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ORIGINAL ARTICLE

Deep-branching Novel Lineages and High Diversity of Haptophytes in the Skagerrak (Norway) Uncovered by 454 Pyrosequencing

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Keywords

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ABSTRACT

Microalgae in the division Haptophyta may be difficult to identify to species by microscopy because they are small and fragile. Here, we used high-throughput sequencing to explore the diversity of haptophytes in outer Oslofjorden, Skagerrak, and supplemented this with electron microscopy. Nano- and picoplanktonic subsurface samples were collected monthly for 2 yr, and the haptophytes were targeted by amplification of RNA/cDNA with Haptophyta-specific 18S ribosomal DNA V4 primers. Pyrosequencing revealed higher species richness of haptophytes than previously observed in the Skagerrak by microscopy. From ca. 400,000 reads we obtained 156 haptophyte operational taxonomic units (OTUs) after rigorous filtering and 99.5% clustering. The majority (84%) of the OTUs matched environmental sequences not linked to a morphological species, most of which were affiliated with the order Prymnesiales. Phylogenetic analyses including Oslofjorden OTUs and available cultured and environmental haptophyte sequences showed that several of the OTUs matched sequences forming deep-branching lineages, potentially representing novel haptophyte classes. Pyrosequencing also retrieved cultured species not previously reported by microscopy in the Skagerrak. Electron microscopy revealed species not yet genetically characterised and some potentially novel taxa. This study contributes to linking genotype to phenotype within this ubiquitous and ecologically important protist group, and reveals great, unknown diversity.

MARINE haptophytes occur in all seas as major components of the pico- and nanoplankton and carry out key processes in global biogeochemical cycles. Besides playing an important role as primary producers (Andersen 2004; Jardillier et al. 2010; Thomsen et al. 1994), haptophyte species display a wide range of additional ecological functions. Both mixotrophic as well as some heterotrophic species exist (Jones et al. 1994; Kawachi et al. 1991) and haptophytes are important bacterial grazers in the ocean (Havskum and Riemann 1996; Unrein et al. 2014). Some haptophytes occur in symbiotic relationships, either with other protists (e.g. Decelle et al. 2012), or cyanobacteria (Thompson et al. 2012). Bloom-forming haptophytes may have large ecological and economical impact. Species within the genera *Prymnesium* and *Chrysochromulina,* in particular *P. parvum* and *P. polylepis* may form toxic blooms that can be harmful to marine biota and farmed fish (Granéli et al. 2012). Blooms of the colony-forming *Phaeocystis* species *P. pouchetii, P. globosa,* and *P. antarctica* may affect fisheries and tourism through the production of scum and acrylic acid (Edvardsen and Imai 2006; Schoemann et al. 2005). *Phaeocystis* spp., together with the calcifying haptophytes (coccolithophores), are also major producers of dimethylsulphoniopropionate and thus play an important role in the global sulphur cycle (Edvardsen and Imai 2006; Malin and Steinke 2004). Furthermore, the production of calcified scales (coccoliths) in the coccolithophores has a major impact on the earth's carbon balance (de Vargas et al. 2007).

Despite the ecological importance of the haptophytes, knowledge on their species biodiversity is limited. Due to small cell size and morphological similarity, identification

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to species level often requires electron microscopy or molecular biological methods, especially among the non-calcifying genera (Edvardsen et al. 2011; Estep and MacIntyre 1989). These are also fragile and may change form drastically upon fixation for microscopy, and may lose appendages and scales essential for morphological identification (Jensen 1998b; Kuylenstierna and Karlson 1994). About 300 species of haptophytes have been described morphologically (Jordan et al. 2004), and from ca. 100 of these the 18S rDNA gene sequence is available (The Protist Ribosomal Reference Database per Feb 2014: Guillou et al. 2013), which is currently the most commonly sequenced marker for haptophytes. However, investigations of environmental water samples using molecular methods have revealed a large diversity of novel haptophyte sequences which suggests that the total number of species is much higher, particularly among the pico-haptophytes (Bittner et al. 2013; Cuvelier et al. 2010; Liu et al. 2009; Moon-van der Staay et al. 2000; Simon et al. 2013). Currently, ca. 650 unique, full-length or partial 18S rDNA haptophyte sequences are included in the PR2 database (Guillou et al. 2013). Some of the environmental sequences may represent novel deep-branching haptophyte lineages. For instance, Clade D (sensu Edvardsen et al. 2000) consists entirely of environmental sequences (Moon-van der Staay et al. 2000), and is sufficiently genetically divergent to be recognised as a separate order of haptophytes once the morphology of these taxa is known (Edvardsen et al. 2011). A third class of haptophytes has been proposed based exclusively on molecular data (Shi et al. 2009), termed HAP-2 by Shalchian-Tabrizi et al. (2011), and recently Simon et al. (2013) recovered yet another deep-branching lineage, termed HAP-3.

In the Skagerrak, the sea separating Norway and Denmark (Fig. S1), a massive, toxic bloom of *Prymnesium polylepis* (previously *Chrysochromulina polylepis*) in 1988 had detrimental consequences for benthic organisms and farmed fish (Dahl et al. 1989; Lekve et al. 2006; Rosenberg et al. 1988). This bloom prompted electron microscopy investigations of the diversity of the order Prymnesiales in Scandinavian waters (Eikrem 1999), in particular "*Chrysochromulina* sensu lato" (e.g. Jensen 1998a); here taken to mean species originally assigned to the genus *Chrysochromulina*, but now divided into five genera based on both molecular data and morphology (Edvardsen et al. 2011). The bloom was also a driving force for isolation of cultures, morphological and molecular characterisation of strains, and phylogenetic inference, resulting in descriptions of new species and revised taxonomy (Edvardsen et al. 2000, 2011; Eikrem 1996; Eikrem and Edvardsen 1999; Eikrem and Throndsen 1999). Today, these studies serve as a valuable reference for interpreting high-throughput sequencing (HTS) data. The major advantage of HTS methods is the sampling depth that can be obtained, and the potential to recover fragile and rare species that may be missed with microscopy methods. However, current HTS technology imposes an upper limit on the length of the marker region of about 350–500 bp, which to a certain extent limits the taxonomic resolution. The variable V4 region of the 18S rDNA is a widely used molecular marker in surveys of protistan diversity in general (e.g. Behnke et al. 2011; Dunthorn et al. 2012; Medlin et al. 2006; Stoeck et al. 2010), and has also been used to target haptophytes in particular (Shalchian-Tabrizi et al. 2011). Calculation of sequence similarity across the V4 region of selected haptophyte 18S rDNA sequences (described in the Methods section) shows that most species of haptophytes can be distinguished based on the V4, except certain groups of closely related species. Considering recent discoveries of haptophyte richness using molecular tools, we expect the haptophyte community in Skagerrak to be more diverse than what was inferred from the earlier microscopy surveys.

The Skagerrak and outer Oslofjorden are characterised by strong seasonal variation in meteorological and hydrological conditions, in particular light and temperature. The water masses are influenced by both saline (Atlantic) and brackish water currents (the Baltic current and runoff from land), resulting in highly variable salinity. Thus, to recover haptophyte species with different environmental preferences, we included samples from all times of the year. We sampled subsurface seawater from the outer Oslofjorden monthly for 2 yr and isolated RNA to target cells that were alive and active at the time of sampling and to avoid extracellular DNA from dead cells (e.g. Not et al. 2009). The haptophytes were targeted by amplification of the variable V4 region of the 18S rRNA with haptophyte-specific primers previously described in Egge et al. (2013). In addition to the samples for sequencing we also collected and analysed samples for electron microscopy. The seasonal dynamics of the haptophyte community will be presented in a following paper.

In this work, we aimed at describing the diversity of haptophytes in the Skagerrak by 454 pyrosequencing, and supplement this with electron microscopy. We addressed the following main questions: What is the OTU and species richness, and taxonomic composition of haptophytes in the Skagerrak? How does the diversity revealed by 454 pyrosequencing compare to previous microscopical observations? Do we recover novel haptophyte lineages and taxa in the Skagerrak by 454 pyrosequencing?

MATERIALS AND METHODS

Study site and sampling

Samples were taken approximately once a month in the period September 2009–June 2011 in the outer Oslofjorden (OF), Skagerrak, at the station OF2 (59.17 N, 10.69E, Fig. S1) with the exception of the September 2009 sampling, which due to strong winds on the sampling day was carried out at the nearby OF1 station (59.25 N, 10.71E). Total daily photosynthetically active radiation at the surface (PAR; mol photons/m^2/d) was obtained from a nearby weather station (Norwegian University of Life Sciences, Ås; 59.66N, 10.77E). Water temperature and conductivity (as a measure of salinity) by depth were measured directly on site using a CTD (Falmouth Scientific Inc., Cataumet,

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MA) attached to a rosette with Niskin bottles. Throughout the study period, daily PAR above water, and average temperature and salinity in the upper mixed layer ranged from 1.3 (December) – 51.6 (June) mol/m^2/d, −1 °C (Jan, Feb) to 17 °C (Aug), and 21–33 PSU, respectively (Egge et al. unpubl. data). The September 2009 and June 2010 samplings were part of the EU project BioMarKs (www.biomarks.org).

From 1-m depth, 20 litres of seawater were collected in Niskin bottles and prefiltered through a 45-μm nylon mesh into hydrochloric acid-washed plastic carboys. To increase recovery of the smallest species and increase diversity, we separated between the nano- and pico-size fractions (3–45 and 0.8–3 μm, respectively, except the October 2009 sample, where the smallest size fraction was 0.45–3 μm; and September 2009 and June 2010, where the nano-size fraction was 3–20 μm; and from which the pico-size fraction is not available). Twenty litres of < 45 μm seawater were filtered by peristaltic pumping (Masterflex 07523-80; ColeParmer, IL), at a rate of 0.5–1 l/min through a 3 μm pore size 142 mm diam. polycarbonate filter (Millipore, Billerica, MA), placed in a Millipore stainless steel tripod, connected to a corresponding filter holder with a 0.8 μm pore size filter. Filtration time was kept to a minimum (20–40 min) to minimise RNA degradation. The filters were carefully removed from the filter holders, cut in four, and each piece was placed in a 5-ml cryo tube, immediately flash frozen in liquid N_2 and kept at −80 °C until RNA extraction.

