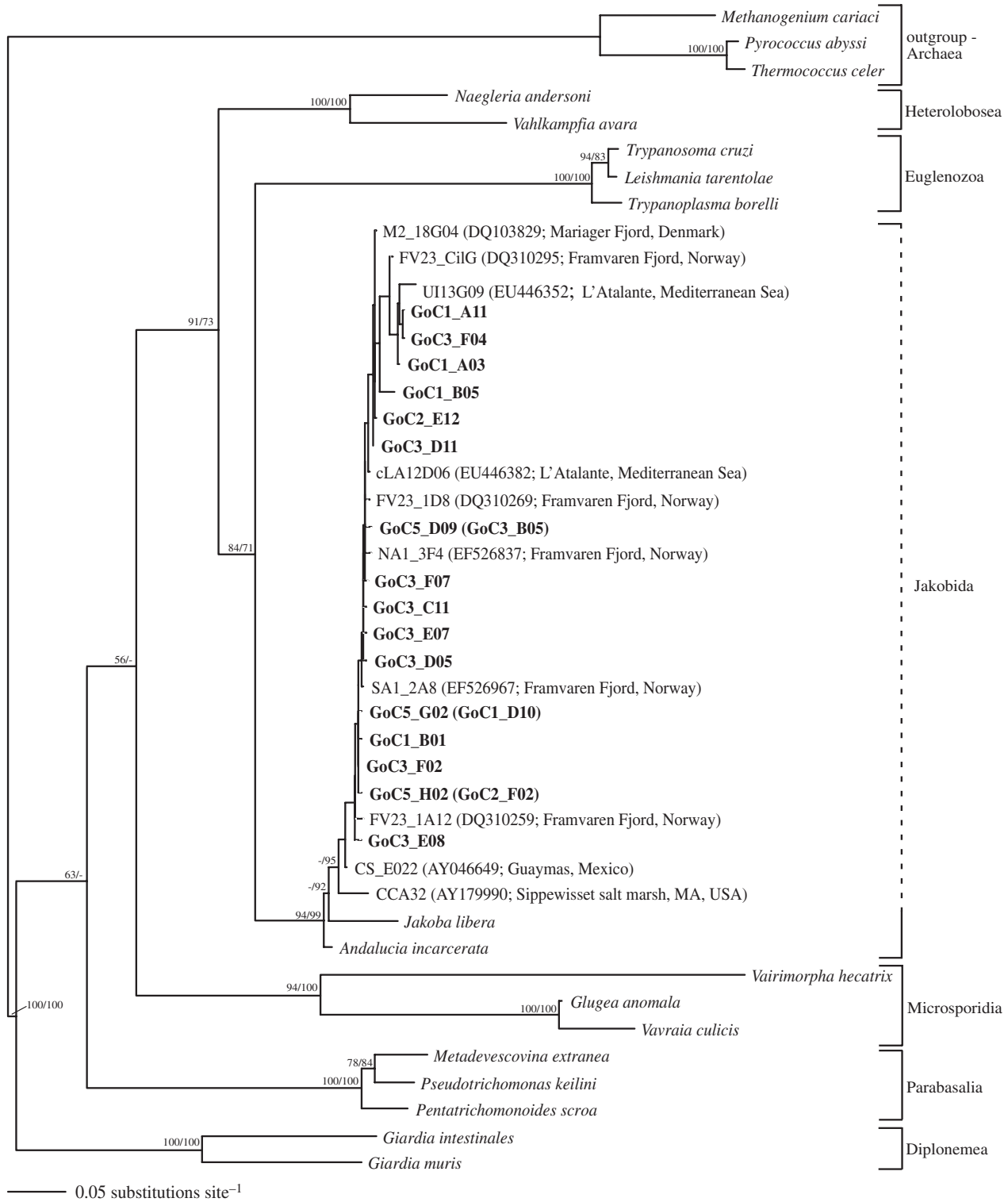

Fig. 11. Phylogeny of Gotland 18S cDNA phylotypes branching within the jakobids. Minimum evolution phylogenetic tree of eukaryotic 18S rRNA showing the position of phylotypes from the oxic–anoxic transition zone (bold GoC4/5/6-clones) and the sulfidic water layer (bold GoC1/2/3-clones) of the Gotland Deep branching within the jakobids. GTR + I + G: (G) = 1.0401, (I) = 0.1325 based on 710 unambiguously aligned positions. Numbers at respective nodes show support values (distance bootstrap values/posterior probabilities). In case a phylotype was found in both clone libraries, one representative sequence of this phylotype from the oxic–anoxic transition zone library is displayed as an individual branch and a representative sequence from the sulfidic water library is given in parentheses

are related to heterotrophic ciliates with a facultative or strictly anaerobic life style like *Trimyema*, *Metopus*, *Pleuronema*, *Prorodon*, *Strombidium*, *Euplotes*.

