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UNCOVERING HIDDEN BIODIVERSITY IN THE CRYPTOPHYTA: CLONE LIBRARY STUDIES AT THE HELGOLAND TIME SERIES SITE IN THE SOUTHERN GERMAN BIGHT IDENTIFIES THE CRYPTOPHYCEAN CLADE POTENTIALLY RESPONSIBLE FOR THE MAJORITY OF ITS GENETIC DIVERSITY DURING THE SPRING BLOOM

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CRYPTOPHYTES
DIVERSITY
PICOPHYTOPLANKTON
NORTH SEA

ABSTRACT. – Cryptophyceae are important group in marine phytoplankton, but little is known about the occurrence and distribution of individual species. Recently, with use of molecular probes and microarray technology, it has been shown that species related to *Teleaulax* spp. or *Chroomonas* spp. (clades 4 or 6) contributed the most to cryptophycean biomass in the North Sea. For the microarray study, the single probe (clade 4/6) recognizes members of both clades 4 and 6 and thus cannot separate them. Therefore, it was unknown as to whether the cryptophyte community was composed of clade 4, clade 6 or both of them. Here, we addressed this question and increased the genetic diversity of our investigations of cryptophycean diversity in the North Sea by sequencing 18S rRNA clone libraries made from fractionated water samples to examine specifically the picoplanktonic fraction because that fraction was studied in detail in the earlier microarray study. We focused on samples from the spring phytoplankton bloom in 2004 because the microarray signals were the strongest at this time. Excluding chimeric sequences, we detected nine cryptophycean OTUs, seven of which fell into the *Teleaulax*/*Plagioselmis* branch, whereas two grouped with *Geminigera* spp. Our results indicate that these OTUs, affiliated with clade 4, may be an important component of cryptophyte community during spring bloom in the North Sea.

INTRODUCTION

Cryptophyceae are unicellular, flagellated algae ubiquitous in marine, brackish and freshwater habitats. They account for substantial source of food for microzooplanktonic ciliates and dinoflagellates (Gustafson *et al*. 2000, Tang *et al*. 2001, Adolf *et al*. 2008). They are prey for the ciliate *Mesodinium rubrum* as a source of photosynthetically active chloroplasts (Gustafson *et al*. 2000), which are ultimately transferred to the dinoflagellate *Dinophysis*. An increase in their abundance has been used to indicate the presence of a *Dinophysis* bloom because the cryptophytes/*Mesodinium* are the preferred prey item of *Dinophysis* (Adolf *et al*. 2008). Such mixotrophic species may successfully grow under nutrient limited conditions, simultaneously influencing composition of other communities (Roberts & Laybourn-Parry 1999).

Taxonomists have described approximately 200 cryptophyte species in over 20 genera (Butcher *et al*. 1967). However, this enormous work has been poorly explored by phytoplankton ecologists because of the difficulty in identifying cryptophytes in either fresh or preserved material. Accurate identification requires extensive studies of their external structures by light, scanning and transmission electron microscopy coupled with detailed analysis of pigments from isolated individuals (Clay *et al*. 1999). Discrimination based on morphological characteristics is also hampered by the fact that the delicate structures of cryptophyte cells are easily destroyed with standard sample fixation using Lugol's iodine solution or formalin. Discoveries of two life stages of cryptophytes with very distinct cell morphologies: haploid and diploid stages of *Proteomonas sulcata* (Hill & Wetherbee 1986), and both crypto-morph and campylo-morph cells within the genus *Cryptomonas* (Hoef-Emden & Melkonian 2003) has further complicated distinguishing of cryptophyte species based solely on morphological features.

As a result of the difficulties in species identification, cryptophytes are either counted together with other nanoflagellates as a one group (Wiltshire & Durselen 2004), enumerated at the class level based on characteristic cell shape in light microscopy (Piwosz *et al*. 2009, Kubiszyn

et al. 2014) or based on orange fluorescence of their phycobilin pigments (Ifremer 2006) detected by epifluorescence microscopy (Silva *et al*. 2009) or flow cytometry (Li & Dickie 2001).