RNA extraction, PCR, and sequencing

Total RNA was extracted with RNA NucleoSpin II (Macherey-Nagel, Düren, Germany). From each sample, two quarters of a 142-mm filter were cut in smaller pieces with flame-sterilised scissors and transferred to an RNase-free 15-ml centrifuge tube (Corning, New York, NY) containing 2.1 ml of the lysis buffer provided in the extraction kit and 21 μl β-mercaptoethanol. To ensure that all the material was washed off the filter, and to facilitate efficient lysis, but without damaging the RNA, the tubes were shaken in a FastPrep-24 bead-beater with a TeenPrep adapter (MP Biomedicals, Illkirch, France) at 2 × 20 s 4,000 beats per minute, with a 20-s break, without beads added to the tubes. The lysate was transferred to three parallel extraction columns, and extraction was performed according to the protocol from the manufacturer. RNA was eluted in 50–60 μl RNase-free water per column, which was run through the column twice, and the eluates were pooled. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA eluates were checked for residual DNA by running standard PCR with universal eukaryote partial 18S rDNA primers 1F and 300R, annealing temperature 50 °C for 35 cycles (see e.g. Edvardsen et al. 2011). If a PCR product could be detected by gel electrophoresis, the RNA eluate was treated with additional DNase (TURBO DNase kit; Ambion, Austin, TX), according to the protocol from the manufacturer. cDNA was reverse-transcribed from RNA using High-Fidelity 1st Strand cDNA Synthesis Kit (Agilent, Santa Clara, CA) with random primers, according to the protocol from the manufacturer. For the synthesis reaction approximately 100 ng of RNA was added to a mix containing 2.0 μl AccuScript RT Buffer 10X, 3 μl random primers (0.1 μg/μl), 0.8 μl dNTP (final conc. 25 mM of each dNTP) and RNase-free water to a total volume of 16.5 μl. The mix was incubated at 65 °C for 5 min before annealing at room temperature for 5 min. Subsequently, 2 μl 100 mM DTT (dithiothreitol, reducing agent), 1 μl AccuScript Reverse Transcriptase, and 0.5 μl RNase Block ribonuclease inhibitor were added. The reaction was incubated at 25 °C for 10 min before cDNA synthesis took place at 42 °C for 75 min in a thermocycler (Mastercycler ep gradient S; Eppendorf, Hamburg, Germany). The synthesis reaction was terminated by incubation at 70 °C for 15 min. RNA was extracted from all samples except that from the October 2009 pico-size fraction, from which DNA was extracted with DNA NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), according to the protocol from the manufacturer. Samples from September 2009 and June 2010 (BioMarKs samples) were prepared as described in Logares et al. (2012).

For PCR amplification we used the primer pair Hap454 described in Egge et al. (2013), with universal eukaryote forward primer 528Flong: 5′-GCGGTAATTCCAGCTCCAA-3′, and haptophyte-specific reverse primer PRYM01 + 7: 5′-GATCAGTGAAAACATCCCTGG-3′. Calculation of a sequence similarity matrix for the region amplified with this primer pair (BioEdit, Hall 1999) (Table S1) based on the reference alignment (described below) shows that this primer pair amplifies a region that distinguishes between most haptophyte species for which the 18S rDNA sequence is available, except for a few very closely related species. Fusion primers for pyrosequencing were designed according to the protocol by Roche by adding adaptors (adaptor A (5′-3′): CCATCTCATCCC TGCGTGTCTCCGAC, key (TCAG), and multiplex identifiers (MIDs) or 'tag', to the forward primer, and adaptor B (5′-3′): CCTATCCCCTGTGTGCCTTGGCAGTC), to the reverse primer. The primers were RP-cartridge purified after synthesis (Eurogentec, Seraing, Belgium). PCR was performed with the fusion primers directly, on an Eppendorf thermocycler (Mastercycler, ep gradient S, Eppendorf) in 25-μl reactions containing 5X Phusion GC buffer, 0.4 μM of each primer, 0.2 mM dNTP, DMSO 3%, 0.5 U polymerase (Phusion, Finnzymes, Vantaa, Finland) and 1 μl of the cDNA synthesis reaction described above. The PCR-programme was as follows: initial denaturation step at 98 °C for 30 s, then 30 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 10 min. PCR was run in four separate reactions for each sample. The four PCR reactions were pooled and purified with AMPure beads (BeckmanCoulter, Brea, CA), according to the protocol from the manufacturer. The purified amplicons were quantified with Quant-iT dsDNA Broad-Range Assay Kit (Invitrogen, Carlsbad, CA), and pooled to give equal concentrations prior to sequencing.

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Emulsion PCR and 454 pyrosequencing of the amplicons was performed at the Norwegian Sequencing Centre at the Department of Biosciences, University of Oslo (www.sequencing.uio.no), using GS-FLX Titanium technology. The amplicons were prepared for sequencing with Lib-L chemistry and were sequenced unidirectionally, from the forward primer, on 2 × ½ of a 454 life sciences FLX Titanium sequencing plate (454 Life Sciences, Branford, CT). The sff-files are submitted to the Sequence Read Archive/European Nucleotide Archive with study accession number PRJEB5541 (http://www.ebi.ac.uk/ena/data/view/PRJEB5541).

Bioinformatic filtering

The flowgrams were denoised with AmpliconNoise (Quince et al. 2011). Reads with mismatches with barcode and forward primer were removed from the dataset, and default settings were used as described in the documentation for the programme (Quince 2011), previously tested on haptophyte 18S rDNA V4 pyrosequencing data (Egge et al. 2013). Putative chimeras were identified by Perseus integrated in AmpliconNoise and removed. The haptophyte reads were identified by classifying the denoised reads with 'classify.seqs' against the Silva Eukarya database v. 102 (Quast et al. 2013), using mothur v. 1.28 (Schloss et al. 2009). The haptophyte reads were selected using get.lineage, and aligned using align.seqs in mothur with default settings, against a template alignment. The template alignment comprised all available unique haptophyte 18S rDNA sequences downloaded from EMBL (all publicly available as of January 1, 2012, omitting duplicates, in total 646 sequences), aligned in MAFFT v.6 with the Q-INS-i strategy (Katoh and Toh 2008). Reads that did not align in the targeted region, and/or were shorter than 365 bp were removed. Uncorrected pairwise distances between the aligned reads were calculated with the dist.seqs-command, with settings calc = onegap (a string of gaps counting as a single gap). Considering the high similarity between some haptophyte species over the V4 region (Table S1), the reads were clustered at a 99.5% similarity threshold, with the average-neighbour algorithm. This gave 1,042 unique $OTUs_{99.5\%}$. OTUs that contained only one read (singletons) and OTUs that contained only two reads that were both from the same sample were removed (altogether 608 OTUs).

All known members of Prymnesiophyceae have a six A homopolymer (position 751–756 in reference sequence AJ004866 *P. polylepis* 18S rDNA), but we observed that this was reduced to five in several OTUs. Too short or long homopolymers is a common error with 454 pyrosequencing (e.g. Gilles et al. 2011). To avoid spurious OTU richness because of differences in homopolymer length, the alignment of unique $OTUs_{99.5\%}$ was visually inspected in BioEdit, and OTUs that only differed in the length of the homopolymers were grouped together. The OTU with homopolymer lengths matching the majority was taken to be the representative. Altogether 38 OTUs were grouped with others.

Taxonomic assignation

To ensure that the closest relatives of all unique Oslofjorden $OTUs_{99.5\%}$ (hereafter "OF OTUs") were included in the alignment for phylogenetic analysis, all unique OTUs were BLASTed (Altschul et al. 1997) against the NCBI-nr database (per June 2013), and all Sequence Read Archive (SRA) projects with the substrings "protist" or "plankton" in the title. The BLAST results are provided in Table S2. This also served as an additional manual chimera check; OTUs where one part had > 3 mismatches to the best hit, but where this part had a 100% match to a different sequence, and which occurred only in one sample, were considered to be chimeras not detected by Perseus, and removed from the dataset (240 of the 396 remaining OTUs, most of which occurred as fewer than 50 reads).

To be able to interpret the diversity and species composition revealed by 454 pyrosequencing in light of previous reports from Skagerrak/Kattegat, we compared the result of the BLAST search to the Skagerrak phytoplankton checklist assembled by Kuylenstierna and Karlson (2006), and observations by Jensen (1998a) and Eikrem (1999) (Table S3).

Phylogenetic placement of Oslofjorden OTUs

For construction of phylogenetic trees, the OF OTUs were aligned to a reference alignment using the add-in function in MAFFT, with method Multi-INS-fragment (Katoh and Frith 2012). The reference alignment was a subset of the template alignment described above, and consisted of selected representative sequences of each cultured species with available 18S rDNA sequences, environmental sequences representing putative novel clades; clades D, E (Edvardsen et al. 2000, 2011), HAP-2 (Shalchian-Tabrizi et al. 2011), HAP-3 (Simon et al. 2013) and Clade F, HAP-4 and HAP-5 (defined in this study), and the nearest BLAST hit in NCBI-nr to each unique OTU. Where the nearest BLAST hit was not already present in the alignment, the sequence was added to the existing alignment using the add-in function in MAFFT for full-length sequences, with method L-INS-1 (Katoh and Frith 2012). The reference alignment was edited by eye in BioEdit. In total the reference alignment comprised 281 sequences.

Maximum likelihood phylogenetic trees of the reference sequences were constructed both including and excluding the OF OTUs, using RAxML v. 7.3.2 (Stamatakis 2006), with substitution model GTRCAT and 100 bootstrap runs. RAxML was run on the Lifeportal on the University of Oslo computer cluster (www.lifeportal.uio.no).

