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ORIGINAL PAPER

Molecular diversity and temporal variation of picoeukaryotes in two Arctic fjords, Svalbard

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Abstract Picoeukaryotes (protists <3 μm) form an important component of Arctic marine ecosystems, although knowledge of their diversity and ecosystem functioning is limited. In this study, the molecular diversity and autotrophic biomass contribution of picoeukaryotes from January to June 2009 in two Arctic fjords at Svalbard were examined using 18S environmental cloning and size-fractioned chlorophyll *a* measurements. A total of 62 putative picoeukaryotic phylotypes were recovered from 337 positive clones. Putative picoeukaryotic autotrophs were mostly limited to one species: *Micromonas pusilla*, while the putative heterotrophic picoeukaryote assemblage was more diverse and dominated by uncultured marine stramenopiles (MAST) and marine alveolate groups. One MAST-1A phylotype was the only phylotype to be found in all clone libraries. The diversity of picoeukaryotes in general showed an inverse relationship with total autotrophic biomass, suggesting that the conditions dominating during the peak of the spring bloom may have a negative impact on picoeukaryote diversity. Picoplankton could contribute more than half of total autotrophic biomass before and after the spring bloom and benefited from an early onset of the growth season, whereas larger cells dominated the bloom itself.

Keywords Arctic · Environmental cloning · Spring bloom · Picobiliphytes · Picoplankton

Introduction

In the Arctic, picoplankton can contribute significantly to primary production although larger diatoms dominate the spring bloom (Degerlund and Eilertsen 2010). In a study of the Arctic Ocean from June to August, picoeukaryotes contributed 36% of autotrophic biomass (Booth and Horner 1997), whereas in the Canadian Arctic from August to September, 11–72% of total chlorophyll *a* could be obtained from intact cells passing through a 1-μm filter (Smith et al. 1985). However, temporal variation plays an important role as small cells in general appear to be most important when total chlorophyll concentrations are low (Brewin et al. 2010), and in the Barent Sea, 46% of total primary production was attributed to cells <10 μm during the entire bloom period, despite accounting for only 19% of the chlorophyll *a* during the peak of the bloom (Hodal and Kristiansen 2008). The near-absence of marine cyanobacteria in the Arctic makes eukaryotes the dominant component of autotrophic picoplankton, thus underlining the importance of this relatively unknown ecosystem component (Gradinger and Lenz 1995; Booth and Horner 1997; Li 1998; Sherr et al. 2003).

An Arctic strain of *Micromonas pusilla* has been shown to be an abundant marine picoautotroph throughout the Arctic (Not et al. 2005; Lovejoy et al. 2007), although other Mamiellophyceae (previously known as Prasinophyceae, Marin and Melkonian 2010), Haptophyta, Cryptophyceae

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and several Bacillariophyceae (diatoms) phylotypes of Arctic picoeukaryotes have also been recovered (Lovejoy et al. 2006). Stramenopiles and Alveolata are common phylotypes in Arctic picoeukaryotic clone libraries, and as seen elsewhere, the putative heterotrophic uncultured marine stramenopiles (MAST) and marine alveolate group (MAG) I and II are numerous (Massana et al. 2004b; Lovejoy et al. 2006; Guillou et al. 2008).

In spite of giving insight into the hidden world of Arctic picoeukaryotes, molecular studies have not yet addressed their seasonal variation. A recent study on the molecular diversity of freshwater protists using high-throughput sequencing showed that temporal variation can be of utmost importance as many protist phylotypes showed a high temporal turnover (Nolte et al. 2010). Using traditional environmental cloning in a temporal study of picoeukaryote diversity in the English Channel, Romari and Vaulot (2004) also found high temporal variability, which was also the case for a molecular study of small (<5 μm) photosynthetic eukaryotes in the Gulf of Naples (McDonald et al. 2007). In the English Channel, the community appeared stable at class/division level except during the diatom summer bloom where picoeukaryote diversity decreased. A study by Medlin et al. (2006) at Helgoland also found high temporal variation at a monthly basis, but identified an annual pattern in the community composition, suggesting seasonality in the picoeukaryotic community. As the Arctic is subject to extreme seasonal variation, it is an ideal location to investigate the influence of seasonality on picoeukaryote diversity. Additionally, as the Arctic is predicted to show amplified responses to global warming (IPCC 2007), obtaining baseline data on Arctic picoeukaryotes is of importance for future comparisons.

In this study, the molecular diversity and temporal variation of eukaryotic picoplankton were examined using environmental 18S clone libraries from an open-ended shallow fjord with partial ice cover throughout winter (Adventfjorden) and a deep two-silled fjord with annual fast ice (Billefjorden) in Isfjorden, western Spitsbergen. In addition, size-fractioned chlorophyll *a* measurements were used to assess the contribution of picoplankton to the total autotrophic biomass. To the best of our knowledge, this is the first molecular study of Arctic picoeukaryote diversity including seasonal variability.

Materials and methods

Study sites and sampling

Seawater samples were collected from three sampling stations: Adventfjorden at 15 m depth (78°16 N 15°30 E) and Adolfbukta in Billefjorden at 15 and 150 m depth (78°38 N 16°32 E, Fig. 1). For each station, two clone libraries were made from samples collected in winter-early spring (defined as January to March, called 'winter' henceforth) and one or two clone libraries from samples collected in spring-early summer (April to June, called 'spring', Table 1). This seasonal division was used because autotrophic biomass first started increasing in April, i.e. 'spring' (see Results). Sampling of chlorophyll *a* and ice thickness was more frequent. The fjords were sampled using a 5- or 10-L Niskin bottle (KC Denmark A/S, Silkeborg, Denmark). The samples were kept cold and dark until further processing, which was done within 36 h of sampling. Filtrations were done with a vacuum pump or a Pump drive PD 5001 with Pump head C4 (Heidolph, Schwabach, Germany). In Billefjorden ice thickness, freeboard and snow thickness were measured at three points in a triangular pattern, each point 10 m apart.

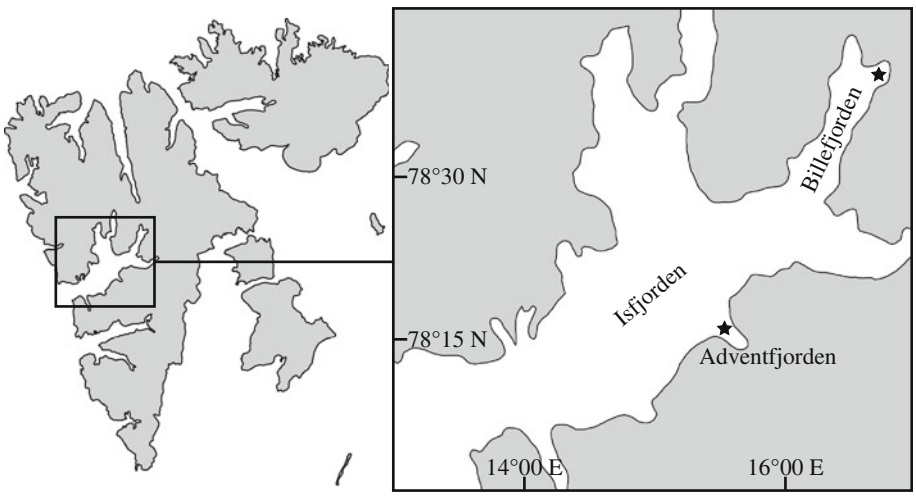

Fig. 1 The study took place on Svalbard. Samples were collected from Adventfjorden (78°16 N 15°30) and Billefjorden (78°38 N 16°32 E), which are part of the Isfjorden system. The sampling locations are marked with *stars*

Table 1 Observed and estimated OTU number and number of clones sampled for each clone library

Station (m)	Date	OTUs found	Clones sampled
Adventfjorden 15	20–02–2009	10	26
Adventfjorden 15	25–03–2009	12	42
Adventfjorden 15	19–05–2009	5	24
Adventfjorden 15	12–06–2009	14	32
Billefjorden 15	14–01–2009	17	24
Billefjorden 15	30–03–2009	14	35
Billefjorden 15	14–05–2009	9	22
Billefjorden 15	10–06–2009	9	26
Billefjorden 150	14–01–2009	20	39
Billefjorden 150	14–05–2009	20	31
Billefjorden 150	10–06–2009	15	36

Chlorophyll *a*

Seawater samples for chlorophyll *a* measurements were collected in 10-L plastic bottles, which were rinsed with distilled water between sampling. A volume of 0.60–1.70 L of water was filtered on 3-μm isopore membrane polycarbonate filters (Millipore, Billerica, USA) for non-picoplanktonic autotrophic biomass and a similar volume on 0.7-μm GF/F glass microfiber filters (Whatman, Maidstone, England) for total autotrophic biomass using a vacuum pump. The 0.7-μm filtrate was refiltered through 0.22-μm Durapore membrane hydrophilic PVDF filters (Millipore, Billerica, USA) to test whether filtration through 0.7 μm was adequate for total chlorophyll *a* measurements. All filtrations were replicated three times, and filters were stored in aluminium foil at −80°C. The equipment used for filtration was rinsed with distilled water between uses. Chlorophyll *a* was extracted from the filters in 100 mL methanol for 20–24 h at +5°C (Holm-Hansen and Riemann 1978) and the concentration measured on a 10-AU-005-CE Fluorometer (Turner Designs, Sunnyvale, USA).