One solution to the problems of species discrimination is to use molecular techniques. Hoef-Emden *et al*. (2002), Hoef-Emden & Melkonian (2003), and Marin *et al*. (1998) have constructed a molecular phylogeny of the group using 18S rDNA markers and have recovered 6 distinct molecular clades, which roughly correspond to families in the Cryptophyceae, although it is clear that a taxonomic revision is necessary (Clay *et al*. 1999, Table I). Metfies & Medlin (2007) demonstrated the effectiveness of molecular DNA oligonucleotide probes to identify these clades. Subsequently, their probes were applied to field material in a microarray format (Metfies *et al*. 2010), by using the Chemscan, a solid phase cytometer (Töbe *et al*. 2003, Medlin & Schmid 2009) and in CARD-FISH (Piwosz *et al*. 2016). Recently, a new heterotropic clade in the Cryptophyta has been documented from clone library sequences taken from freshwater samples (Shalchian-Tabrizi *et al*. 2008, Piwosz *et al*. 2016).

Our previous microarray study in Helgoland in south German Bight showed clades 4 & 6 to contribute the most to the cryptophyte abundance with an early spring bloom over three consecutive years (Metfies *et al*. 2010). These two clades are targeted by a single probe, probe 4/6 (Metfies & Medlin 2007). The aim of this study was to complement presence/absence and relative abundance data from the microarray with information on the phylogenetic diversity of cryptophytes, and to resolve whether the microarray signal was caused by the presence of clade 4 or clade 6 or both. We prepared clone libraries from the same subsamples. Our data indicate that species from clade 4 were likely the dominant cryptophytes in Helgoland during the spring bloom in 2004.

MATERIAL AND METHODS

Sampling site and strategy: The Helgoland time series station is located at 54°11.3'N, 7°54.0'E in the southern German Bight of the North Sea (Fig. 1). Samples are taken from the surface for biotic and abiotic analyses on a weekly basis since 1962. Diatoms and dinoflagellates are counted and identified to the lowest possible taxonomic level, but Cryptophyceae are lumped with other flagellates (Wiltshire & Durselen 2004).

Environmental samples from which clone libraries were made, had been collected as a part of three year (Feb. 2004-Dec. 2006) study on cryptophytes in Metfies *et al*. (2010). Phytoplankton biomass was collected by filtration of 1-1.5 L of water passed through 10 and 3 µm Isopore TCTP membrane filters and the fractionated seawater collected onto a 0.2 µm Isopore GTTP membrane filter (Millipore, Schwalbach, Germany). DNA was