Electron microscopy

From each sampling date, samples for scanning (SEM) and transmission (TEM) electron microscopy were collected and prepared as follows. Water samples 1–3 litre were prefiltered through a 45-μm mesh gauze and concentrated ca. 100X with tangential flow filtration (TFF; Vivaflow 200, Sartorius AG, Goettingen, Germany), or ca.

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50X with gravitational filtration on 3-μm polycarbonate filter. For additional examinations of coccolithophores, 100 ml of < 45 μm prefiltered seawater was filtered onto polycarbonate filters (2-μm pore size, 13 mm diam., Whatman, Maidstone, UK), placed on filter holders. The filters were rinsed with 10–20 ml alkaline distilled water, air dried and placed on stubs.

Scanning electron microscopy

A volume of 100 μl each of 2% osmium tetroxide (Sigma-Aldrich, St. Louis, MO) and TFF-concentrated water sample was placed on round glass coverslips dipped in poly-L-lysine. The cells were left to settle in a moist chamber over night. The samples were transferred to a critical point holder and rinsed 3 × 10 min in 0.1 M cacodylate buffer with pH 8 (Agar Scientific Ltd., Essex, UK). The samples were dehydrated in an ethanol series starting at 70%, proceeding through 90% and 96% for 10 min at each step. The dehydration was concluded with 4 × 10 min in 100% ethanol and was transferred to the critical point dryer (BAL-TEC 030 CPD; Technion, Haifa, Israel), where the ethanol was replaced with carbon dioxide. The samples were then mounted on stubs and coated with ca. 7 nm platinum in a sputter coater (Cressington 308R Desktop Advanced Coating Systems; Ted Pella Incorporated, Redding, CA). The samples were viewed in a Hitachi S-4800 scanning electron microscope (Hitachi High-Tech, Tokyo, Japan) at the Electron Microscopy unit, Department of Biosciences, University of Oslo.

Transmission electron microscopy

Whole mounts were prepared from a drop of the TFF-concentrated samples placed on a copper grid covered with a carbon coated polyvinyl formvar film. The samples were fixed in the vapour of 2% osmium tetroxide for 1 min and left to air dry for 1 h. Subsequently they were rinsed in distilled water, air dried, and either contrasted with a saturated solution of uranyl acetate (Merck, Darmstadt, Germany), or shadow casted with platinum at a 30° angle in an Edwards Speedivac 12 E6 coating Unit (Edwards, Crawley, West Sussex, UK).

Thin sections were prepared of TFF-concentrated samples fixed for 1 h in 2% glutaraldehyde final concentration (Electron Microscopy Sciences, Hatfield, PA) and centrifuged (Eppendorf, model 5810R) at 400 *g* for 20 min at 15 °C. The resulting pellet was rinsed 2 × 5 min in sterile filtered seawater and 3 × 5 min 0.1 M cacodylate buffer (pH 8) and subsequently fixed for 1 h in a mixture of 1% osmium tetroxide, 1% potassium ferricyanide (Sigma-Aldrich) in 0.1 M cacodylate buffer, final concentrations. Subsequently, the samples were rinsed 3 × 5 min in 0.1 M cacodylate buffer before they were dehydrated in an ethanol series starting at 15 min in 30%, proceeding through 50%, 70%, 90%, and 96%. The dehydration was concluded with 4 × 15 min in 100% ethanol, followed by 2 × 5 min in 100% propylenoxide. The cells were left overnight at room temperature in 1:1 propylenoxide and epoxy resin (EM Bed-812 based on EPON-812; Sigma-Aldrich), rinsed 3 × 1 h in 100% epoxy resin and polymerised for 12 h at 60 °C. Sectioning was carried out on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria). The thin sections were stained 20 min in saturated aqueous uranyl acetate and 3 min in lead citrate (Reynolds 1963). The thin sections and whole mounts were viewed in a Phillips CM 100 (Hillsboro, OR) at the Electron Microscopy Unit, Department of Biosciences, University of Oslo.

RESULTS AND DISCUSSION

454 pyrosequencing recovers unprecedented diversity of haptophytes in Skagerrak

The phytoplankton communities in the Oslofjorden and Skagerrak have been studied by microscopy throughout the 20th century, especially larger nano- and microplankton, dominated by diatoms and dinoflagellates, which may be identified under the light microscope (e.g. Hasle and Smayda 1960). Most haptophytes are small and fragile and can only be identified to higher taxonomic levels under the light microscope (phylum – genus) and require electron microscopy for certain species identification. To achieve the sampling depth and potentially high taxonomic resolution that can be obtained with HTS techniques, we did 454 pyrosequencing of samples collected monthly for 2 yr, targeting the haptophytes with haptophyte-specific primers. Overall, after rigorous cleaning of reads including denoising with AmpliconNoise, 86% of the total reads (406,928 reads) were assigned to Haptophyta, which confirms the specificity of the primer pair. After clustering at 99.5% similarity and removal of dubious reads as described in the Methods section, we obtained 156 unique OTUs (Table S2). The number each OTU is given is according to their normalised total read abundance, where OTU 1 had most reads. The BLAST hits in NCBI-nr of the 25 most abundant OTUs are given in Table 1.

As shown in the inferred phylogenetic trees and from the BLAST results, the phylogenetic diversity of the OF OTUs is impressive (Fig. 1–4; Table S2). All orders of Haptophyta were represented, and within the order Prymnesiales all genera were represented. Prymnesiaceae and Chrysochromulinaceae were the most OTU rich families (Table 2). Further, we recovered a high diversity of novel taxa. Out of the 156 OTUs, only 25 (16%) had > 99.5% sequence similarity with a cultured and genetically characterised species, whereas the majority had better match with environmental clones (Table S2; Fig. 1–4). The highest number of uncultured and genetically uncharacterised taxa was affiliated with Prymnesiaceae and Chrysochromulinaceae (Table 2).

Considering that the material examined by electron microscopy was obtained from much smaller volumes of water than what was processed for pyrosequencing, the aim here is not a direct comparison between HTS and electron microscopy methods. Rather, we consider these methods to give complementary information about the haptophyte community. Thus, the pyrosequencing results are interpreted both in relation to the EM images obtained

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Table 1. The 25 proportionally most abundant haptophyte V4 SSU rRNA OTUs recorded in Skagerrak in the period September 2009–June 2011[a]

OTU id	Percentage of total reads (normalised)	Group	Lowest taxonomic level possible to determine	Best BLAST hit (species and/or environmental clone name)	Acc. no. of best BLAST hit	% match to best BLAST hit	Cultured	Size fraction	Previously observed in Skagerrak
OTU 1	12.60	Calcihaptophycidae	*Emiliania huxleyi*	*Emiliania huxleyi*/*Gephyrocapsa oceanica*	HQ877901.1	99.7	Yes	Both	Yes
OTU 2	11.60	Phaeocystales	*Phaeocystis cordata*	*Phaeocystis cordata*	JX660992.1	100	Yes	Both	No
OTU 3	9.50	Chrysochromulinaceae	*Chrysochromulina simplex*	*Chrysochromulina* cf. *simplex*/Ma135-Pry1-C55	JX680441.1	99.7	Yes	Both	Yes
OTU 4	7.30	Calcihaptophycidae	Clade F	3b-H6	FN690514.1	99.5	No	Both	–
OTU 5	7.10	Chrysochromulinaceae	*Chrysochromulina campanulifera*/*C. cymbium*/*C. strobilus*	Ma135-Pry1-C24	JX680404.1	99.5	No	Both	–
OTU 6	6.40	Prymnesiaceae	*Haptolina ericina*/*H. fragaria*/*H.* cf. *herdlensis*/*H. hirta*	*Haptolina ericina*/*H. fragaria*/*H.* cf. *herdlensis*/*H. hirta*	AM491013.2	99.5	Yes	Both	Yes
OTU 7	5.00	Phaeocystales	*Phaeocystis pouchetii*	*Phaeocystis pouchetii*	AF182114.1	100	Yes	Both	Yes
OTU 8	4.00	Prymnesiaceae	*Imantonia*	*Imantonia* sp. strain RCC 2298	JN934681.1	99.5	Yes	Both	–
OTU 9	3.90	Calcihaptophycidae	*Syracosphaera pulchra*	*Syracosphaera pulchra*/Ma130-Pry1-C40	JX680341.1	100	Yes	Both	No
OTU 10	3.40	Chrysochromulinaceae	*Chrysochromulina campanulifera*/*C. cymbium*/*C. strobilus*	*Chrysochromulina campanulifera*/*C. cymbium*/*C. strobilus*	FN599060.1	99.7	Yes	Both	Yes
OTU 11	2.90	Chrysochromulinaceae	*Chrysochromulina acantha*	*Chrysochromulina acantha* (two mismatches with *C. throndsenii*)	FN599059.1	99.7	Yes	Both	Yes
OTU 12	2.00	Calcihaptophycidae	Clade F	SHAX992	HQ868752.1	99.7	No	Both	–
OTU 13	2.00	Calcihaptophycidae	*Algirosphaera robusta*	*Algirosphaera robusta*	AM490985.2	99.7	Yes	Both	Yes
OTU 14	1.90	Chrysochromulinaceae	Chrysochromulinaceae	DH125-Pry1-C10	JX680365.1	99.7	No	Both	–
OTU 15	1.60	HAP-5	HAP-5	SHAC596	HQ867320.1	99.2	No	Both	–
OTU 16	1.50	Chrysochromulinaceae	Chrysochromulinaceae	FS04GA79_01Aug05_5 m	HM581565.1	99.7	No	Both	–
OTU 17	1.30	Prymnesiaceae	*Prymnesium polylepis*	*Prymnesium polylepis*/Ma135-Pry1-C41	FN551248.1	99.7	Yes	Both	Yes
OTU 18	1.30	Chrysochromulinaceae	Chrysochromulinaceae	7656BH1019_SP6	JX291689.1	100	No	Both	–
OTU 19	1.00	Prymnesiaceae	*Prymnesium kappa*	*Prymnesium kappa*/SGSA635	HQ865286.1	99.7	Yes	Both	Yes
OTU 20	0.84	Chrysochromulinaceae	*Chrysochromulina*	hotxp4f1	EU500066.1[b]	98.4	No	Both	–
OTU 21	0.73	Chrysochromulinaceae	*Chrysochromulina*	Ma135-Pry1-C16	JX680400.1	99.5	No	Both	–
OTU 22	0.73	Phaeocystales	*Phaeocystis globosa*	*Phaeocystis globosa*/Ma135-Pry1-C26	JX680405.1	99.7	Yes	Both	Yes
OTU 23	0.67	Clade D	Clade D	Ma135-Pry1-C7	JX680409.1	99.7	No	Both	–
OTU 24	0.66	Prymnesiales Clade B4	Prymnesiales Clade B4	MO.011.5 m.00022	HM858457.1	99.7	No	Both	–
OTU 25	0.63	Chrysochromulinaceae	Chrysochromulinaceae	SHAX774	HQ868546.1	99.7	No	Both	–

– = not applicable.