DNA extraction

Seawater samples for DNA extraction were collected in 2-L plastic bottles, which were washed with sample water before collection. Containers and filtration units were washed with 1% chlorine for 12–24 h and repeatedly with distilled water between uses. A volume of 2 L of sample water was prefiltered with a 3-μm isopore membrane polycarbonate filter and cells were collected on a 0.22-μm Durapore membrane hydrophilic PVDF filter (both from Millipore, Billerica, USA) on a Pump drive PD 5001 with Pump head C4 (Heidolph, Schwabach, Germany) at 60 rpm. The 0.22-μm filters were cut in half and stored at −80°C until DNA extraction. Divided 0.22-μm filters were incubated in 594 μL CTAB and 6 μL *β*-mercaptoethanol for 45 min at 65°C, being vortexed every 15 min. The samples were frozen for ≥30 min at −80°C, heated at 65°C for 45 min and vortexed every 15 min. After adding 500 μL of chloroform mix (24:1 chloroform to isoamyl-alcohol), the samples were vortexed twice, shaken continuously for 10 min and then centrifuged at 12,000 rpm for 5 min on an Eppendorf Centrifuge 5415D (Eppendorf AG, Hamburg, Germany). The water phases were transferred to new Eppendorf tubes, 500 μL chloroform mix was added again and the procedure repeated. A volume of 4 μL of RNAse was added to the transferred water phase and the samples were incubated for 30 min at 37°C. The samples were centrifuged briefly, and two-thirds of the sample volume of ice cold isopropanol was added and the samples were kept at −20°C for ≥30 min. The samples were centrifuged at 12,000 rpm for 10 min and washed twice in 600 μL 70% ethanol. Residual ethanol was evaporated by putting opened sample tubes on a heating block at 65°C for a few minutes. A volume of 30 μL Milli-Q water was added to the sample and it was kept at room temperature for one hour before being stored at +5°C overnight. Extracted DNA was then stored at −20 or −80°C until further processing.

Polymerase chain reaction

Polymerase chain reactions (PCR) were carried out on an Eppendorf Mastercycler Ep Gradient S PCR cycler (Eppendorf AG, Hamburg, Germany) in volumes of 25 μL containing 1× buffer with 2.5 mM $MgCl_2$ (5 PRIME, Hamburg, Germany), 0.2 mM dNTP, 0.1 μg BSA, 0.2 μM of each primer and 1 U HotMaster Taq DNA polymerase (5 PRIME, Hamburg, Germany). For colony PCR, only half of the volumes were used per reaction, polymerase was reduced to 0.7 U and replaced with DreamTaq DNA polymerase (Fermentas, Burlington, Canada) and BSA was excluded. The following thermal cycling programme was used: 94°C for 3 min, 30 or 40 cycles of (94°C for 45 s, 57°C for 60 s, 72°C for 120 s) and 72°C for 10 min. An aliquot of 5 μL extracted DNA was amplified by an initial PCR (EukA and EukB primers, 40 cycles). When necessary, the correct band was isolated by gel extraction (21.8 μL PCR product, 0.7% TAE gel, 90 V, 45 min) using Agarose GelExtract Mini Kit (5 PRIME, Hamburg, Germany). A template of 2 μL 1,000-fold dilution of the PCR product or gel extract was used for a nested PCR (30 cycles, Euk528f and EukB primers). DNA was purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, USA). An Eppendorf Centrifuge 5424 (Eppendorf AG, Hamburg, Germany) was used for all post-PCR

centrifugation. The initial PCR was insufficient for amplifying DNA from winter samples, explaining the need for a total of 70 PCR cycles. The nested PCR was also used on spring samples to impose the same PCR bias in all clone libraries.

Cloning

Samples were cloned with CloneJET PCR cloning kit (Fermentas, Burlington, Canada) using TOP10 cells (Qiagen, Hilden, Germany) or 10G cells (Lucigen, Middleton, England) and ampicillin as selective media. Inserts were amplified by PCR (Euk528f and EukB, 30 cycles) and digested with HaeIII (5′-GG↓CC-3′, Fermentas, Burlington, Canada) following the manufacturer's protocol. Clones with unique restriction fragment length polymorphism (RFLP) patterns were grown overnight at 37°C in ~1 mL liquid LB–ampicillin medium, and the plasmids were isolated using the E.Z.N.A. Plasmid Miniprep Kit I (Omega Bio-Tek, Norcross, USA) using 500 µL DNA wash buffer for the second optional wash. The isolated plasmids were sequenced at the CEES DNA lab, Centre for Ecological and Evolutionary Synthesis, University of Oslo, on a 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) using various sequencing primers. Primers used for PCR and sequencing reactions are listed in Table 2 (Elwood et al. 1985; Medlin et al. 1988; Ekelund et al. 2004).

Molecular analyses

The sequences were divided into major taxonomical groups based on an initial NCBI BLAST search (Altschul et al. 1990). Suspected chimeras were checked using Chimera Detection (http://www.35.8.164.52/cgis/chimera.cgi?su=SSU) and KeyDNATools (http://www.keydnatools.com/) and by performing BLASTn searches using different ends of the 18S rDNA sequence. Non-eukaryotic, fungal and <500-bp sequences were discarded. Clones with distinct RFLP patterns and <99% sequence similarity were treated as separate operational taxonomical units (OTUs). Taxonomical subdivisions were aligned against selected 18S sequences from NCBI GenBank in CLC Main Workbench 5 (CLC bio, Århus, Denmark) and alignments were manually adjusted. Alignments are available upon request to NS.

Bayesian analyses were done with MrBayes 3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Bayesian posterior probabilities were calculated with 2,000,000 generations (5,000,000 for Syndiniales and stramenopiles), sampling trees every 50 generations. Tree convergence was checked with AWTY (Wilgenbusch et al. 2004) and burn-in was found manually: the first 101 (Choanoflagellida) or 401 (all other groups) trees were discarded. Posterior probabilities were calculated using a post-burn-in 50% majority rule consensus tree. ModelTest 3.7 (Posada and Crandall 1998) was used to find the best-fit model (TrN + I + G for all alignments, Tamura and Nei 1993) and neighbour-joining bootstrap values (1,000 replicates), using maximum likelihood to estimate all pairwise comparisons, were calculated with PAUP* 4.b10 (Swofford 2003). Rarefaction curves were created using Rarefaction Calculator (http://www.2.biology.ualberta.ca/jbrzusto/rarefact.php). Sequences reported in this study have been deposited in GenBank under accession numbers HQ156808-HQ156856 and HQ156858-HQ156897 and the frequency of each of these in the different clone libraries can be found in the supplementary table supplied online.

Results

Study sites

The fast ice thickness in Billefjorden varied from 72 cm in late February to 100 cm in mid-May and broke up in mid-June just after the final sampling. The snow thickness varied between 12 and 28 cm throughout the season. Adventfjorden was partially ice covered until May, with more complete ice cover in late March and late April. See supplementary material for more detailed information on ice thickness, CTD profiles and nutrient concentrations.

