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Phylogenetic diversity of dinoflagellates in polar regions

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Abstract Because of the limitations of sampling and seasonal study in polar regions, knowledge of dinoflagellate diversity, distribution and ecology are limited. Dinoflagellates have been incidentally reported from polar regions during some seasons and some populations have been reported as components of microalgae. Surveys of molecular diversity link the genotype of dinoflagellates from polar regions with environmental adaptation. In this study, 37 positive clones of dinoflagellates collected from different sites were used for genotype analysis, providing new insights into the biodiversity and distribution of these species based on 18S rRNA sequencing. Diverse genotypes were recorded for the summer season in Kongsfjorden (high Arctic) whilst a single novel genotype of dinoflagellate was recorded from winter samples from the Antarctic Ocean. Data from ice cores suggests that this single dinoflagellate genotype was adapted to extreme cold and clone library screening found that it was occasionally the only microbial eukaryotic genotype found in winter ice cores. The findings of this study could improve our understanding of the diverse dinoflagellate genotypes occurring in these perennially cold microbial ecosystems.

Keywords **bipolar, dinoflagellates, molecular diversity, 18S rRNA**

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0 Introduction

Dinoflagellates display a wide array of nutritional mechanisms, including autotrophy, mixotrophy, osmotrophy and phagotrophy[1]. These protists are an important transition level in pelagic ecosystems. Traditional microscopic approaches for documenting the diversity and abundances of protistan assemblages, have contributed greatly to our current understanding of microbial food web structure and biogeochemical processes. Free-living marine dinoflagellates have been represented by 1 555 species (117 genera)[2]. The most numerous genera are unarmored, gymnodinioid dinoflagellates with sigmoid apical grooves *Gymnodinium* (173 species). These have been recorded since the 1980s, as *Gymnodinium* type–‘84K[3] and as *Gymnodinium* sp.1[4].

Recently, an attempt at phylogenetic-based classification of dinoflagellates has been made, encompassing both living and fossil genera[5]. Meanwhile, revision combined LSU rDNA, ultrastructure characteristics, and chloroplast pigment composition has increased our knowledge[6]. New genera, such as *Karenia*, *Akashiwo*, *Karlodinium*, *Polarella* and *Takayama* have been described from 1993 to 2003 by integrating many different protocols[2, 6−9]. The classification of the dinoflagellates has been subjected to continuous revision with new findings and particularly with the application of recent molecular techniques.

Heterotrophic dinoflagellates are plentiful and appear to be active consumers of bacteria, eukaryotic algae and small protozoa in cold water environments. Nevertheless, dinoflagellates have comparatively few representatives in polar waters in contrast to temperate and tropical seas[10−12]. Some species, such as *Protoperidinium islandicum*, *P.*

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saltans and *P. thulesense* exhibit a bipolar distribution[10], *P. americanum*, *P. gvenlandicum*, *Dinophysis braarudii*, *Gyrodinium lachryma* and *Torodinium robustum* could also be considered as conventionally bipolar species. Data on the biogeography of dinoflagellates recorded from the Arctic have been summarized but not all range types found have been discussed in detail[10].

Around 200 planktonic dinoflagellates have been recorded from the Arctic, while seven biogeographic range types have been described [12]. Dinoflagellates from Antarctic waters have been described in detail[13] and revised taxonomies, such as for *Polarella* have been published with more recent examination of material[7]. The latest novel taxa of dinoflagellate, *Dinophyceae* sp. was found to be ecologically significant in some Antarctic marine ecosystems using integrated protocols. It was found to occur in high numbers in slush, water and sea-ice clone libraries from the Ross sea, Antarctic[14].

In this research the phylogeny of dinoflagellates from different clone libraries of the both polar region ecosystems were analyzed. 37 positive clones of dinoflagellates were used for the phylogeny analysis.New information regarding biodiversity was found for these species based on 18S rRNA sequencing. Full–length sequences were used to examine the genetic diversity present in the polar regions.

The most common classification from the literature was adopted and updated according to recent findings. Increased SSU rRNA information can help to link the genotype of gymnodininoid dinoflagellates in polar regions with their ecology and potential seasonal importance in these extreme ecosystems.

1 Materials and methods Sampling and environmental data

Arctic samples were collected from the Kongsfjoden coastal ecosystem of the high Arctic in August, 2006. Related dinoflagellate clones were selected from different environments, such as glacial melt water and sea water. Libraries NPK2 (14 clones) and NPK60 (2 clones) of sea water were constructed from samples taken at depths of 2 m and 200 m from the same sea station (78°59.29′ N, 11°39.60′ E). Library NPK97 (7 clones) was constructed from glacial melt water sampled from Austre BrØggerbreen, one of the glacier surrounding Kongsfjorden, Svalbard (Figure1 A). Antarctic samples were collected from ice cores during the Australian sea–ice cruise in September, 2007. The ice samples used in this study were drilled from the scientific sea–ice cruise sampling stations during Aug – Oct 2007 (Figure1 B). Cores were immediately transferred to sterile plastic bags in which they were melted the same day at 4℃ in an equivalent volume of sterile seawater (Sigma Chemical Co., St. Louis, MO, USA). Samples were then filtered onto 0.2 μm pore size filters (Millipore) which were subsequently stored at –80℃ until processed.