[a]The raw.sff-files have been deposited in the Sequence Read Archive (SRA) with accession number: PRJEB5541 (http://www.ebi.ac.uk/ena/data/view/PRJEB5541).

[b]Flagged as chimeric in the Protist Ribosomal Reference database (http://ssu-rrna.org/), and therefore not included in the phylogeny.

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from the same samples, and observations from previous electron microscopy surveys.

From previous surveys of Scandinavian waters, altogether 85 morphological species of haptophytes have been reported (Eikrem 1999; Jensen 1998a; Kuylenstierna and Karlson 2006), however, currently the 18S rDNA sequence is available from only 30 of these (Table S3). HTS nevertheless reveals a higher species richness of haptophytes than previously reported from Skagerrak. Of the species previously reported with known 18S rDNA sequence, eight were not recovered by 454 sequencing (Table S3). Conversely, of the sequenced and morphologically described species we detected, three had not previously been reported from the Skagerrak. Interestingly, two of these were proportionally abundant OTUs; *Phaeocystis cordata* (OTU 2) and *Syracosphaera pulchra* (OTU 9), whereas the third was the freshwater species *Chrysochromulina parva* (OTU 122) (Table S2).

Of the 156 OTUs, 21 (13%) and 38 (24%) OTUs were found uniquely in the pico- and nanoplanktonic size fractions, respectively (Table S2). Of the 38 OTUs found uniquely in the nano-size fraction most were affiliated with coccolithophores or Phaeocystales, whereas the OTUs found only in the pico-size fraction were mostly affiliated with Chrysochromulinaceae, and novel clades consisting of environmental sequences only. The only cultured species found exclusively in the pico-size fraction was *Chrysochromulina rotalis*. Size fractionation by in-line filtration obviously does not separate the pico- and nanoplanktonic populations perfectly, but the size fraction in which an OTU is predominantly found can give an indication of its cell size range.

Observations by electron microscopy

All samples analysed by 454 pyrosequencing were also examined qualitatively by either TEM or SEM or both. The haptophyte species observed by electron microscopy in this study are listed in Table 3 and described more in detail under each taxonomic group below.

Support for novel classes of haptophytes

HAP-3 (Fig. 1)

Several of the OTUs matched environmental sequences that form novel clades. We detected four OTUs which with good support grouped together with an environmental sequence isolated from surface waters of the Marmara Sea, Turkey, previously suggested to represent a putative new class-level clade of haptophytes; HAP-3 (Simon et al. 2013). In our phylogenetic analysis, this clade also included the environmental clone SHAX445, isolated from 10 m depth in the Saanich Inlet, British Colombia, Canada (Orsi et al. 2012). This clade has only ca. 90% sequence similarity to representatives from either of the two formally described classes of haptophytes, and its position in the haptophyte phylogeny is unstable. In our analysis this clade was placed sister to Pavlovophycae, but the placement was not supported (45%). Simon et al. (2013) found it to either branch as a sister group of Pavlovophyceae or at the base of both Pavlovophyceae and Prymnesiophyceae, but neither of the placements were supported. Nevertheless our results confirm the existence of this clade, and indicate that it consists of several species.

HAP-4 and HAP-5 (Fig. 1)

The phylogenetic analyses suggest the existence of two additional new classes, here termed "HAP-4" and "HAP-5". Six OF OTUs were affiliated with HAP-4, which consists of four environmental sequences previously isolated from the Caribbean Sea (Edgcomb et al. 2011), at 2,500 m depth in the Sargasso Sea (Countway et al. 2007), 120 m depth in the Saanich Inlet, Canada (Orsi et al. 2012), and the North Pacific (Frias-Lopez et al. 2009). While the support for this clade was good (97%), its placement in the haptophyte tree was uncertain. Furthermore, there was considerable divergence also within the clade; the environmental sequences in this clade were only 89–95% similar over the part of 18S rDNA they overlapped.

The two environmental clones SHAC596 isolated from 10 m in the Saanich Inlet (Orsi et al. 2012), and FS01AA17_01Aug05_5 m from the Florida Strait (Cuvelier et al. 2010), formed a clade with weak support in the tree without OF OTUs (Fig. S2A), here termed HAP-5. The 15th most abundant OTU (OTU 15) in the dataset was 99.2% similar to SHAC596, which in turn was 90–94% similar to members of Prymnesiophyceae and ca. 89% similar to members of Pavlovophyceae, as determined by a manual BLAST search against NCBI-nr. OTUs 55, 107 and 121 were also placed in this clade, but with no support (Fig. 1). Over the entire 18S rDNA the divergence between the established classes Pavlovophyceae and Prymnesiophyceae is ca. 6% (Edvardsen et al. 2000). Although the divergence between sequences will depend on the region of the 18S rDNA under consideration, it seems these environmental lineages are divergent enough to warrant the erection of new classes once the morphologies of these species are known.

Evidence for novel deep-branching lineages has also been found from cultured strains. The marine strain CG5, isolated from South Africa, was here placed among the HAP-clades but with no certain position in the haptophyte phylogeny. A description of this new species, possibly representing a new haptophyte class, is underway (Sym et al. unpubl. data).

Diversity within established clades

Class Pavlovophyceae (Fig. 1)

Only four OTUs were placed within the Pavlovophyceae (OTU 46, 105, 136 and 154). This class is much less diverse than Prymnesiophyceae, with only 13 formally described species, of which all are genetically characterised in the 18S rDNA (e.g. Bendif et al. 2011). However, as for the Prymnesiophyceae, the species richness of Pavlovophyceae is probably higher than what is currently described. All four OTUs were placed as sister groups to

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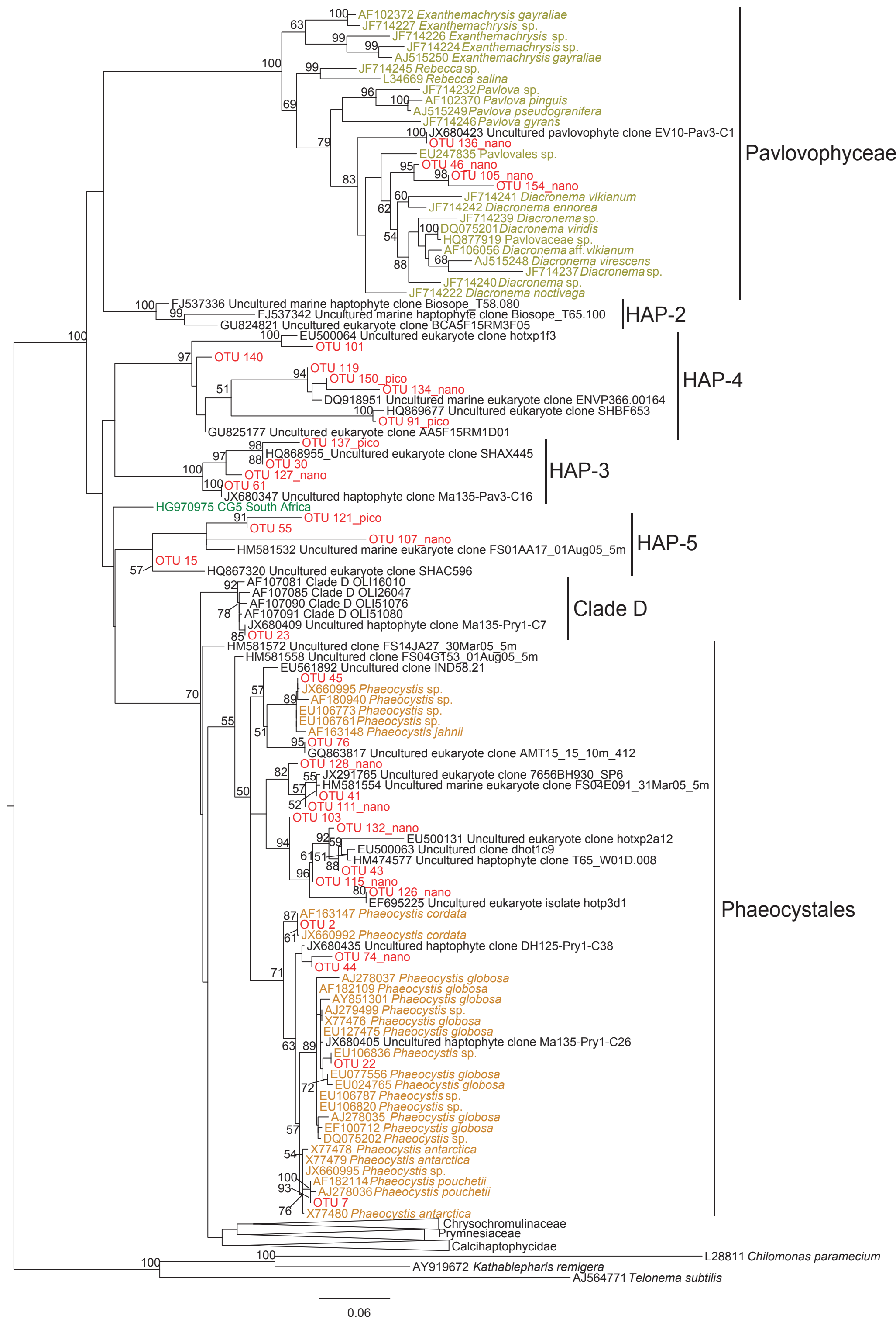

Figure 1 Maximum likelihood (RAxML) tree based on nuclear 18S rDNA sequences of cultured and environmental sequences of the Haptophyta, including 454 pyrosequencing OTUs$_{99.5\%}$ of the V4 region from the haptophyte community in Oslofjorden. Maximum likelihood bootstrap values > 50 are shown above or below the branches. *Chilomonas paramecium* (L28811), *Kathablepharis remigera* (AY919672) and *Telonema subtilis* (AJ564771) were used as outgroups. Scale bar represents number of substitutions/site. Pavlovales, HAP-3, HAP-4, HAP-5, Clade D and Phaeocystales are shown in detail, other groups are collapsed. OTUs from this study are in red, cultured strains are in other colours, and environmental haptophyte sequences are in black. OTUs labelled "nano" were only found in the 3–45 μm size fraction, whereas OTUs labelled "pico" were only found in the 0.8–3 μm size fraction.