Chlorophyll *a*

In Adventfjoden at 15 m, the phototrophic biomass was low during winter and early spring (0.02–0.03 µg Chl *a* L^{-1}),

Table 2 Primers used for PCR and sequencing

Primer	Sequence	References
EukA	5′-AACCTGGTTGATCCTGCCAGT-3′	Medlin et al. (1988)
EukB	5′-TGATCCTTCTGCAGGTTCACCTAC-3′	Medlin et al. (1988)
Euk528f	5′-GCGGTAATTCCAGCTCCAA-3′	Elwood et al. (1985)
ND5F	5′-GGTGGTGCATGGCCGTTC-3′	Ekelund et al. (2004)
ND7R	5′-GAACGGCCATGCACCACC-3′	Ekelund et al. (2004)
ND8R	5′-TCTGAGAATTTCACCTCT-3′	Ekelund et al. (2004)

pJETf and pJETr primers (specific for the pJET cloning vector) were also used

increased from April and reached a maximum of 5.7 μg Chl *a* L^{-1} in May (Fig. 2). Picoplankton made up over half of the total autotrophic biomass in April, but this decreased to 10–16% in May when total chlorophyll *a* was highest. In June, total chlorophyll *a* dropped to 0.3 μg L^{-1}, and picoplankton again made up a substantial amount (62%) of the autotrophic biomass. In Billefjorden at 15 m, the autotrophic biomass also increased from April, but the initial concentrations were lower (for both total and picoplanktonic chlorophyll *a*) and values >1 μg L^{-1} were not reached until June (Fig. 2). In April and May, picoplankton made up 24–51% of total autotrophic biomass, but this decreased to 12% in June when total chlorophyll *a* was at its highest. Chlorophyll *a* concentration of the refiltrate (0.22–0.7 μm) was on average 1.0% of the total (>0.7 μm) with a maximum of 2.8%.

Molecular diversity

The 11 clone libraries yielded 337 clones belonging to 62 different putative picoeukaryotic phylotypes (Tables 1, 3) when excluding ciliate, dinoflagellate, rhizarian (all three groups unlikely to be picoplanktonic, see Discussion), metazoan, fungal, chimeric, non-eukaryotic and sequences of poor quality (<500 bp). Of the phylotypes, >75% belonged to stramenopiles and Syndiniales (MAG-I and MAG-II). Only two phylotypes of Mamiellophyceae were found. Four sequences clustered within the novel lineage picobiliphytes (Not et al. 2007b). Two sequences belonging to Haptophyta and three to Choanoflagellida were also found, as well as two unidentified Alveolata (sister to Dinokaryota) and a single Cryptophyceae. Additionally, 72 clones representing 18 phylotypes of Ciliophora, Dinokaryota and Rhizaria were found as well as 9 phylotypes of Metazoa (see supplementary material).

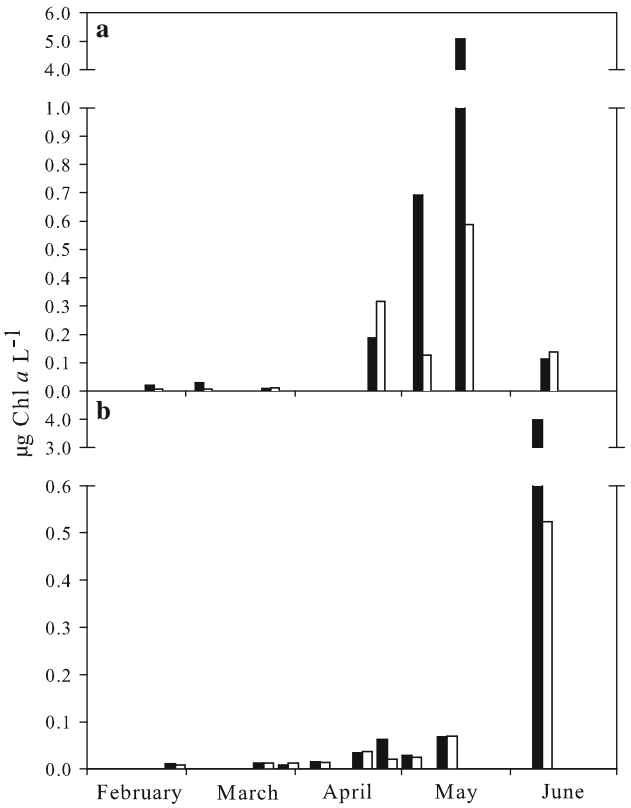

Fig. 2 **a** Chlorophyll *a* concentrations in Adventfjorden 2009 at 15 m and **b** in Billefjorden 2009 15 m for phytoplankton >3 μm (*black bars*) and picoplankton (*white bars*). In Adventfjorden, the bloom occurred in mid-May and chlorophyll *a* decreased drastically afterwards, and in Billefjorden, the bloom occurred in June

Rarefaction curves showed Billefjorden at 150 m to generally have high molecular diversity (Fig. 3). For both fjords at 15 m, the lowest picoeukaryote diversity was found at the peak of the phytoplankton bloom, although the diversity was also relatively low in Billefjorden 15 m just before the bloom. Following the decline in chlorophyll *a* in Adventfjorden, molecular diversity rose again. Most phylotypes showed ≥95% similarity to sequences already deposited in GenBank; some stramenopiles and picobiliphytes had lower similarities (Fig. 4). There was no relationship between the seasonal distribution of a phylotype (whether it was found in winter, spring or both seasons) and its similarity to deposited sequences (ANOVA, $F = 0.43$, $P = 0.65$).

Mamiellophyceae

Both prasinophyte phylotypes clustered within the Mamiellales (Fig. 5). The most widespread one (100609_23) was identified as *M. pusilla* and clustered within the Arctic subclade as defined by Lovejoy et al. (2007). It was found in all clone libraries except from Billefjorden 150 m in January and May. The other prasinophyte phylotype, 010809_04, formed a sister group to the genus

Table 3 Number of operational taxonomical units (OTU) found for different taxonomical groups based on partial 18S sequencing

Taxonomic group	Number of OTUs	Proportion (%)
Syndiniales	27	44
Stramenopiles	21	34
Picobiliphytes	4	6
Choanoflagellida	3	5
Mamiellophyceae	2	3
Haptophyta	2	3
Unidentified Alveolata	2	3
Cryptophyceae	1	2

Sequences were defined as different OTUs if they had different RFLP patterns and <99% sequence similarity. Ciliate, dinoflagellate, rhizarian, fungal, metazoan and non-eukaryotic sequences are not included

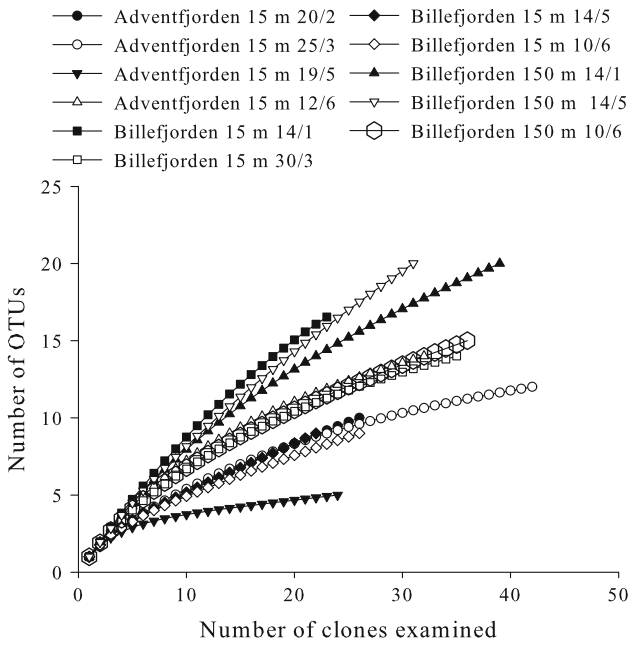

Fig. 3 Rarefaction curves of putative picoeukaryotic OTUs for the eleven clone libraries. The number of OTUs was found by the restriction enzyme HaeIII, and treating sequences with >99% similarity as belonging to the same phylotype. Ciliophora, Dinokaryota and Rhizaria are not included

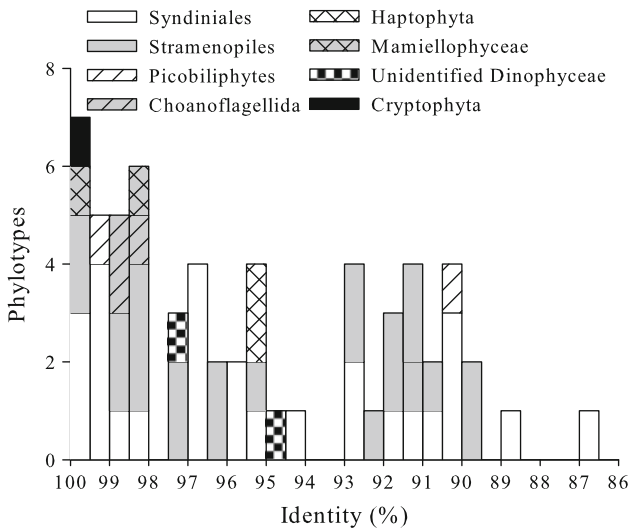

Fig. 4 Novelty histogram of phylotypes from this study by taxonomic affiliation. Phylotypes are binned by 0.5% identity to sequences in GenBank

Crustomastix with DSGM-81, although with limited support. It was only observed in spring in Adventfjorden.