However, in the sulfidic layer below the chemocline, the ciliate diversity decreased considerably, possibly because of a significant drop of bacterial abundances below the chemocline (Fig. 3). Such a correlation was also observed in the anoxic Cariaco Basin (Taylor et al. 2006). Indeed, recent analyses have identified bacterial abundance as a major biotic factor structuring protistan communities in an anoxic Norwegian Fjord (T. Stoeck, A. Behnke & V. Edgcomb unpubl. data). Instead, phylotypes that branched in a clade with jakobid flagellates accounted for almost a third of all phylotypes and 71% of all sequences below the chemocline. We note that the environmental sequences from the Gotland Deep were relatively distant to previously described and sequenced jakobids. Thus, we cannot exclude the possibility that these distant sequences may belong to different taxonomic groups. Whether the respective organisms do share similar metabolic and ultrastructural characters with jakobids needs to be determined using appropriate *in situ* techniques and microscopy (Massana et al. 2002) and/or cultivation. However, considering the generally high sequence divergence within the jakobids — e.g. *Jakoba libera* (GenBank accession AF411288) and *Andalucia* (*Jakoba*) *incarcerata* (GenBank accession AY117419) shared only 91.0% sequence similarity, and *Jakoba libera* (GenBank accession AF411288) and *Andalucia godoyi* (GenBank accession AY965870) shared only 81.7% — it seems highly likely that the sequences recovered from the Gotland samples indeed represent jakobids.

Jakobids are common members of anoxic protistan communities (Luo et al. 2005, Behnke et al. 2006, Zuendorf et al. 2006), while they are either very rare or missing from clone libraries from oxygenated freshwaters (Lefranc et al. 2005) or marine systems (Countway et al. 2007). Reasons for the differences in jakobid diversity between samples from the transition zone and those from below the chemocline of the Gotland Deep remain speculative as their physiology, ecology and taxonomic diversity are hardly known, underlining our poor knowledge of the occurrence and role of this group in anoxic aquatic systems.

The high proportion of fungi is less surprising, as they constituted a large proportion of clone libraries constructed from anoxic sample material in previous studies. For example, fungi accounted for 26% of all phylotypes retrieved from intertidal sediments (Dawson & Pace 2002), for 17% in an anoxic deep-sea halocline (Alexander et al. 2009) and 16% in a sulfidic spring (Luo et al. 2005). Even in an anaerobic sandy aquifer, fungi accounted for the largest proportion of taxa in the eukaryote community (Brad et al. 2008). Although no strictly anaerobic fungi have been described to date, several members of this group are capable of anaerobic growth and to withstand prolonged periods of anoxia (Tsuruta et al. 1998). For example, some ascomycetes (Sonderegger et al. 2004) and some basidiomycetes (Fell et al. 2001) are capable of fermentation; and some of these taxa have been isolated from anaerobic deep-sea environments (Nagahama et al. 2003). Thus, we assume that, in contrast to previous assumptions (Mansfield & Barlocher 1993), fungi may indeed play an important role in ecosystem processes in anaerobic systems. This assumption needs to be verified in future process-oriented studies.

Choanoflagellates and heterotrophic chrysophytes (stramenopiles) also accounted for a relatively large proportion of the Gotland Deep cDNA clone libraries. As ecological studies on these organisms usually focus on oxygenated environments (Marchant 1985), little is known about the diversity and ecology of these taxa in oxygen-depleted environments. Several environmental diversity surveys reported 18S rDNA sequences that fall within the choanoflagellates (Moon-van der Staay et al. 2001, Zuendorf et al. 2006) and heterotrophic chrysophytes (Luo et al. 2005, Zuendorf et al. 2006). Previously, we also isolated *Spumella*-like flagellates from a 220 m sample from the Gotland Deep (Stoeck et al. 2008). Choanoflagellates have also been isolated and cultured from sulfidic, anoxic marine waters (Marchant et al. 1987). The high sequence divergence specifically of the Gotland choanoflagellate sequences indicates that oxygen-depleted environments are promising targets to discover novel heterotrophic nanoflagellates (HNFs). Studying their ecological role in these systems will help to elucidate the trophodynamics in anaerobic food webs, of which little is known (Fenchel & Finlay 1995) compared to oxygenated marine waters (Azam et al. 1983).