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members of the genus *Diacronema* in our phylogeny. None of these OTUs matched any known species, and may represent novel species of the genus *Diacronema*, or new genera. OTUs 46, 105 and 154 are < 98% similar to any published environmental sequence, and may thus represent taxa never detected before by 18S rDNA environmental sequencing. OTU 136 was identical to an uncultured clone isolated from a freshwater lake in France (Simon et al. 2013), which was ca. 97% similar to members of the genus *Diacronema*, and branched off as a sister clade. Due to the influence of the river Glomma, Norway's longest river with outlet in outer Oslofjorden, the local phytoplankton flora may at times include both brackish and freshwater species (Throndsen et al. 2007). Kuylenstierna and Karlson (2006) did not include any members of Pavlovophyceae in their checklist for Skagerrak or Kattegat, but a strain of *Diacronema ennorea* (UIO 021) was isolated by Jahn Throndsen from the Oslofjorden and the 18S rDNA has been sequenced (Egge et al. 2013).

Clade D (Fig. 1)

Clade D has been shown to be a well-supported clade consisting only of environmental sequences, but with an uncertain position in Prymnesiophyceae in 18S rDNA trees (Edvardsen et al. 2000; Moon-van der Staay et al. 2000). We detected one member of this clade, OTU 23, which was identical to an environmental sequence from the Marmara Sea, Turkey (JX680409), previously shown to nest within this clade (Simon et al. 2013). Members of Clade D were here placed basally within class Prymnesiophyceae and are expected to represent a novel order within this class (Edvardsen et al. 2000).

Order Phaeocystales (Fig. 1)

Altogether 15 OTUs nested within order Phaeocystales, of which four matched cultured species (Table 2). Interestingly, the overall second most abundant OTU (OTU 2) was identical to *P. cordata*, which has not previously been recorded in the Skagerrak (Eikrem, Edvardsen, pers. commun.; Kuylenstierna and Karlson 2006). Over the course of this study, OTU 2 was proportionally abundant in April and May (Egge et al. unpubl. data). *Phaeocystis*-like cells have previously been observed to reach maximum abundance in spring in Skagerrak (April 1991, Kuylenstierna and Karlson 1994). *Phaeocystis cordata* does not form colonies (Zingone et al. 1999), therefore it is easily overlooked, and difficult to distinguish from swarmers of *P. globosa* and *P. pouchetii*. The colony-forming *P. pouchetii* and *P. globosa* are both previously reported from Skagerrak (Table S3), and known to constitute large components of the phytoplankton community during spring in North-Eastern Atlantic waters (e.g. Christaki et al. 2014; Paasche 1960). These two species were also detected in our study by 454 pyrosequencing (OTU 7 and OTU 22, respectively). As *P. globosa* and *P. pouchetii* are both < 97% similar to *P. cordata* in the V4 region, we are confident that *P. cordata* was not confounded with these species in the taxonomic assignation. Members of *Phaeocystis* were detected by electron microscopy in this study, but they could not be identified to species (Fig. 5A–C).

OTU 45 matched a newly genetically characterised free-living strain isolated from the North East Atlantic (strain PCC559, Decelle et al. 2012), placed within the *Phaeocystis jahnii*-clade.

Our analyses indicate the existence of novel clades or species complexes within Phaeocystales. Together with environmental sequences isolated from the Pacific (North and South), OTU 43, OTU 103, OTU 115, OTU 126 and OTU 132 formed a well-supported clade (94%). Further, OTU 41, 111 and 128 formed a clade with environmental sequences from the Florida Strait and Northern Pacific. OTU 44 and 74, together with an uncultured clone isolated from the South Atlantic were placed as a sister clade to the *P. pouchetii-antarctica-globosa*-clade. This group was not supported (42%), and the OTUs may therefore belong to either *P. globosa* or to the *P. pouchetii* + *P. antarctica* clade. According to Medlin and Zingone (2007) several *Phaeocystis* species have been recognised that are not yet formally described.

Order Prymnesiales

In total, 93 (60%) OTUs were placed within the order Prymnesiales, of which 13 matched cultured species (Table 2). Within this order we detected members of several well-supported groups consisting of only environmental sequences, but the phylogenetic placement of these clades relative to described genera in Prymnesiales was uncertain. For instance, nine environmental sequences including one isolated from freshwater (Lake Finsevatn, Norway, Shalchian-Tabrizi et al. 2011) formed a well-supported group (Fig. S2C), which was named Clade B3 in an earlier study (Simon et al. 2013). Seven OF OTUs were affiliated with this group, but the group itself was no longer supported once the OF OTUs were included in the tree (Fig. 2). Interestingly, two of the OTUs detected in Clade B3 (OTU 88 and 92) were only found in the pico-size fraction, and the environmental sequences HM581601 and EF173004 were obtained from a flow cytometry-sorted picoplanktonic sample (Cuvelier et al. 2010), and a sample prefiltered through 2 μm filter (Not et al. 2007), respectively. This could indicate that this clade is predominantly picoplanktonic, which might explain why it so far has no cultured and described species. Thirteen OF OTUs were placed in another novel clade within Prymnesiales here termed Clade B4, which had good support in the reference tree (Fig. S2C). However, the placements of these OTUs were for the most part not supported (Fig. 3), and the placement of the clade itself was uncertain.

Order Prymnesiales – family Chrysochromulinaceae (Fig. 2)

Several OTUs matched environmental sequences placed in or near the clade containing *Chrysochromulina leadbeateri* and *C. simplex*. This clade has previously been shown to contain undescribed diversity (Edvardsen et al. 2011; Moon-van der Staay et al. 2000). Altogether 31 OTUs

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Figure 2 Maximum likelihood (RAxML) tree based on nuclear 18S rDNA sequences of cultured and environmental sequences of the Haptophyta including 454 pyrosequencing $OTUs_{99.5\%}$ of the V4 region, as in Fig. 1. Family Chrysochromulinaceae is shown in detail, other groups are collapsed.

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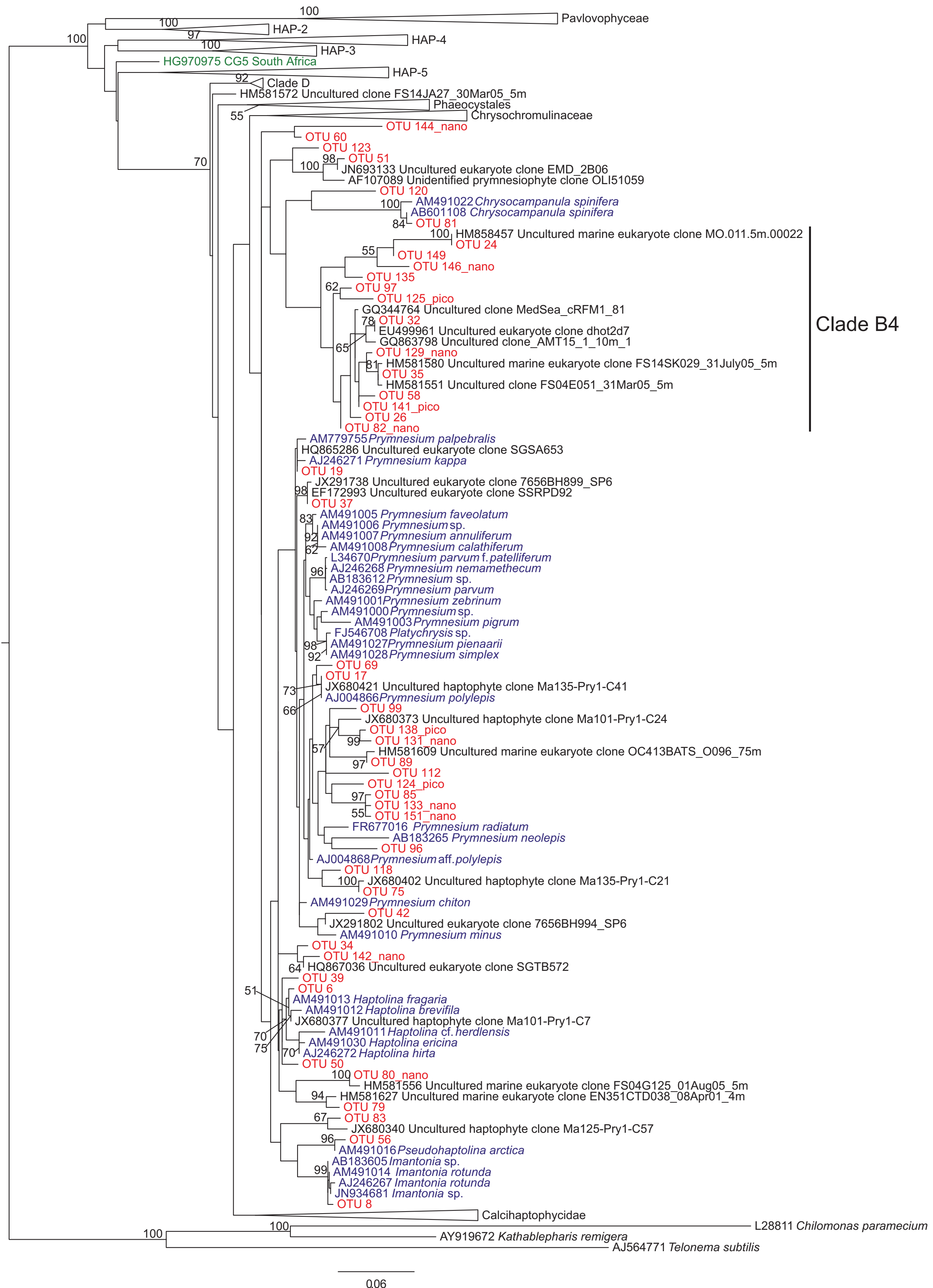

Figure 3 Maximum likelihood (RAxML) tree based on nuclear 18S rDNA sequences of cultured and environmental sequences of the Haptophyta including 454 pyrosequencing $OTUs_{99.5\%}$ of the V4 region, as in Fig. 1. Family Prymnesiaceae is shown in detail, other groups are collapsed.