Stramenopiles

Of the 21 stramenopile phylotypes obtained, 18 clustered within a MAST clade, two clustered within the Chrysophyceae and one could not be assigned to any specific clade (Fig. 6). Those within MAST-1A, MAST-1B, MAST-1C, MAST-3 and MAST-4 clades matched the probes designed by Massana et al. (2002, 2006b), except for 010609_04 (MAST-1A, 1 mismatch). MAST-1A, B and C clades are in accordance with Massana et al. (2006a), while the remaining MAST clades are as defined by Massana et al. (2004b) and Kolodziej and Stoeck (2007). Of the 21 stramenopile phylotypes, 16 were absent from Adventfjorden (including the entire MAST-1C and MAST-4 clade). The phylotypes were evenly distributed between those found in winter, spring or both of these seasons (8, 7 and 7 phylotypes, respectively). The vast majority of closely related environmental sequences from other studies were from picoplanktonic size fraction and they were geographically diverse. The phylotype 100609_22 (MAST-1A) was found in all clone libraries and two other phylotypes, 130609_13 (MAST-1B) and 100609_12 (*Paraphysomonas*), were found in both winter and spring at all stations, although they were not present in all clone libraries.

Syndiniales

Ten phylotypes clustered within MAG-I and 17 within MAG-II (Fig. 7), both clades as defined by Skovgaard et al. (2005). Two phylotypes (190609_12 and 300709_06) could not be assigned to any specific clade, but showed weak support as a sister group to Dinokaryota together with their respective sister taxa (clade A). Both of these phylotypes were only found in one clone library each. MAG-I phylotypes were more often found in winter (90% of phylotypes) than in spring (30%). MAG-II did not exhibit such a temporal pattern (70 and 65%, respectively). The majority of the closest related environmental sequences stemmed from picoplanktonic size fraction (<3 μm), and they were collected from geographically diverse regions. Two of the related environmental sequences were from ship ballast water (BW-dinoclone1 and BW-dinoclone22). Phylotypes 210609_12 and 100609_24, belonging to MAG-I and MAG-II respectively, were found in both winter and spring at all stations, although not in all clone libraries.

Picobiliphytes

In Fig. 8, four phylotypes clustered within the clade defined as picobiliphytes by Not et al. (2007b). One of the phylotypes was found in both winter and spring and at all three sampling sites, while the others had a more limited distribution. Most of the closest related environmental sequences from other studies are from picoplanktonic size fractions (<3 μm) and were collected from the North

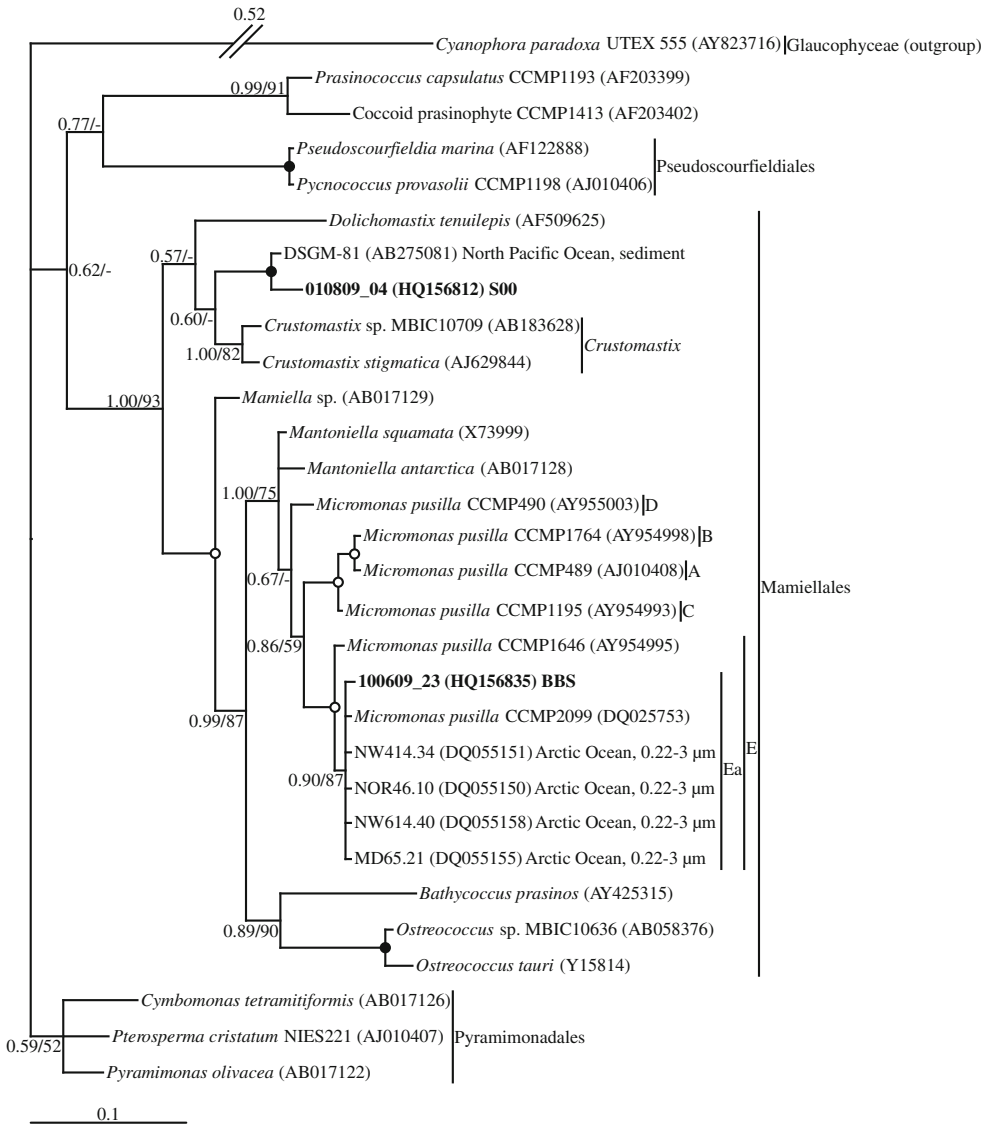

Fig. 5 Phylogenetic tree of Mamiellophyceae based on Bayesian inference. The clades *A–E* are the different clades of *M. pusilla* as defined by Slapeta et al. (2006), while Ea is the Arctic subclade investigated by Lovejoy et al. (2007). Phylogenetic trees based on Bayesian inference with posterior probability and bootstrap support values in per cent at relevant nodes. *Filled circle* indicates 1.00/100% and *open circle* indicate ≥0.95/≥95%. *Hyphen* indicates posterior probability <0.50 or bootstrap <50%. Sequences from this study are in *bold*. The three-character code after the accession number tells the seasonal distribution of phylotypes at each of the three sampling stations: Adventfjorden 15 m (1st letter), Billefjorden 15 m (2nd letter) and Billefjorden 150 m (3rd letter). For each sampling station, phylotypes were categorized as found in winter (*W*), spring (*S*), both seasons (*B*), or absent (*0*); for example, W0B means a phylotype was found in winter in Adventfjorden at 15 m, absent from Billefjorden at 15 m and found in spring and winter in Billefjorden at 150 m. Winter was defined as January to March and spring was defined as May and June. For environmental sequences from other studies, location of sampling and size fraction of clone library is given if applicable

Atlantic or Arctic regions. Two phylotypes (100609_15 and 210609_18) represented a distinct subclade within one of the clades (subclade A) and contained 4 identical mismatches to the 18-bp picobiliphyte probe PICOBI01 designed by Not et al. (2007b). Phylotype 130609_16 had no mismatches while 100609_40_pJETf_b had 1 mismatch to the same probe. None of the mismatches were similar to mismatches reported by Not et al. (2007b) for sequences within the picobiliphyte clade. The 18-bp picobiliphyte probe PICOBI02 also by Not et al. (2007b) had 6 mismatches with each of the 4 phylotypes.