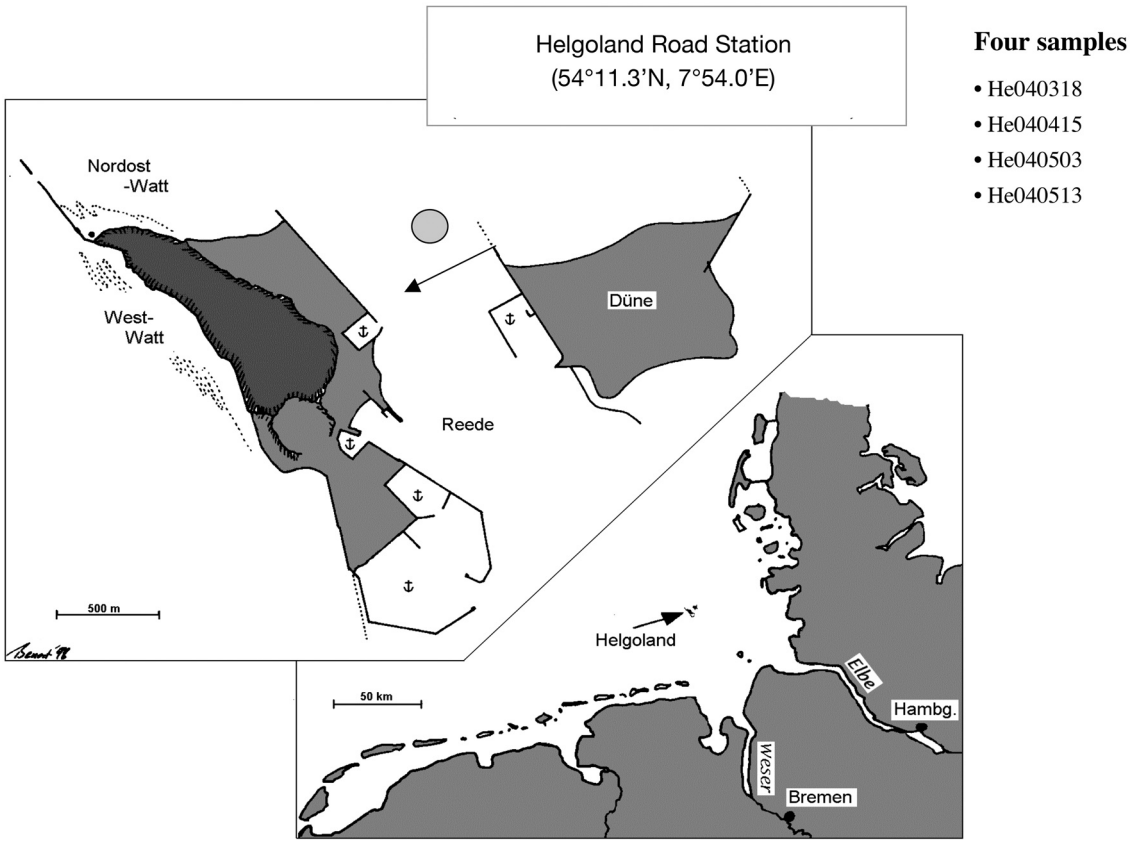

Fig. 1. – Map of the sampling site at Helgoland.

extracted from filters with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) (Metfies *et al*. 2010). We focused on samples that showed high signals of cryptophytes: from 18.03.2004, 15.04.2004, 03.05.2004 and 13.05.2004, as determined by the phylochip analysis from Metfies *et al*. (2010) (Fig. 2). These samples were additionally recounted by light microscopy to screen for abundance of cryptophytes.

Gene Amplification and Cloning: Partial (~850 bp) 18S rRNA genes were amplified with general eukaryotic primers 82F (5'-GAA ACT GCG AAT GAA TGG CTC-3') and 690R (5'-ATC CAA GAA TTT CAC CTC TGA-3'). 45 PCR cycles were performed (1 min at 94 °C, 2 min at 54 °C, and 2 min at 72 °C, initial denaturation: 5 min at 94 °C, final extension: 10 min 72 °C), with use of the Eppendorf Taq polymerase. PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and cloned into the pCR®4-TOPO® vector with use of a TOPO TA XL zero background kit (Invitrogen). 96 clones from each sampling date were selected and purified with R.E.A.L. Prep 96 Plasmid Kit (Qiagen). They were sequenced with the vector specific M13 reverse primer on the ABI 3730 system (Applied Biosystem) using the BigDye Terminator Mix. Quality of the sequences was monitored based of a chromatogram with the SeqMan™ II 5.07© software (DNASTAR Inc.). Sequences longer than 400 bp were compared against GenBank database with use of WU-BLAST2 algorithm (Lopez *et al*. 2003). 32 clones were identified as cryptophycean and were subsequently sequenced with the M13 forward primer. The chimeric sequences were detected manually by separate BLAST search of the sequences obtained with forward and reverse primers. Only 9 sequences of the 32 clones were not chimeric and they were deposited in the EMBL Nucleotide Sequence Database under accession numbers FN689828 to FN689834 and FN689839 to FN689840. They were aligned on-line with the SINA web aligner against the SILVA database (Pruesse *et al*. 2007), and the alignment was refined manually in the software ARB (Ludwig *et al*. 2004) Bootstrapped Maximum Likelihood (ML) trees were then calculated using the RAxML algorithm with the CAT model of rate heterogeneity on a subset of 74 sequences on a dedicated web server (Stamatakis *et al*. 2008). The resulting best result tree were imported into FigTree with bootstrap values placed at the nodes with > 50 % support.