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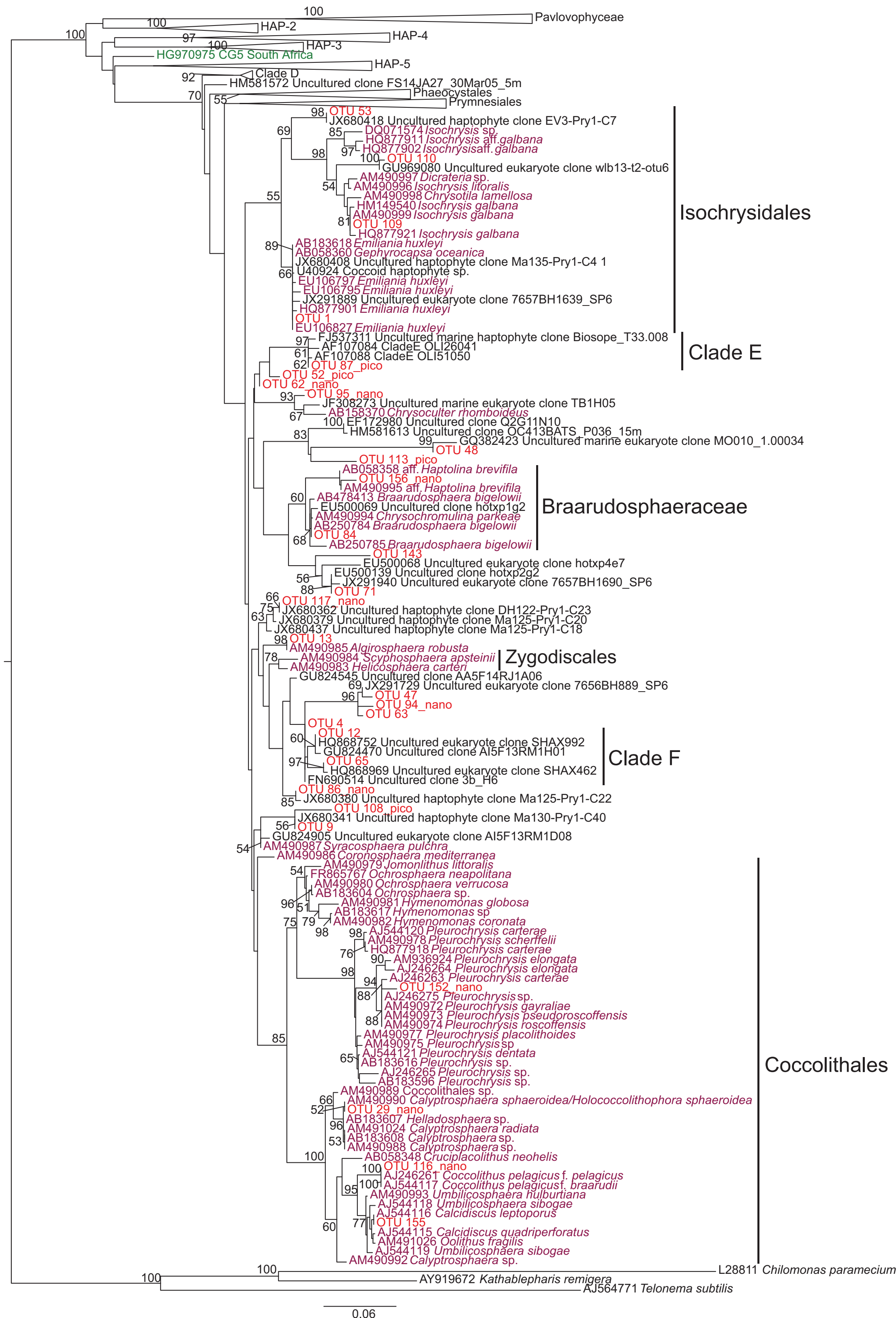

Figure 4 Maximum likelihood (RAxML) tree based on nuclear 18S rDNA sequences of cultured and environmental sequences of the Haptophyta including 454 pyrosequencing $OTUs_{99.5\%}$ of the V4 region, as in Fig. 1. Subclass Calcihaptophycidae is shown in detail, other groups are collapsed.

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Table 2. Distribution of number of unique haptophyte V4 SSU rRNA OTUs recorded in Skagerrak among the major haptophyte groups

Group	Best blast-match with environmental sequence	Best blast-match with genetically characterised species	Total unique OTUs
Chryosochromulinaceae	32	8	40
Prymnesiaceae	48	5	53
Calcihaptophycidae	21	8	29
Phaeocystales	11	4	15
Pavlovophyceae	4	0	4
Clade D	1	–	1
HAP-3	4	–	4
HAP-4	6	–	6
HAP-5	4	–	4
Total	131	25	156

Table 3. List of species or cell types observed in electron microscope in surface samples from the Skagerrak in the period September 2009–June 2011

	Prymnesiales		Coccolithales		Isochrysidales
Phaeocystales	Prymnesiaceae	Chrysochromulinaceae	Syracosphaeraceae	Rhabdosphaeraceae	Noelaerhabdaceae
Phaeocystis sp.	*Chrysocampanula spinifera*	*Chrysochromulina* cf. *camella*	*Syracosphaera* cf. *anthos*	*Algirosphaera robusta*	*Emiliania huxleyi*
	Chrysochromulina mactra[a]	*Chrysochromulina* cf. *ephippium*	cf. *Syracosphaera*	*Calciosolenia brasiliensis*	
	Haptolina ericina	*Chrysochromulina leadbeateri*	*Syracosphaera marginiporata*	Calyptrosphaeraceae	
	Haptolina cf. *fragaria*	*Chrysochromulina* cf. *scutellum*		*Corisphaera* sp.	
	Haptolina hirta	*Chrysochromulina simplex*			
	Cf. *Prymnesium*	*Chrysochromulina throndsenii*			
	Prymnesium polylepis authentic type	Prymnesiales sp. nov. 1			
	P. polylepis deviant type	Prymnesiales sp. nov. 2			
	Cf. *Prymnesium minus*				

[a]Cell shape and length of flagella and haptonema indicate that this species belongs to family Prymnesiaceae.

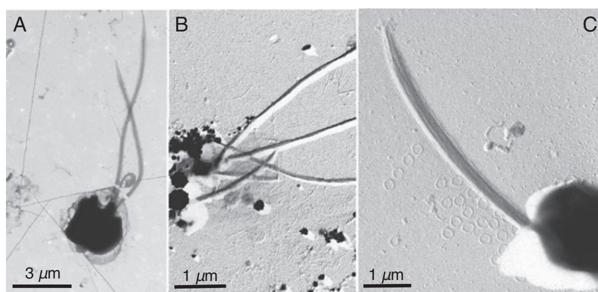

Figure 5 Electron microscopy images of Phaeocystales. **A.** *Phaeocystis* sp. with haptonema and flagella. **B.** Pentagonal star (ejectosome) formed by *Phaeocystis* sp. **C.** Detached scales from the cell body covering of *Phaeocystis* sp.

nested within this clade, among them the overall third most abundant OTU (OTU 3), which had 2 bp mismatches with *C. simplex* (pos. 892 and 918 in *C. simplex* AM491021.2). Cells of *C. simplex* are covered by numerous plate scales without a spine, which were observed in the electron microscope (Fig. 6C). *Chrysochromulina leadbeateri* is thought to constitute a species complex due to high variation in morphology of cell shape and body scales (Edvardsen et al. 2011). However, the 18S rDNA has been sequenced from only two strains of *C. leadbeateri,* isolated from the same bloom event (Lofoten, N Norway, 1991, B. Edvardsen pers. commun.), and they are identical in this region (strains UIO035 and TJE: AM491017). Some of the OTUs that nest within this clade but that are distinct from the *C. leadbeateri* 18S rDNA sequence may represent members of the *C. leadbeateri*-complex. Cells of *C. leadbeateri* were also observed in EM.