Choanoflagellida

Three phylotypes clustered within the Choanoflagellida (Fig. 9). The support for phylotype 100609_37 as a member of Choanoflagellida was limited, especially when considering that Metazoa clustered within the Choanoflagellida.

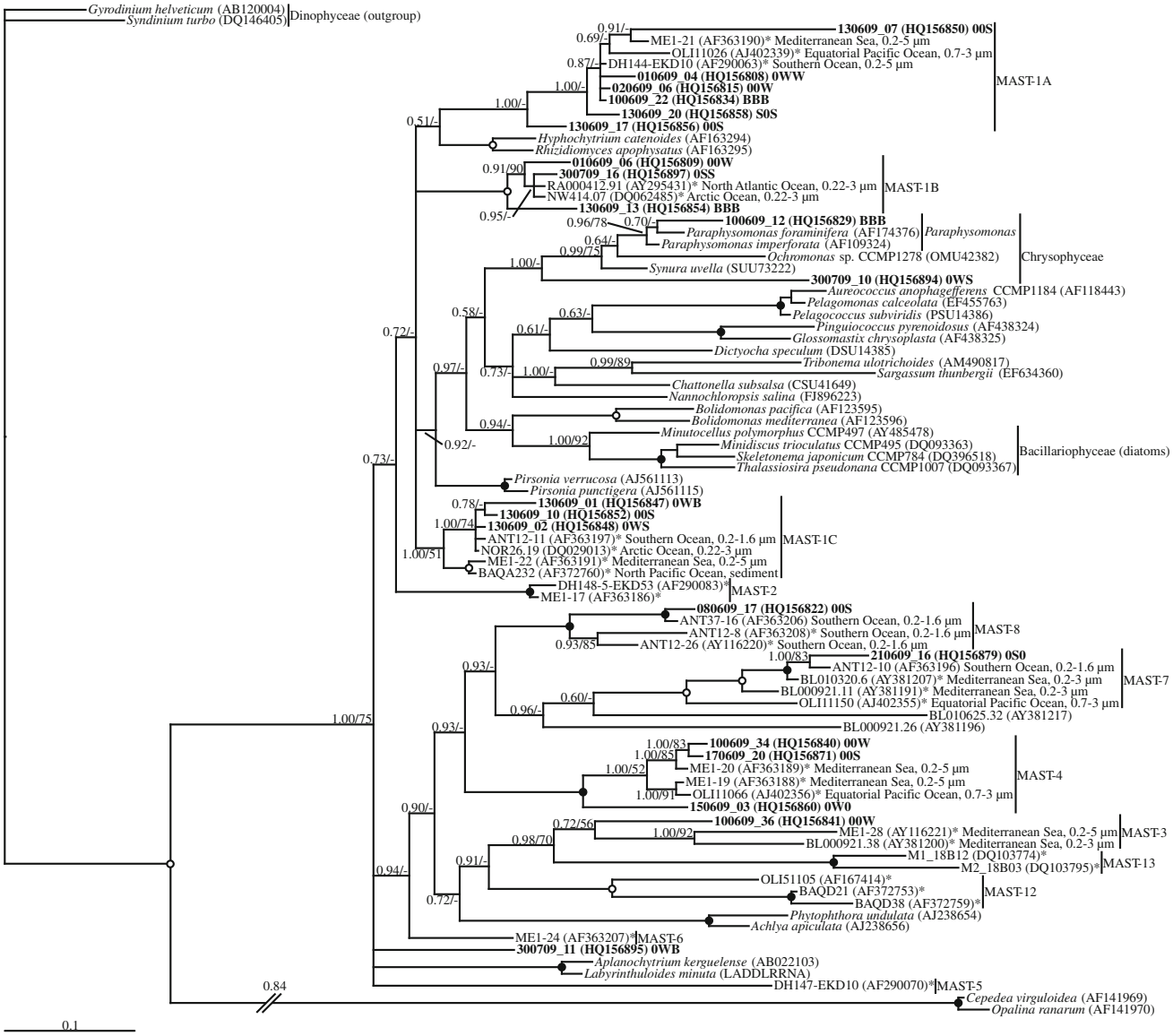

Fig. 6 Phylogenetic tree of stramenopiles based on Bayesian inference. Sequences marked with an *asterisk* (*) are used by Massana et al. (2004b), or Massana et al. (2006a), Kolodziej and Stoeck (2007) to define the respective clades. The explanations are given in Fig. 5

Two of the phylotypes were only represented by one clone each, while the last phylotype (230609_07) was present in 6 of 11 clone libraries.

Haptophyta and Cryptophyceae

The two haptophycean phylotypes both clustered within the genus *Chrysochromulina* (Fig. 10). Both phylotypes were only represented by one clone each in spring in Billefjorden at 150 m. The single cryptophyceaen phylotype obtained in this study showed 99.38% similarity to *Geminigera cryophila* (accession number AB058368). It was only represented by a single clone in June in Billefjorden at 15 m.

Ciliophora, Dinokaryota, and Rhizaria

Among the Ciliophora, six phylotypes clustered within Spirotrichea, 170609_14 within Litostomatea, and 230609_05 together with RD010517.29 and *Urotricha* sp. as sister to Prostomatea and Oligohymenophorea (Fig. A5, supplementary material). Ciliophora were found at all sampling stations but only in spring. All the related environmental sequences had been found in a <5-µm fraction.

Six phylotypes clustered within Dinokaryota (Fig A6, supplementary material). Dinokaryota and its sister group Clade A were more often found in winter (87.5% of phylotypes) than in spring (37.5%).