RESULTS AND DISCUSSION

Pigmented cryptophytes contributed throughout the whole year to the phytoplankton community at that site, with peaks observed in early spring and late summer (Metfies *et al*. 2010). Clade 2 and clades 4/6 (annotation after Metfies & Medlin 2007), generally contributed the most to cryptophyte numbers (Metfies *et al*. 2010, Fig. 2). Clade 2 contains four genera: *Rhinomonas*, *Rhodomonas*, *Pyrenomonas* and *Storetula*; and clades 4 and 6, which are detected by a single probe Crypt4/6, contain eight genera: *Plagioselmis*, *Teleaulax*, *Geminigera* (Clade 4), and *Komma*, *Chroomonas*, *Hemiselmis*, *Plagiomonas*, *Protomonas*, and *Falcomonas* (Clade 6). The aim of our study was to resolve which of these genera (those from clade 4 or those from clade 6) were present in the samples collected during the spring maximum in 2004 and which Metfies *et al*. (2010) detected, by the microarray analysis, to be the dominant signal in the picoplanktonic fraction.

In the phylogenetic tree presented in Figure 3A, there are 79 cryptomonad sequences, including the clone library sequences from this study. The cryptomonads are rooted with their sister group, the Katablepharids.

In the cryptomonad tree, the heterotropic taxa are the first divergences in the tree. The first two clades belong to *Goniomonas* spp., which are followed by a new clade of freshwater heterotrophic taxa again known only from clone library sequences (Shalchian-Tabrizi *et al*. 2008, Piwosz *et al*. 2016). The next divergences are the pigmented lineages of which there are both freshwater and marine representatives of both pico and nano size ranges. Clade 1 diverges first, followed by clade 2. The final divergence

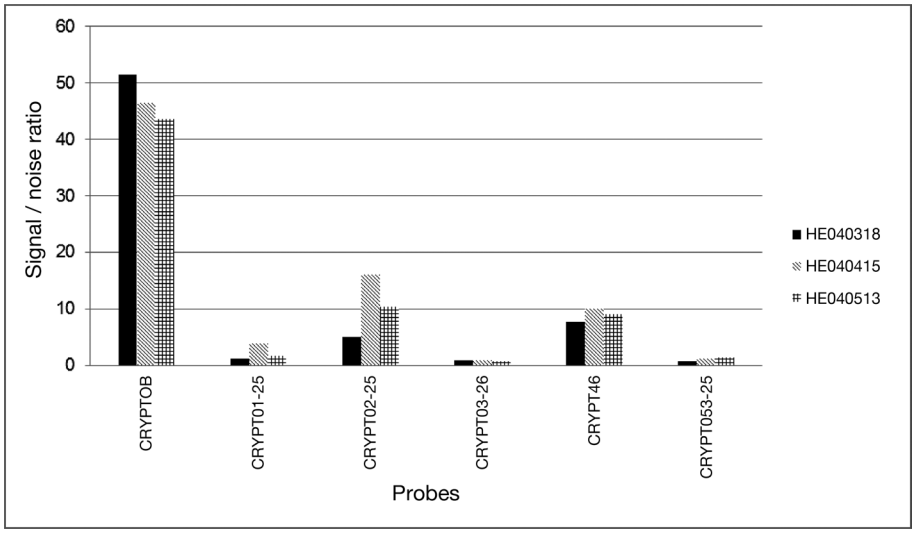

Fig. 2. – Phylochip data on the occurrence of cryptophytes in the samples of March 18, April 15, and May 13 2004 analysed by clone library sequencing in this study. The results have been extracted from a PHYLOCHIP analysis focussing on the assessment of cryptophytes in the North Sea at the island of Helgoland between 2004 and 2006 (Metfies *et al*. 2010).