By contrast, only two OTUs (5 and 10) were affiliated with the *C. strobilus-cymbium-campanulifera* clade (Table 1; Fig. 2). These species are identical in the 18S rDNA V4 region, and cannot be separated with our primers. Except for one deletion (T instead of TT around pos. 608 of *C. cymbium* AM491018.1), OTU 10 was identical to these three species, and also to seven environmental sequences (among them JX291812, included in the refer-

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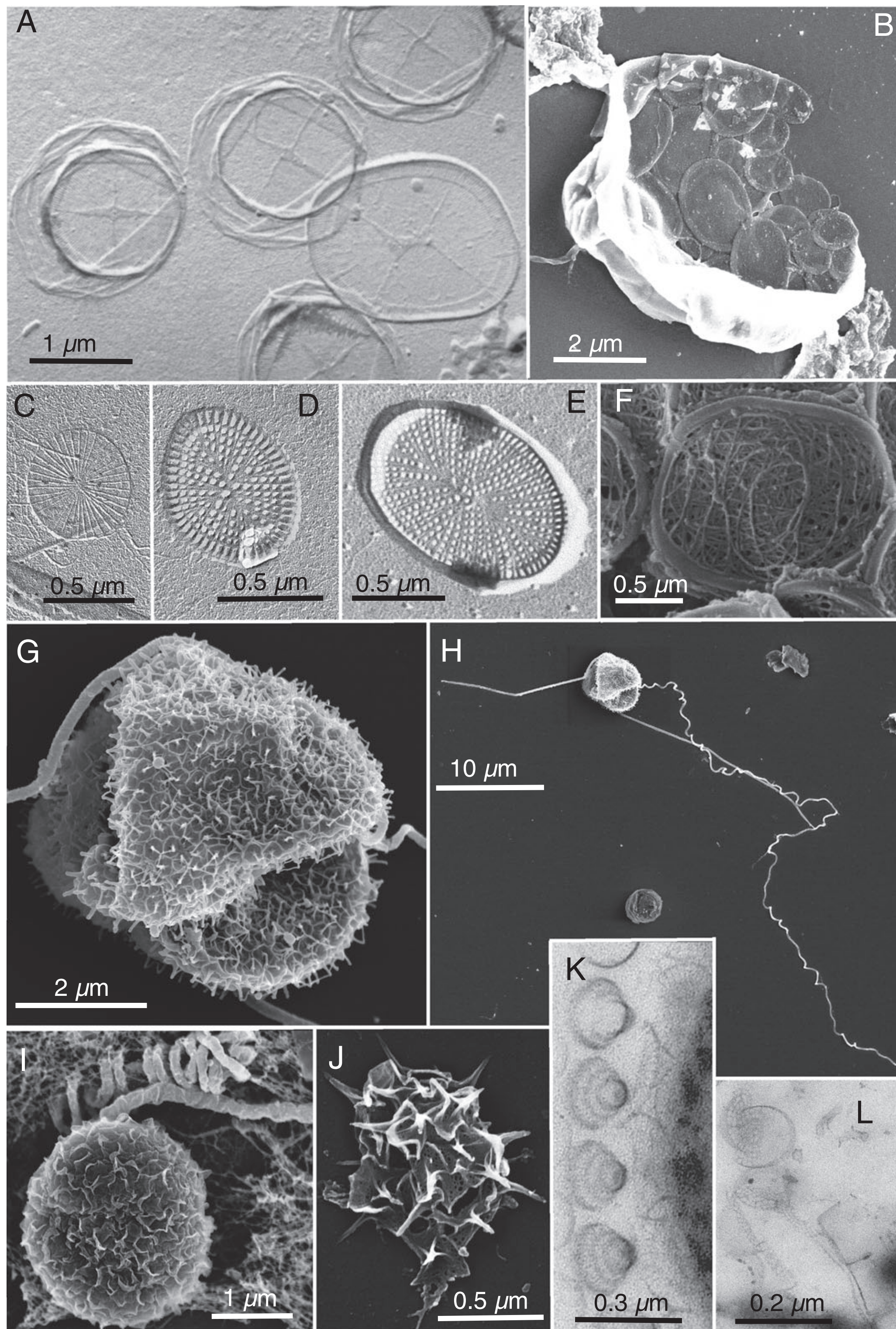

Figure 6 Electron microscopy images of Prymnesiales. **A.** *Chrysochromulina mactra* detached body scales. **B.** Scale covering of undescribed Prymnesiales-like cell. **C.** *Chrysochromulina simplex* body scale. **D.** Small plate scale of *Prymnesium polylepis* authentic type. **E.** Large plate scale of *Prymnesium polylepis* authentic type. **F.** Plate scale details of *Haptolina ericina*. **G.** Cell of *Chrysochromulina* cf. *ephippium* covered by outer layer of spine scales. **H.** *Chrysochromulina* cf. *ephippium* with flagella and extended haptonema. **I.** Undescribed Prymnesiales-like cell with scales and haptonema. **J.** Close up of body scales from previous image. **K.** Plate and cup scales of *Chrysochromulina* cf. *camella*. **L.** Plate scales and scales with upright rims of *Chrysochromulina* cf. *throndsenii*.

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ence tree). It was therefore not possible to give OTU 10 a certain phylogenetic placement, and it may thus represent one or more of these three species. Nevertheless, the total diversity in this particular clade of *Chrysochromulina* may be higher than what can be resolved in this study. The species in this clade are covered by cup-formed body scales, as is *Chrysochromulina camella*, a species not yet genetically characterised in the 18S rDNA. *Chrysochromulina* scales resembling most those of *C. camella* were observed in the electron microscope (Fig. 6K). We might thus speculate that OTU 5 could represent *C. camella*.

The clade containing *C. rotalis*, *Chrysochromulina throndsenii*, *Chrysochromulina acantha* and *C. parva* also contained several environmental sequences and species with identical sequence in the V4 region. For instance OTU 11, which had 1 mismatch with *C. acantha* and sequences of two endosymbionts of the foraminifera *Globigerinella siphonifera* (AF166376 and AF166377), could not be reliably placed, and it might indeed represent more than one species. Further, *C. acantha* and *C. throndsenii* are only 2 bp different in the V4 region, and might not be possible to separate. *Chrysochromulina throndsenii* was observed with electron microscopy in this study (Fig. 6L). Members of the *Chrysochromulina* genus can be recognised by their saddle-shaped cells (Edvardsen et al. 2011). Morphologically described saddle-shaped species that have been observed in Skagerrak, but are not yet sequenced, include *Chrysochromulina alifera* and *Chrysochromulina ephippium* (Eikrem and Moestrup 1998; Jensen 1998a). *Chrysochromulina ephippium* was also observed in this study (Fig. 6G,H). Thus, these are possible candidates for OF OTUs placed within *Chrysochromulina*, but without match to a cultured species.

Order Prymnesiales – family Prymnesiaceae (Fig. 3)

Five OTUs were affiliated with the genus *Haptolina*. OTU 6 had one mismatch with *Haptolina fragaria*, *H. ericina*, *H.* cf. *herdlensis*, *H. hirta*, and two environmental sequences, and may thus represent one or several of these species. Seventeen OTUs were affiliated with *Prymnesium*, of which *P. polylepis* and *P. kappa* were the only cultured and sequenced species detected. OTU 8 matched a strain of *Imantonia* isolated from the Bering strait. OTU 56 had four mismatches with *Pseudohaptolina arctica*, which suggests that it could represent a new species in the genus *Pseudohaptolina.* OTU 81 matched *Chrysocampanula spinifera. Chrysocampanula spinifera, Haptolina ericina, H. fragaria, H. hirta* and *P. polylepis* were also detected in EM (Table 3; Fig. 6D–F).

The high number of novel sequences within Prymnesiales is consistent with the diversity of *Chrysochromulina* (sensu lato) observed in previous surveys of the Skagerrak and Kattegat, both formally described and nondescribed forms (Backe-Hansen and Throndsen 2002; Eikrem 1999; Jensen 1998a). Jensen (1998a) recorded 32 morphological species of *Chrysochromulina* sensu lato and in addition scales of 20 nondescribed forms with morphologies resembling this group. Many of these undescribed forms were quantitatively important contributors to the *Chrysochromulina* population (Jensen 1998a). Of the morphological forms reported by Jensen, 16 have until now been genetically characterised (Table S3). Four of these were not detected by 454 pyrosequencing in this survey; *Prymnesium minus*, *P. chiton*, *Haptolina brevifila* and *C. throndsenii*, although we detected OTUs closely matching sequences of *H. brevifila* and *C. throndsenii* (Table S3). Unreported and undescribed Prymnesiales-like cells were detected in SEM, such as the *Chrysochromulina*-like cell in Fig. 6I,J and the scale covering in Fig. 6B. Further, we detected *Chrysochromulina mactra* in TEM (Fig. 6A). This seems to be a common species in Skagerrak, but it has not yet been genetically characterised. The morphology of this species (rounded cell shape and similar length of flagella and haptonema) indicates that it belongs in Prymnesiaceae (Edvardsen et al. 2011).

A high novel diversity within Prymnesiales has similarly been revealed by recent studies targeting the 28S rDNA gene; by 454 pyrosequencing of samples from the Mediterranean (Bittner et al. 2013) and by cloning and Sanger sequencing of oceanic picohaptophytes (Liu et al. 2009).

Subclass Calcihaptophycidae (Fig. 4)

'Calcihaptophycidae' has been introduced as a subclass comprising the monophyletic entity of potentially calcifying haptophytes (de Vargas et al. 2007), i.e. including noncalcifying species phylogenetically affiliated with coccolithophores, such as *Isochrysis*. This category is convenient for novel environmental sequences affiliated with the coccolithophores, as it is not known whether they represent actually calcifying species. However, in our analysis the Calcihaptophycidae was not supported either with or without OF OTUs included in the trees (Fig. 4, S2D). A clade consisting entirely of environmental sequences, which is here termed Clade F, branched off before the clade of described orders of coccolithophores (except Braarudosphaeraceae), although this placement was not supported. Clade F was distinct from the previously inferred Clade E (Moon-van der Staay et al. 2000), which also so far only consists of environmental sequences. Interestingly, OTU 4, which matched environmental sequence 3b-H6 in Clade F, isolated from sea ice in the Baltic (Majaneva et al. 2011), was proportionally dominating in the reads from samples taken April 2010 and March 2011 and detected in both the pico- and nano-size fractions (Egge et al. unpubl. data). Although not much is known about the cell size of this putative novel species, assuming that the high proportion of reads corresponds to high proportion of biomass, this indicates that it constitutes an important part of the haptophyte spring community. Further, this shows that even seemingly abundant species in the environment remain to be cultured and genetically characterised. In total six OF OTUs were affiliated with Clade F. We detected one member of Clade E, OTU 87, which was only found in the pico-size fraction. Further, OTU 95 fell in a clade with the cultured species *Chrysoculter rhomboideus*, a sister-taxa to members of Clade E, and may represent a novel species of *Chrysoculter*. Two OTUs were placed in the *Braarudosphaera bigelowii/Chrysochromulina*

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parkeae clade. *Chrysochromulina parkae* has been suggested to be a life cycle stage of *B. bigelowii* or a sibling species of *B. bigelowii* (Hagino et al. 2013).