Interestingly, sequences retrieved from a number of anoxic sites such as the Venezuelan Cariaco deep-sea basin (Stoeck et al. 2003b), a hydrothermal deep-sea site at the Mid-Atlantic ridge (López-García et al. 2003), a deep-sea basin in the eastern Mediterranean (Alexander et al. 2009), marine fumaroles and methane cold-seep sediments near Japan (DSGM-58), from a coastal Pacific site (Dawson & Pace 2002) and a salt marsh sample (Stoeck & Epstein 2003) in the USA, a sulfide-rich spring in Oklahoma (Luo et al. 2005) and sediment from a lake in France (Lefranc et al. 2005) did not fall into Gotland Deep phylotypes on a 95% sequence similarity level but showed a higher sequence divergence. Thus, at least on a relatively low taxonomic level, corresponding to 18S rDNA sequence similarity, the protistan community in the Gotland Deep was distinct from protistan communities at permanently

anoxic sites. This may be explained by a combination of (1) methodological artifacts like PCR and primer bias (Stoeck et al. 2006) and the construction and analysis of RNA-derived clone libraries as opposed to DNA-derived clone libraries (Stoeck et al. 2007b), and (2) environmental selection of evolutionary lineages.

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Appendix 1. Taxonomic assignment of each phylotype from Fig. 5 with the sequence abundance per phylotype, a representative sequence from the Gotland Deep libraries and their according GenBank accession numbers. –: no phylotype isolated. **Bold** letters indicate shared phylotypes

	Oxic–anoxic transition zone			Sulfidic waters			
	No. of forward sequenced clones:	288		No. of forward sequenced clones:	288		
	No. of complete sequenced clones:	171		No. of complete sequenced clones:	237		
	No. of detected phylotypes:	41		No. of detected phylotypes:	50		
Phylotype no.	No. sequences	Representative	GenBank accession no.	No. sequences	Representative	GenBank accession no.	Group
1	8	**GoC5_D12**	FJ153718	4	**GoC2_A03**	FJ153644	**Choanoflagellida**
2	1	**GoC5_E11**	FJ153721	1	**GoC3_E12**	FJ153680	**Ascomycota**
3	0	–	–	1	GoC2_A04	FJ153645	Ascomycota
4	12	GoC5_A12	FJ153712	0	–	–	Ascomycota
5	2	GoC5_E06	FJ153719	0	–	–	Ascomycota
6	11	**GoC5_C01**	FJ153714	6	**GoC2_B10**	FJ153649	**Ancyromonadidae**
7	4	GoC4_C09	FJ153698	0	–	–	Stramenopiles
8	2	GoC5_C06	FJ153715	0	–	–	Stramenopiles
9	0	–	–	6	GoC2_D01	FJ153652	Chloroplastida
10	0	–	–	1	GoC1_A03	FJ153625	Jakobida
11	2	**GoC5_D09**	FJ153717	40	**GoC3_B05**	FJ153669	**Jakobida**
12	0	–	–	1	GoC3_E07	FJ153678	Jakobida
13	0	–	–	1	GoC3_F04	FJ153682	Jakobida
14	0	–	–	6	GoC3_F02	FJ153681	Jakobida
15	0	–	–	1	GoC3_D11	FJ153676	Jakobida
16	0	–	–	1	GoC3_F07	FJ153683	Jakobida
17	1	**GoC5_G02**	FJ153725	52	**GoC1_D10**	FJ153634	**Jakobida**
18	0	–	–	1	GoC2_E12	FJ153658	Jakobida
19	0	–	–	3	GoC3_D05	FJ153675	Jakobida
20	0	–	–	3	GoC2_C09	FJ153651	Ciliophora
21	0	–	–	1	GoC3_A11	FJ153667	Ciliophora
22	0	–	–	2	GoC1_G08	FJ153640	Ciliophora
23	9	GoC6_F09	FJ153741	0	–	–	Ciliophora
24	18	GoC5_F07	FJ153724	0	–	–	Ciliophora
25	3	GoC6_F10	FJ153742	0	–	–	Ciliophora
26	14	GoC4_B10	FJ153696	0	–	–	Ciliophora
27	8	GoC4_G06	FJ153704	0	–	–	Uncultured Fungi
28	0	–	–	11	GoC3_E08	FJ153679	Jakobida
29	0	–	–	1	GoC1_A11	FJ153626	Jakobida
30	1	**GoC5_H02**	FJ153727	28	**GoC2_F02**	FJ153659	**Jakobida**
31	0	–	–	20	GoC1_B01	FJ153627	Jakobida
32	0	–	–	1	GoC2_A11	FJ153647	Cercozoa
33	1	GoC4_B04	FJ153695	0	–	–	Cercozoa
34	9	**GoC5_A08**	FJ153711	1	**GoC1_B09**	FJ153629	**Choanoflagellida**
35	17	GoC5_F01	FJ153722	0	–	–	Ascomycota
36	0	–	–	3	GoC2_D07	FJ153653	Ancyromonadidae
37	0	–	–	4	GoC1_F05	FJ153638	Stramenopiles
38	1	GoC4_D05	FJ153700	0	–	–	Stramenopiles
39	8	GoC5_A03	FJ153710	0	–	–	Uncultured Fungi
40	3	GoC6_G03	FJ153743	0	–	–	Ciliophora
41	13	GoC4_D09	FJ153701	0	–	–	Ciliophora
42	1	GoC5_E08	FJ153720	0	–	–	Ciliophora
43	1	GoC5_H05	FJ153729	0	–	–	Ciliophora
44	1	GoC6_B10	FJ153733	0	–	–	Ciliophora
45	0	–	–	1	GoC3_B04	FJ153668	Chloroplastida
46	0	–	–	1	GoC3_C11	FJ153673	Jakobida
47	0	–	–	1	GoC3_C12	FJ153674	Stramenopiles
48	1	GoC4_F05	FJ153702	0	–	–	Choanoflagellida
49	0	–	–	1	GoC1_A01	FJ153624	Choanoflagellida
50	0	–	–	1	GoC1_B05	FJ153628	Jakobida
51	0	–	–	1	GoC3_C07	FJ153671	Chloroplastida
52	1	GoC4_H08	FJ153708	0	–	–	Ciliophora
53	0	–	–	1	GoC2_E03	FJ153655	Choanoflagellida
54	1	GoC6_A05	FJ153730	0	–	–	Ciliophora