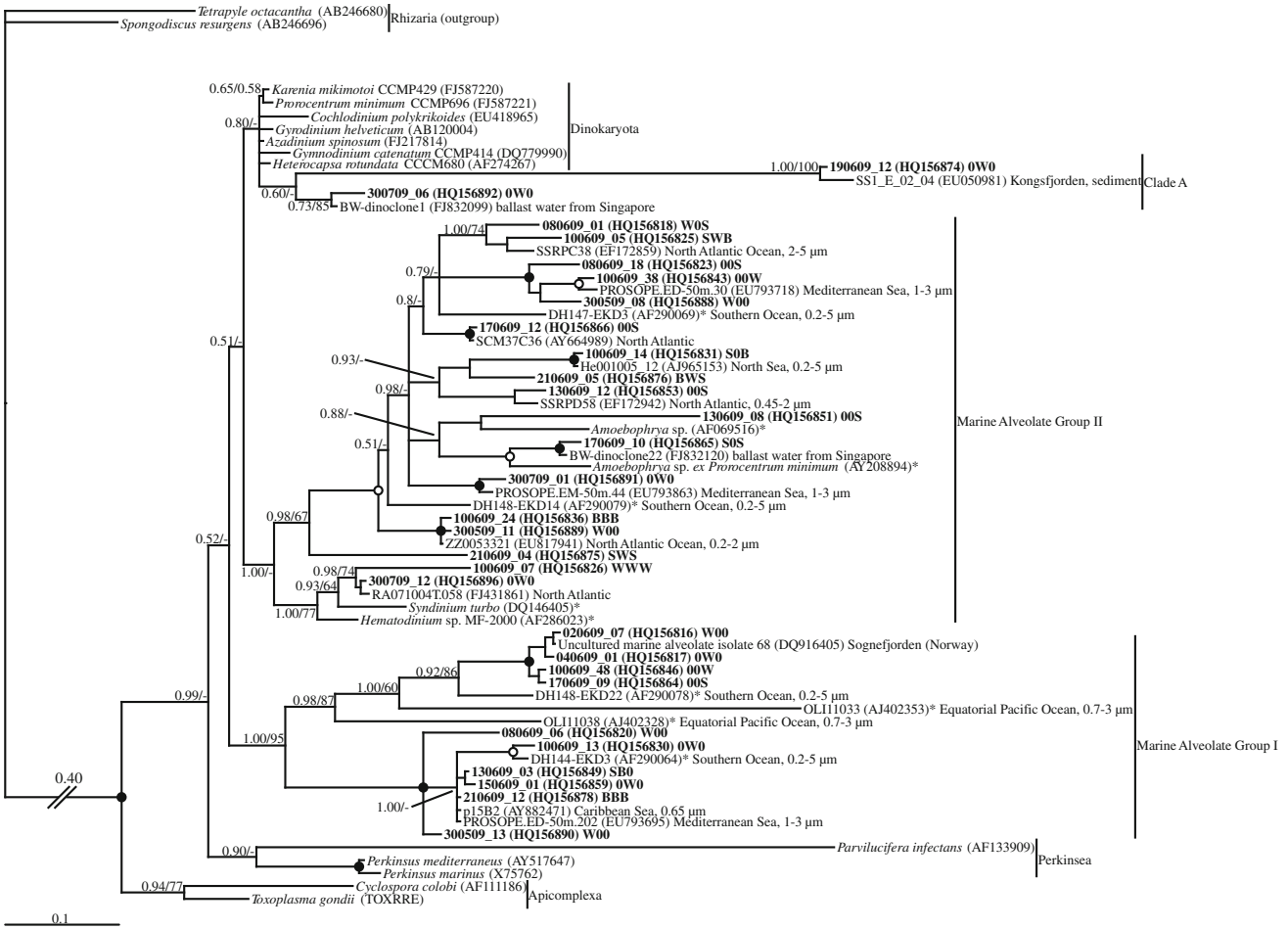

Fig. 7 Phylogenetic tree of Syndiniales. Sequences marked with an *asterix* (*) are used by Skovgaard et al. (2005) to define the respective clades; these clades have been expanded to include other phylotypes in this study. See supplementary material for a phylogeny including Dinokaryota sequences obtained in this study. The explanations are given in Fig. 5

Of the four Rhizaria phylotypes found, one sequence, 170609_19, clustered within the Nassellaria, while the remaining three sequences did not cluster with morphologically well-characterized Rhizaria (Fig. A7, supplementary material). Instead, they clustered with sequences of unknown cellular identity. However, group A had a bootstrap support of 79% as a monophyletic clade, although being unresolved in the Bayesian analysis. All phylotypes were found in winter and two were also found in spring. The related environmental sequences were geographically diverse and half of them were obtained from <3-µm fractions.

Discussion

Heterotrophs dominated picoeukaryote diversity throughout the Arctic spring

Stramenopiles (including MAST) and Syndiniales (i.e. MAG-I and II) accounted for >75% of the phylotypes found, comparable to other picoplankton communities both within and outside of Arctic waters (Romari and Vaulot 2004; Massana et al. 2004a; Lovejoy et al. 2006; Medlin et al. 2006; Not et al. 2007a, 2009). The MAST clades described so far (MAST-1A, MAST-1B, MAST-1C, MAST-2, MAST-3, MAST-4 and MAST-12) appear to be strictly heterotrophic based on incubations, grazing experiments and taxonomical affiliations (Massana et al. 2002, 2004b, 2006a, b; Kolodziej and Stoeck 2007; Massana et al. 2009). MAG-I and II are part of Syndiniales, which are obligate marine parasitoids (i.e. parasites that kill or castrate their host) able to produce picoplanktonic spores (Guillou et al. 2008). Of the five phylotypes found at both seasons and all stations, four belonged to MAST (100609_22 and 130609_13, Fig. 6) and MAG (100609_24 and 210609_12, Fig. 7). The last phylotype (100609_12, Fig. 6) most likely belong to the strictly heterotrophic genus *Paraphysomonas* (Caron et al. 1999). So not only do heterotrophs dominate picoeukaryote diversity, they also contain the phylotypes with the widest spatial and temporal

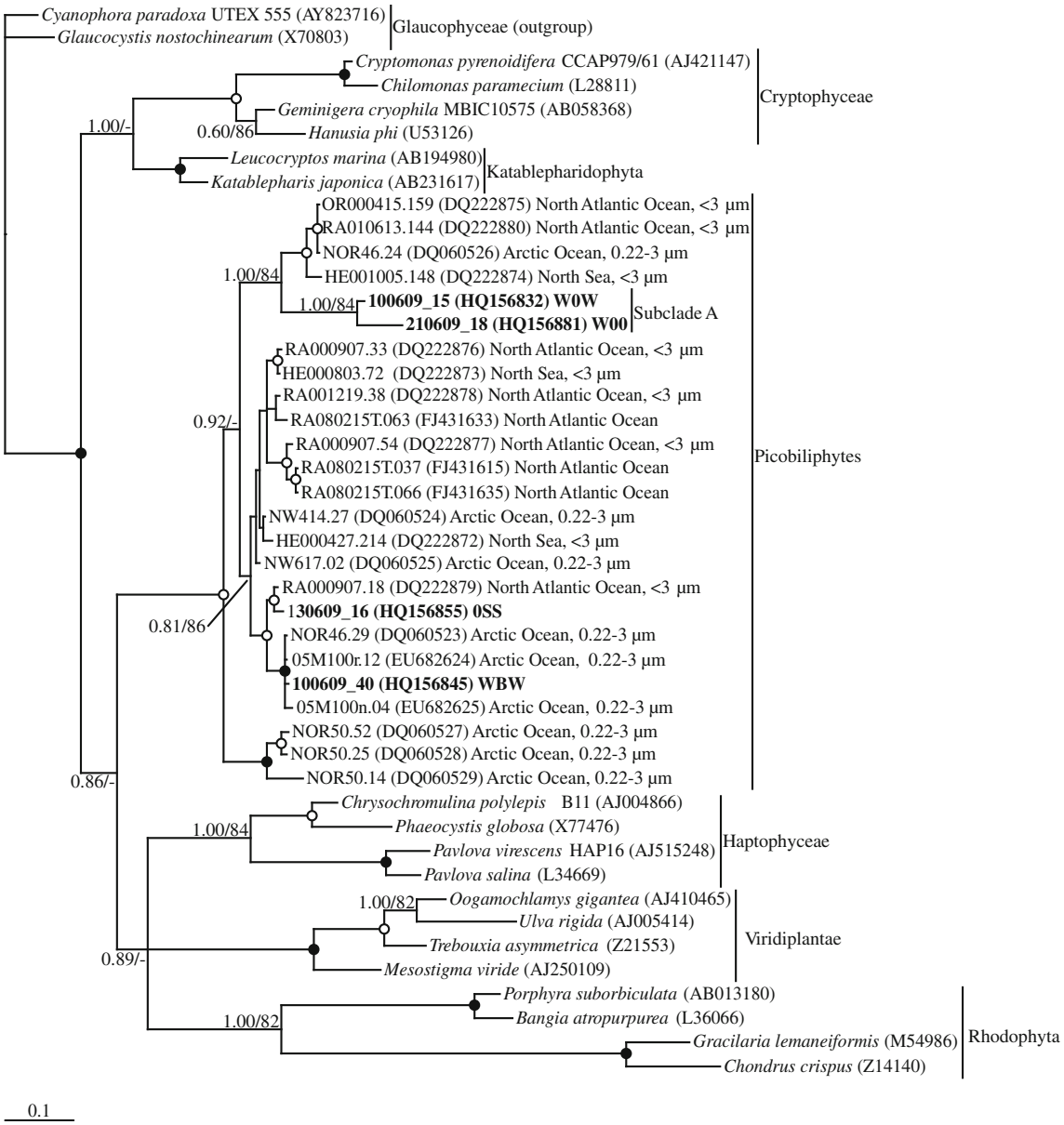

Fig. 8 Phylogenetic tree of picobiliphytes based on Bayesian inference. Sequences marked with *asterix* (*) are used by Not et al. (2007b) to define the respective clades; these clades have been expanded to include other phylotypes in this study. The explanations are given in Fig. 5

distributions. Thus, the diversity of the Arctic picoeukaryotic community is dominated by organisms with a heterotrophic or parasitic lifestyle throughout the investigated period.