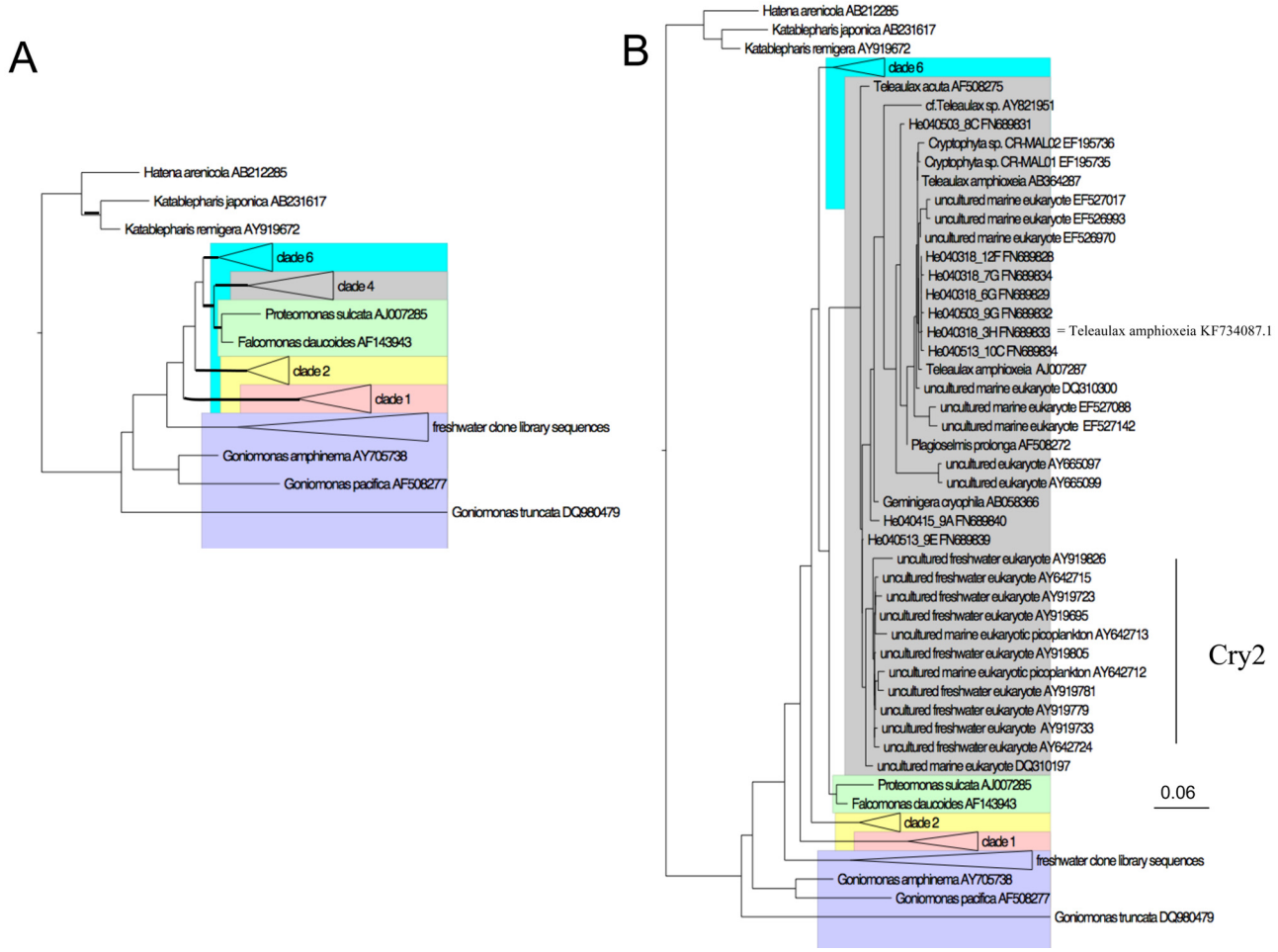

Fig. 3. – Maximum likelihood (ML) tree of 18S rDNA sequences of cryptophytes and ketablepharids. Clades were named after Metfies & Medlin (2004) or after Shalchian-Tabrizi *et al*. (2008) – (CRY2). **A**: Clades collapsed. Thicker bars represent clades with a greater than 90 % BT support in the ML analysis. **B**: Clade 4 open and sequences with the notation 'He' are the clone library sequences determined in this study. The bar indicates 6 % sequence divergence.

splits clade 6 from clade 4, which is sister to a clade with one representative of clade 5 (*Proteomonas*) and 1 representative of clade 7 (*Falcomonas*). Clade 4 splits into two major sub-clades, one of which CRY2 was discovered in the study by Shalchian-Tabrizi *et al*. (2008). The other sub-clade contains our clone library sequences and many known species. Bootstrap support is only shown for the major clades. Although the bootstrap support for the sister relationships among the clades is low, the clades themselves are strongly supported and thus there is no doubt as to the alliance of the members of each clade.