Appendix 1 (continued)

Phylotype no.	No. sequences	Representative	GenBank accession no.	No. sequences	Representative	GenBank accession no.	Group
55	1	GoC4_A05	FJ153691	0	–	–	Ciliophora
56	1	GoC5_C07	FJ153716	0	–	–	Basidiomycota
57	0	–	–	1	GoC2_E06	FJ153656	Basidiomycota
58	1	GoC4_A07	FJ153692	0	–	–	Basidiomycota
59	1	GoC6_A10	FJ153731	0	–	–	Ciliophora
60	1	GoC6_D07	FJ153736	0	–	–	Stramenopiles
61	0	–	–	1	GoC3_B08	FJ153670	Stramenopiles
62	0	–	–	1	GoC1_E06	FJ153637	Stramenopiles
63	1	GoC6_H03	FJ153746	0	–	–	Basidiomycota
64	0	–	–	1	GoC1_D05	FJ153632	Ciliophora
65	0	–	–	1	GoC3_G07	FJ153687	Ciliophora
66	0	–	–	2	GoC1_E02	FJ153636	Ciliophora
67	0	–	–	11	GoC3_E04	FJ153677	Fungi (incertae sedis)
68	1	GoC4_A01	FJ153690	0	–	–	Ciliophora
69	0	–	–	1	GoC1_C05	FJ153631	Ciliophora
70	1	GoC6_G10	FJ153745	0	–	–	Chytridiomycota
71	0	–	–	1	GoC2_A07	FJ153646	Cercozoa
72	0	–	–	1	GoC1_F06	FJ153639	Cercozoa
73	1	GoC4_A11	FJ153693	0	–	–	Cercozoa
74	0	–	–	2	GoC3_C08	FJ153672	Choanoflagellida
75	1	GoC6_C10	FJ153735	0	–	–	Dinoflagellata
76	2	GoC6_F02	FJ153738	0	–	–	Perkinsidae
77	0	–	–	1	GoC1_H06	FJ153642	Perkinsidae
78	0	–	–	2	GoC2_C01	FJ153650	Stramenopiles
79	0	–	–	1	GoC2_F04	FJ153660	Cryptophyta
80	0	–	–	1	GoC2_H04	FJ153666	Cryptophyta
81	0	–	–	1	GoC1_B10	FJ153630	Centrohelida
82	3	GoC5_G11	FJ153726	0	–	–	Ciliophora
83	0	–	–	1	GoC1_G12	FJ153641	Lobosea
84	2	GoC4_G12	FJ153706	0	–	–	Ciliophora

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