The MAST clades 1A, 1B, 1C, 2, 3 and 7 have previously been found in Arctic open-ocean environments in late August and early October, MAST-3 being especially abundant (Lovejoy et al. 2006), while MAST clades 1A, 3 and 7 have been found in the North Water in August (Hamilton et al. 2008). MAST-4 has been suggested to be mostly absent from polar waters below 5°C (Rodriguez-Martinez et al. 2009), but have previously been found in the Arctic, at temperatures >4°C, in late August (Massana et al. 2006b). In the fjords of this study, MAST-2 was not found, but MAST-4 was. The latter clade was found during winter in Billefjorden, where temperatures were around −1.5°C, indicating a distribution not as limited by cold temperatures as previously suspected. MAST-3 was not abundant, only being found once in winter in Billefjorden at 150 m depth. Furthermore, although the MAST-3 phylotype from this study did match the probe by Massana et al. (2006b), it only clustered within the MAST-3 clade with weak support (Fig. 6).

In line with other studies (Lovejoy et al. 2006; Guillou et al. 2008; Hamilton et al. 2008), MAG-I and II were ubiquitous in the clone libraries, with MAG-II being more

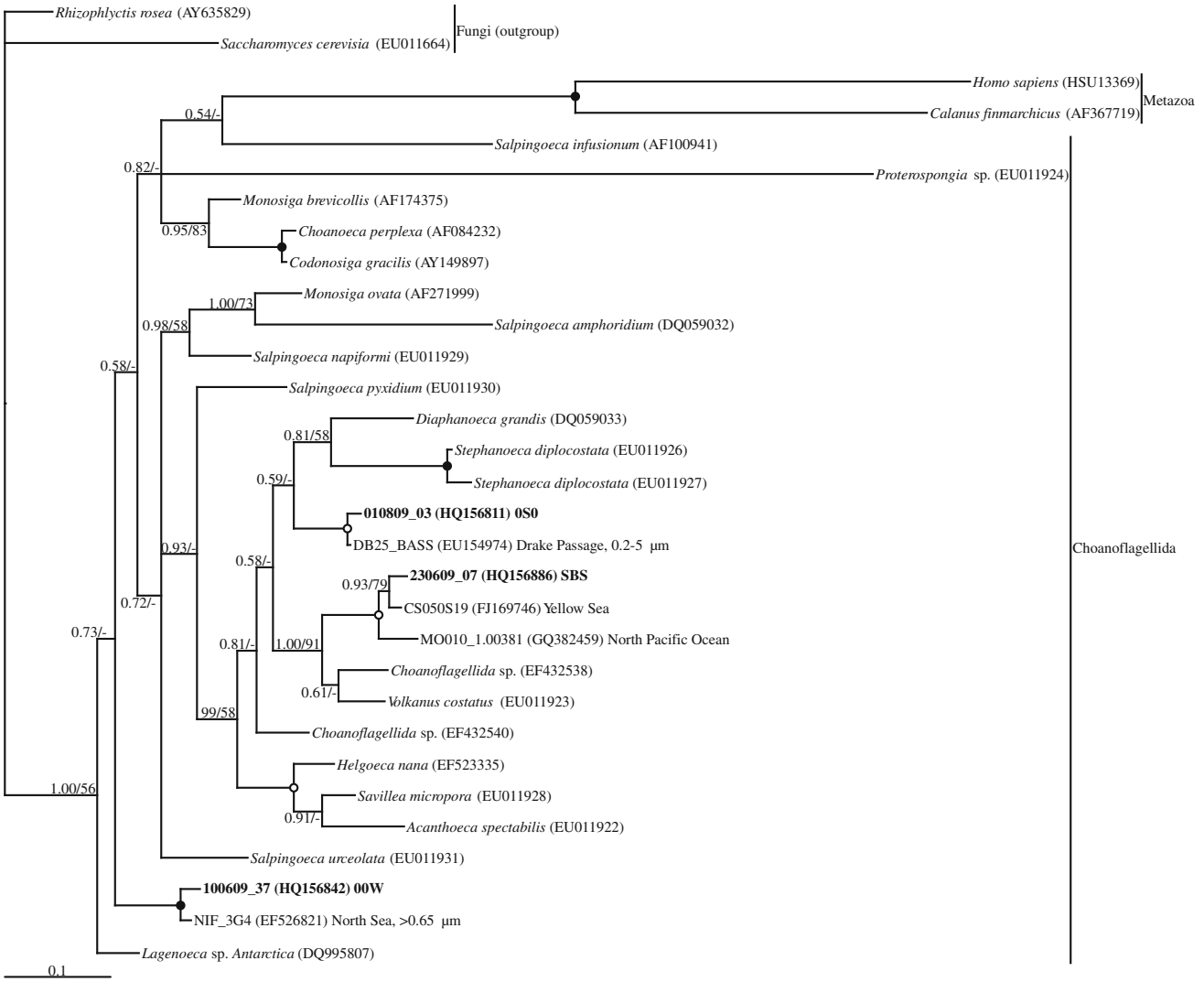

Fig. 9 Phylogenetic tree of Choanoflagellida based on Bayesian inference. The explanations are given in Fig. 5

diverse and widely distributed. Syndiniales can play a role stronger than herbivory in regulating the abundance of their host species (Chambouvet et al. 2008), but the ecological impact of each phylotype is bound to be strongly linked to its choice of host species. However, inferring host species based on phylogenetic data can be difficult for Syndiniales, as they in general do not appear to co-evolve with their host, and some may be opportunistic in host choice (Guillou et al. 2008). The association of MAG-I phylotypes with winter libraries could indicate that their hosts are most abundant in winter.

Autotrophs and other protists

The widespread abundance (especially in spring) of *M. pusilla* in our clone libraries indicates that it is likely to be an important picoplanktonic primary producer in the investigated fjords, as found elsewhere in the Arctic (Lovejoy et al. 2007). In fact, of the putative autotrophic phylotypes found, *M. pusilla* was the only one to have a widespread distribution supportive of significant primary production.

Picobiliphytes were originally thought to be photosynthetic (Not et al. 2007b), although a newer study using whole-genome shotgun sequencing has challenged this notion (Yoon et al. 2011). In the investigated fjords, their distribution does not support extensive phototrophy, as they were all absent from Adventfjorden in spring and 2 of the 4 phylotypes were also absent from Billefjorden in spring. Indeed, Hamilton et al. (2008) found picobiliphytes in the North Water to be associated with deep Arctic waters with low chlorophyll concentration, making them unlikely primary producers of importance in the Arctic.

No picoplanktonic Bacillariophyceae were found, although they have previously been recovered from the Arctic (Lovejoy et al. 2006) and several picoplanktonic