Out of 350 clones picked from the four clone libraries, we found only nine cryptophycean sequences that were not chimeric. They were similar (> 97 %) to species from clade 4: *Plagioselmis prolonga*, *Teleaulax amphioxeia* or *Geminigera cryophila*. Phylogenetic analysis grouped seven sequences on one branch together with *Plagioselmis prolonga* and *Teleaulax amphioxeia*, whereas the remaining two sequences were at the base of clade 4 diverging just before *Geminigera cryophila* (Fig. 3B). This suggests that the spring peak of cryptophytes detected by the Crypt46 probe (Fig. 2) reflected more individuals from clade 4 than from clade 6. This is in agreement with light microscopy based studies that often reported presence of *Plagioselmis* and *Teleaulax* ssp. in marine samples collected during the phytoplankton bloom (Mackiewicz 1991, Novarino 2005, Cerino & Zingone 2006) as well as in next generation sequencing studies (Masana *et al*. 2015). In contrast, representatives of other clades and thus other genera have been observed in higher numbers at coastal sites (Medlin & Schmid 2010) or in other seasons (Cerino & Zingone 2006). However, we did not find any sequence that would affiliate with clade 2. Thus our study suggests that cryptophytes from clade 4 have a higher abundance in the picoplanktonic fraction than clade 6. This could come from several sources: cells from clade 4 are more fragile and ruptured when passed through the 3 mm filter and their DNA was collected onto the 0.2 mm filter (Masana *et al*. 2015) or sexual reproduction was taking place and the larger cell had divided by meiosis into smaller celled gametes, which were collected in the picoplankton fraction. Certainly pico-sized crypto-

phytes have been identified with FISH probes (Medlin & Schmidt 2006, Piwosz *et al*. 2016) so cryptophyte cells in this size fraction are not unknown (see below) and not always the case of cells being ruptured.

Molecular studies of planktonic diversity have shown unexpected novelty within marine picoplanktonic groups (Lopez-Garcia *et al*. 2001, Moon-Van Der Staay *et al*. 2001). However, cryptophycean sequences retrieved from marine samples seemed to originate from individuals of described species (Shalchian-Tabrizi *et al*. 2008). In contrast, sequences of freshwater origin formed two novel branches, one within clade 4 (CRY 2 in the Fig. 3B) and one that diverges before clade 1 (Shalchian-Tabrizi *et al*. 2008) for two invasions of freshwater. In our tree there is one additional uncultured marine clade at the base of the freshwater clade with high bootstrap support (Fig. 3B). Novel heterotrophic clades have also been detected in freshwaters (Piwosz *et al*. 2016) but our sequences fell into the pigmented group of cryptophytes. Perhaps marine cryptophytes have been relatively well characterized by conventional methods. On the other hand, clone library analyses are known to provide only a very limited view on the diversity and composition of phytoplankton communities, which is furthermore biased towards sequences that are easier to amplify via PCR. This could account for the few numbers of cryptophyte sequences recovered here and it is possible than clade 4 is easier to amplify than clade 6. Moreover, a PCR bias could lower the observed diversity, especially that relatively many cycles were used to obtain the PCR product and we obtained many chimeric sequences, of which part of them likely represent other taxa because none of them matched even partially with our sequences, not even clade 6. As a consequence, information on distribution, seasonality and importance of cryptophytes in global cycles is still very scarce. The application of new high throughput methodologies, such as next generation 454-pyrosequencing using cryptophyte specific primers, combined with direct enumeration of specific clades of cryptophytes by fluorescence in-situ hybridization, provide the possibility to gain more comprehensive information on the diversity and composition of cryptophyte and the total phytoplankton communities (Masana *et al*. 2015, Nolte *et al*. 2010, Piwosz *et al*. 2015). NGS sequencing avoids any kind of cloning bias and should be able to resolve finally whether clade 4 or clade 6 is the dominant cryptophyte clade in this area of the North Sea.

From our studies we identified clade 4 instead of clade 6 to be potentially an important component of phytoplankton bloom in the Helgoland in 2004. Further studies are needed to fully elucidate dynamics and distribution of this, and other cryptophyte clades, and their contribution to both pico- and nanophytoplankton.

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