Three OTUs were affiliated with *Isochrysis*, the noncalcifying sister genus to *Emiliania* and *Gephyrocapsa*. OTU 109 was identical to *Isochrysis galbana*, whereas OTU 53, which was identical to the environmental clone EV3-Pry1-C7, branched off as a sister clade to *Isochrysis*. This sequence was isolated from a freshwater lake in France (Simon et al. 2013). The most abundant OTU (OTU 1) in the dataset was identical to *Emiliania huxleyi* (and *Gephyrocapsa oceanica*), which was frequently observed in the EM preparations (Fig. 7C). *Syracosphaera pulchra* is the only member of genus *Syracosphaera* with a known 18S rDNA sequence, to which OTU 9 was identical. By SEM we observed, for the first time in Skagerrak, *Syracosphaera marginiporata* (Fig. 7B) and *Syracosphaera anthos* (Fig. 7E). *Algirosphaera robusta* (OTU 13) was detected both with SEM (Fig. 7A) and 454 pyrosequencing. OTUs identical to sequences of *Calyptrosphaera sphaeroidea*, *Calcidiscus leptoporus,* and *Coccolithus pelagicus* were recovered, all of which have previously been reported from microscopy surveys (Table S2). In this study, also *Corrisphaera* sp. (Fig. 7D) was recorded in SEM. The total number of coccolithophore species previously observed in Skagerrak by microscopy methods was higher (44 spp.) than what we recovered here by 454 (29 OTUs, Table 2). Some species may have identical 18S rDNA V4 region (such as *E. huxleyi* and *G. oceanica*) or be joined in a common haplo-diploid life cycle with distinct morphologies, which shows the need for combining molecular and microscopical methods.

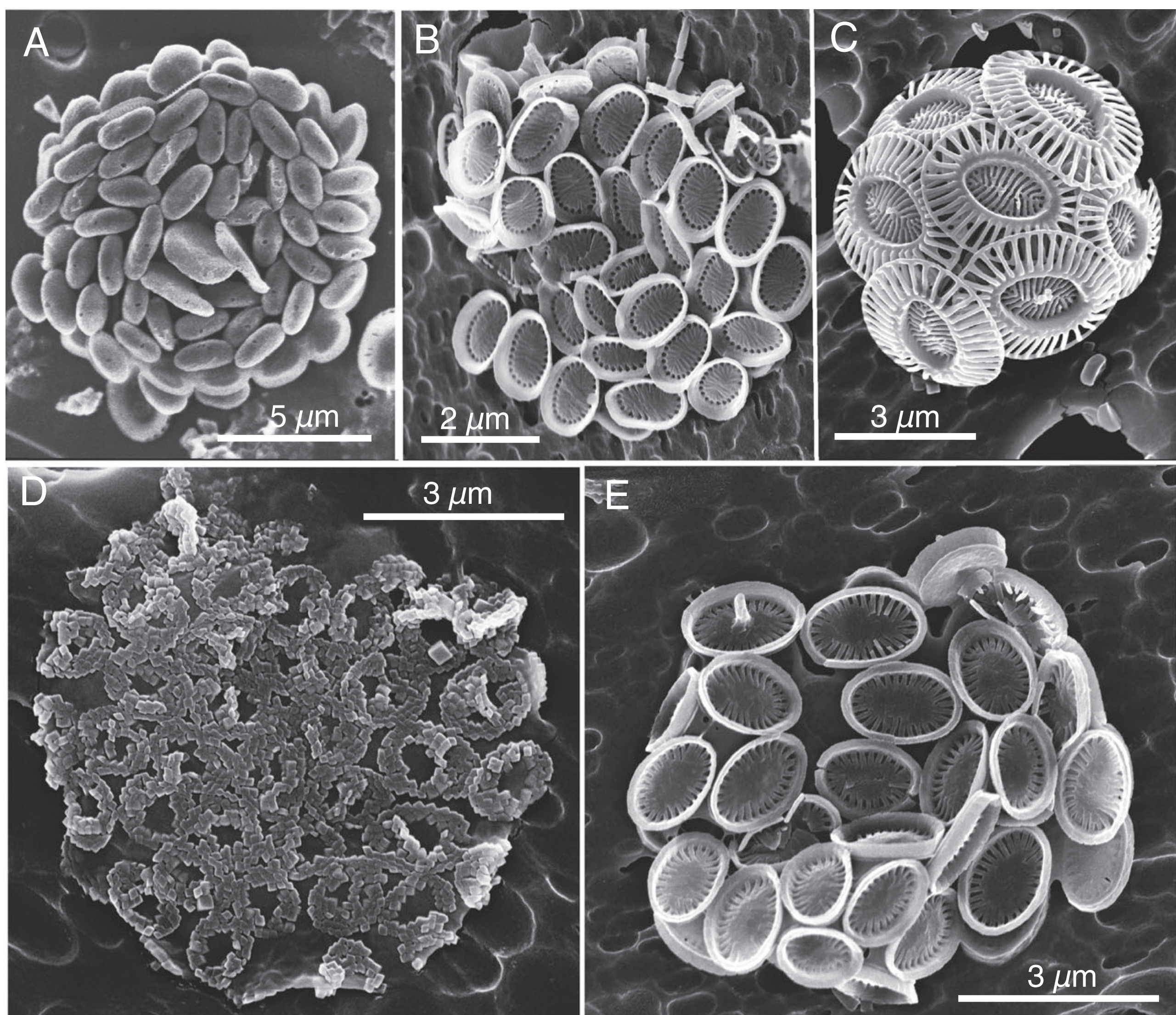

Figure 7 Electron microscopy images of Coccolithales and Isochrysidales. **A.** *Algirosphaera robusta* (Photo: Shuhei Ota). **B.** *Syrachophaera marginiporata.* **C.** *Emiliania huxleyi*, type A. **D.** *Corrisphaera* sp. incomplete coccoliths. **E.** *Syracosphaera anthos.*

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Methodological considerations

Diversity lost in the sampling process?

By prefiltering through a 45-μm mesh we could miss out on haptophytes attached to particles or marine snow, in symbiosis with organisms > 45 μm, or species forming colonies, such as *Phaeocystis* spp. However, prefiltration was performed to prevent metazoan, ciliate or dinoflagellate sequences from dominating the pyrosequencing results due to their large cell size and putatively high rRNA content. Further, haptophyte community composition may vary by depth (e.g. Bittner et al. 2013). For instance some coccolithophores may dwell deeper in the water column (Malinverno et al. 2003). Thus, the samples included in this study, obtained from 1 m depth, may under-represent the full haptophyte diversity in the water column. The diversity of all protist groups in samples taken at the deep chlorophyll maximum from the same sampling cruises will be presented in a following study (Gran Stadniczeňko et al. unpubl. data).

Chimeras

The proportion of unique OTUs flagged as chimeras was here very high (> 50% of unique OTUs). However, most of these were represented as < 50 reads each, and thus did not constitute a large proportion of the total number of reads. A mechanism for chimera formation with a cDNA protocol with random primers was suggested in Egge et al. (2013). Under cDNA synthesis, random primers may anneal within the target region, and cDNA strands that only cover a part of the target region may be created. If this part is copied in the first round of the PCR, it might act as a long, nonspecifically binding primer in subsequent rounds of the PCR, and thus create chimeras. Chimeras between closely related taxa may not be detected by chimera check programmes (e.g. Perseus) and in this case an additional thorough manual check by BLAST, and visual inspection of alignments were needed.

Concluding remarks

Pyrosequencing revealed higher species richness of haptophytes than previously observed with electron and light microscopy in the Skagerrak. Novel lineages were detected, ranging from species to class level. In particular, we observed high, uncultured diversity within the order Prymnesiales. Considering that some closely related species have identical 18S V4 rDNA regions, the true richness may be even higher. At this point, we can only speculate about the possible ecological functions of these environmental clades. This study in particular highlights the lack of overlap between morphological descriptions and genetic characterisation of haptophytes. Our work contributes to linking genotype to phenotype within this ecologically important protist group, and reveals great, unknown diversity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Map of the Skagerrak and the Kattegat off the coasts of Norway, Sweden and Denmark. The OF2 sampling site is indicated by a star. The arrows show a simplified picture of the general circulation pattern in the Skagerrak. AW = Atlantic Water; CNSW = Central North Sea Water; JCW = Jutland Coastal Water; BW = Baltic Water; NCC = Norwegian Coastal Current. Adapted from Lekve et al. (2006).

Figure S2. Maximum likelihood (RAxML) reference tree based on 281 nuclear 18S rDNA sequences of cultured strains and environmental clones of the Haptophyta, 454 pyrosequencing OTUs not included. Maximum likelihood bootstrap values > 50 are shown above or below the branches. *Chilomonas paramecium* (L28811), *Kathablepharis remigera* (AY919672) and *Telonema subtilis* (AJ564771) were used as outgroups. Scale bar represents number of substitutions/site. **A.** Pavlovales, HAP-3, HAP-4, HAP-5, Clade D, Phaeocystales, **B.** Chrysochromulinaceae, **C.** Prymnesiaceae, **D.** Calcihaptophycidae.

Table S1. Sequence similarity matrix (%) between the haptophyte sequences included in the reference tree (Fig. S2), trimmed down to the region spanned by the OF OTUs.

Table S2. Best BLAST matches of haptophyte V4 SSU rRNA OTUs recorded in the Skagerrak in the period September 2009–June 2011.

Table S3. Morphological species observed by microscopy in the Skagerrak in previous investigations.

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