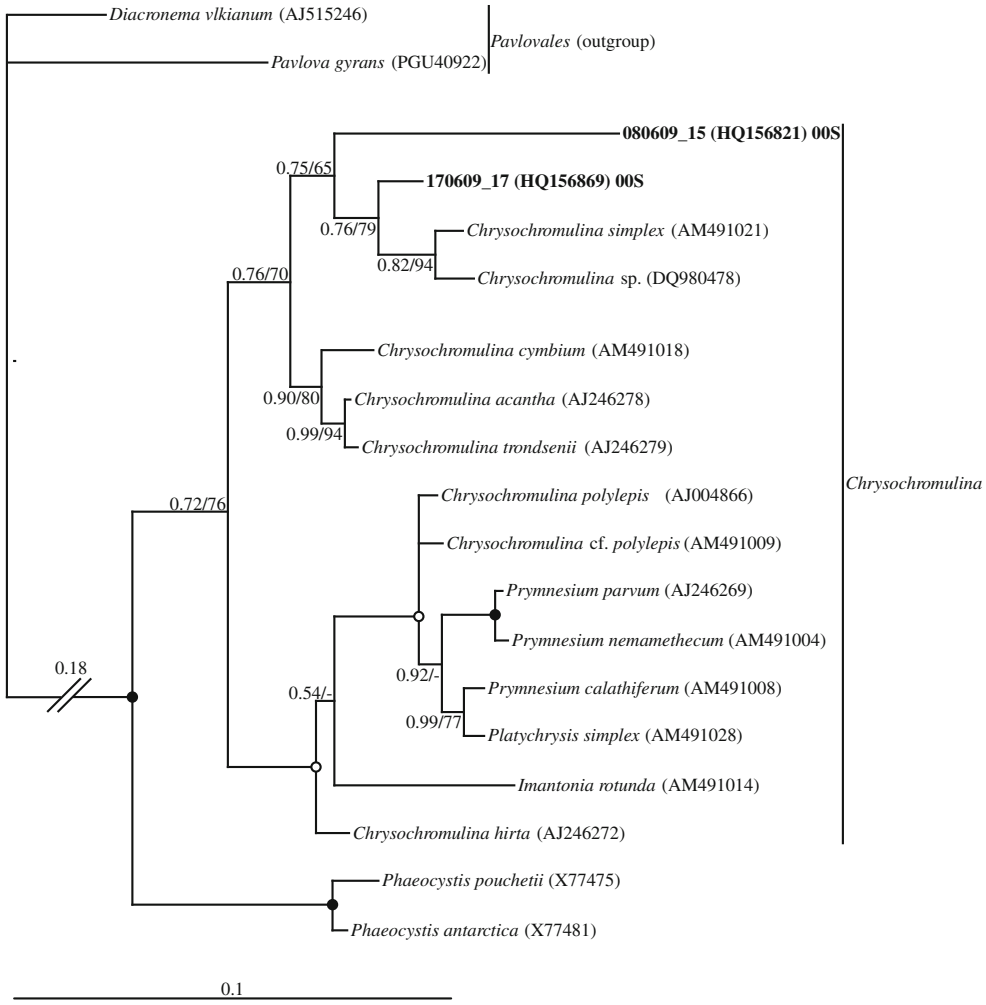

Fig. 10 Phylogenetic tree of Haptophyta based on Bayesian inference. The explanations are given in Fig. 5

bacillariophyceaen species have been described, many belonging to the genus *Minidiscus* (Vaulot et al. 2008; Kaczmarska et al. 2009).

Temporal patterns of picoeukaryote diversity

The inverse relationship between picoeukaryote diversity and autotrophic biomass indicates that the conditions found during the spring bloom have a negative impact on picoeukaryote diversity; likewise, a negative correlation between phytoplankton biomass and diversity has also been found for larger protists (Moustaka-Gouni 1993). Although single picoeukaryote species may flourish, the negative impact from the Arctic spring bloom on the picoeukaryote diversity suggests an important connection between the diatom bloom and the picoeukaryote community, which requires further investigation. A similar result was found in a study from the English Channel where the lowest picoeukaryote diversity was found at the peak of the diatom-dominated spring bloom (Romari and Vaulot 2004). Piquet et al. (2010) have suggested that salinity, influenced by glacial melt water, may play an important role in shaping the microbial eukaryotic community of Arctic fjords, but clogging of the 3-μm prefiltration filter due to a high concentration of phytoplankton could potentially withhold some picoplanktonic species, thus decreasing the picoplanktonic diversity observed during the bloom.

Although the biomass of picoautotrophs also increased during the bloom, it is unclear to what degree this picoeukaryote bloom influenced the heterotrophic picoeukaryotes. For instance, phylotypes of the MAST-1C and MAST-4 clades, which have been observed to ingest *M. pusilla* in experimental set-ups (Massana et al. 2009), were not found in Adventfjorden in spring.

Despite the proliferation of marine picoeukaryote molecular diversity studies over the past years (e.g. Diez et al. 2001; Moon-van der Staay et al. 2001; Romari and Vaulot 2004; Massana et al. 2004a; Lovejoy et al. 2006; Medlin et al. 2006; Worden et al. 2006; Not et al. 2007a, 2009; Piquet et al. (2010)), many novel sequences were

found in this study: of 62 sequences, 26 (42%) had <93% similarity with sequences available in GenBank. Fjords may contain more novel sequences than the open ocean due to the focus on open oceans in earlier molecular studies, and Arctic regions have not yet been well studied. More novel sequences were not found as a consequence of sampling in winter, as there was no relationship between seasonal distribution of a phylotype and its similarity to previously deposited sequences. Thus, it is the location rather than the addition of winter sampling that best accounts for the novelty found in this study. That this little sampled environment provided many novel sequences supports the idea of endemic Arctic picoeukaryotes as discussed by Lovejoy and Potvin (2011).

It proved difficult to obtain amplified 18S rDNA PCR products from the winter samples, as a total of 70 PCR cycles were required. Potentially, this could have caused a severe PCR bias. However, the diversity dominated by stramenopiles and Syndiniales in this study is in concordance with other molecular studies, which suggests limited additional PCR bias. In addition, Not et al. (2009), using a PCR-free approach for investigating the molecular diversity of picoeukaryotes, found that PCR steps do not impose major biases.

Size fractions

Mamiellophyceae (Slapeta et al. 2006), stramenopiles (Kaczmarska et al. 2009), Syndiniales (Guillou et al. 2008) and picobiliphytes (Not et al. 2007b) all have picoplanktonic species, while Choanoflagellida (Nitsche et al. 2007), Haptophyta (Rhodes and Burke 1996) and Cryptophyceae (Taylor and Lee 1971), as well as several MAST cells (Massana et al. 2002, 2006b; Kolodziej and Stoeck 2007; Massana et al. 2009), can have dimensions that may facilitate occasional passage through a 3-μm filter. This is not the case for Rhizaria, Ciliophora, or Dinokaryota as we currently understand the range of cell sizes in each of these groups.

A recent molecular study found that, although present in the <0.8-μm fraction, Ciliophora and Rhizaria were absent from the 0.8- to 3-μm fraction and the occurrence of Dinokaryota greatly reduced (Not et al. 2009). This indicates that extracellular DNA is the source of these sequences. Despite being represented by a total of 18 phylotypes and 72 clones, sequences belonging to Ciliophora, Dinokaryota and Rhizaria may not represent picoplankton in this study, although as yet undescribed picoplanktonic members of these groups may exist. Sampling of 'too large' organisms is a well-known phenomenon when working with size-fractioned clone libraries (Lovejoy et al. 2011); this was also evident from the fact that 9 metazoan phylotypes were recovered from the fjords.

The absence of Choanoflagellida, Cryptophyceae and Haptophyta from most clone libraries could suggest a limited ecological impact. However, both Haptophyta and Cryptophyceae have been observed to contribute significantly to phytoplankton in Adventfjorden (Dobrzyn et al. 2009) and elsewhere in the Arctic (Booth and Smith 1997). While Choanoflagellida, *G. cryophila* and *Chrysochromulina* spp. can all have dimensions that may facilitate passage through a 3-μm filter (Taylor and Lee 1971; Rhodes and Burke 1996; Nitsche et al. 2007), the prefiltration may have reduced their frequency in the clone libraries if the organisms in the fjords are not strictly picoplankton. These organisms may play an important ecological role in the investigated ecosystems, but are unlikely to be important members of the picoplankton size fraction. Finally, the relative low occurrence of Haptophyta may be explained by poor amplification during PCR, as they are in general underrepresented in environmental clone libraries (Liu et al. 2009; Marie et al. 2010).

GF/F filters proved sufficient to estimate total chlorophyll *a* as refiltration with 0.22-μm filters only yielded 1% additional chlorophyll *a* on average, but picoplanktonic autotrophic biomass may still have been underestimated as the fraction of phytoplankton >3 μm could have been overestimated as a result of the 3-μm filter clogging during filtration, thus retaining smaller particles.

Open versus ice-covered ecosystems

The less extensive ice cover likely caused the bloom to start earlier and higher pre-bloom chlorophyll *a* concentrations in the open Adventfjorden compared to the ice-covered Billefjorden. As the ratio of picoplanktonic to total chlorophyll *a* was greatest before the bloom, as seen in other studies (Hodal and Kristiansen 2008; Brewin et al. 2010), it seems that picophytoplankton benefit from conditions that support early growth. Indeed, Li et al. (2009) found that warming of the surface water in the Canada Basin caused an increase in picophytoplankton. The predicted warming of the Arctic may therefore result in pelagic picophytoplankton forming an increasingly important component of Arctic marine ecosystems. This could have serious cascading effects on higher trophic levels.

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