Positive clones, SS7, SA7 and SB7 were collected from the same ice station (65°34' S, 121°31' E). SS7 (0.2–3 μm) and SA7 (≥ 3.0 μm) were enriched as different size partitions from bottom ice (0–5 cm of the ice core) (Table 1), SB7 with size of≥ 3 μm was enriched from the same ice core but from the 5–10 cm section. SA9 represented the size class of ≥3 μm from another station (65°20' S, 118°34' E).

Figure 1 Sample sites used in this study. (A) Kongsfjorden (Arctic) sample sites. NPK97 from glacial melt water was constructed from site I, and NPK2 & 60 of sea water were constructed from site II in Kongsfjorden (Arctic). (B) SIPEX Ice core sample sites (Antarctic). SS7, SA7 and SB7 were constructed from ice station 7, while SA9 was from ice station 9 during the Antarctic sea ice cruise.

Table 1 Stations, sample sites and temperature of water sample for the clone libraries

Clone library	Sample Site	Characteristics		Temperature	Sample time (local)
		sample	Size fraction		
NPK97	78°55.3′N, 11°48. 43 ′E	500 mL Glacial melting water	⩾ 0.2 μm	−2℃	25/08/2006
NPK2	78°59.29′N, 11°39.60′E	500 mL Sea surface water	⩾ 0.2 μm	8.5℃	22/08/2006
NPK60	78°59.29′N, 11°39.60′E	500 mL 200 m depth sea water	⩾ 0.2 μm	5.3℃	22/08/2006
SS7	65°34' S, 121°31 ' E	0–5 cm of the Ice core	0.2–3 μm	−15.1℃	22/09/2007
SA7	65°34' S, 121°31' E	0–5 cm of the Ice core	⩾3.0 μm	−15.1℃	22/09/2007
SB7	65°34' S, 121°31' E	5–10 cm of the Ice core	⩾ 3.0 μm	−15.1℃	22/09/2007
SA9	65°20' S, 118°34' E	0–5 cm of the Ice core	⩾ 3.0 μm	−12.9℃	28/09/2007

DNA extraction. Sample filters were thawed on ice. The microbial organisms were rinsed from the filters and then digested using lysozyme (final concentration, 1 mg/mL) and proteinase K (0.2 mg/mL). Lysates were recovered and nucleic acids extracted with phenolchloroform–isoamyl alcohol (25:24:1), followed by the chloroform–isoamyl alcohol (24:1) protocol.

DNA amplification, cloning and sequencing. Eukaryotic 18S rRNA genes were amplified using PCR with eukaryote–specific primers EukA and EukB[15]. Amplified rRNA gene products from several individual PCRs were pooled[16]. Polymerase chain reaction was performed with an initial 'hot start' for 10 min at 95℃, proceeded by 35 cycles at 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1 min; followed by a final extension at 72 ℃ for 15 min. 100 μl PCR products were cleaned using a Qiagen purification kit, and then cloned with the cloning kit (pGEM–T, Promega) following the manufacturer's directions. Almost complete 18S rDNA gene sequences were obtained from selected clones. Clones were grown overnight in SOC–Amp media, and plasmids isolated using the QIAprep kit (Qiagen).

Full length sequencing was done using an ABI 3730 Sequencer with four conserved primers: two internal to the PCR products (570 F: 5'–CCA GCA GCC GCG GTA ATT C–3'; 905 F: 5'–GTC AGA GGT GAA ATT CTT GG –3') and two targeted to the plasmid (M13F and M13R).

Phylogenetic analysis. The closest match to each sequence was obtained from NCBI blast. Poor–quality sequences and suspected chimeras were checked using BLAST with sequence segments separately and then using the Chimera check program of the Ribosomal Data Project II. The sequences that passed Chimeric screening were phylogenetically grouped and aligned using Clustal X v.1.83; alignments were manually checked using the 'multicolor sequence alignment editor' of Hepperle (2003)[17]. Some ambiguously aligned positions were removed manually. Phylogenetic analyses were conducted using various modules from the Phylip 3.62 package, using neighbor joining (NEIGHBOR) and maximum likelihood (mL). Support for trees was obtained by bootstrapping 1 000 datasets (NJ) and 100 datasets (mL).

Nucleotide sequence accession numbers. Gene sequences reported in this study have been deposited in the GenBank database, the related GenBank numbers are shown in Figure 2. The alignments are available from the authors upon request.

2 Results

Clone library NPK97 from glacial melt water was constructed from site I, and NPK2 & 60 from sea water were constructed from site II in Kongsfjorden, Arctic (Figure 1A). SS7, SA7 and SB7 were constructed from ice station 7, while SA9 was from ice station 9 on the SIPEX Antarctic Ice core sample sites during winter season (Figure1 B). Positive clone sequences of gymnodinioid dinoflagellates used into this study came from different environments in polar ecosystems (Table 1). The temperature of the sampling environments showed significant differences due to distinct sampling seasons. Molecular phylogenies were suggested from the data set of 18S rRNA with full length of 1 796 bp. The tree topology was obtained from maximum likelihood and neighbor– joining analysis using a data set of almost full length 18S rRNA. Bootstrap values of > 50% are indicated in Figure 2.

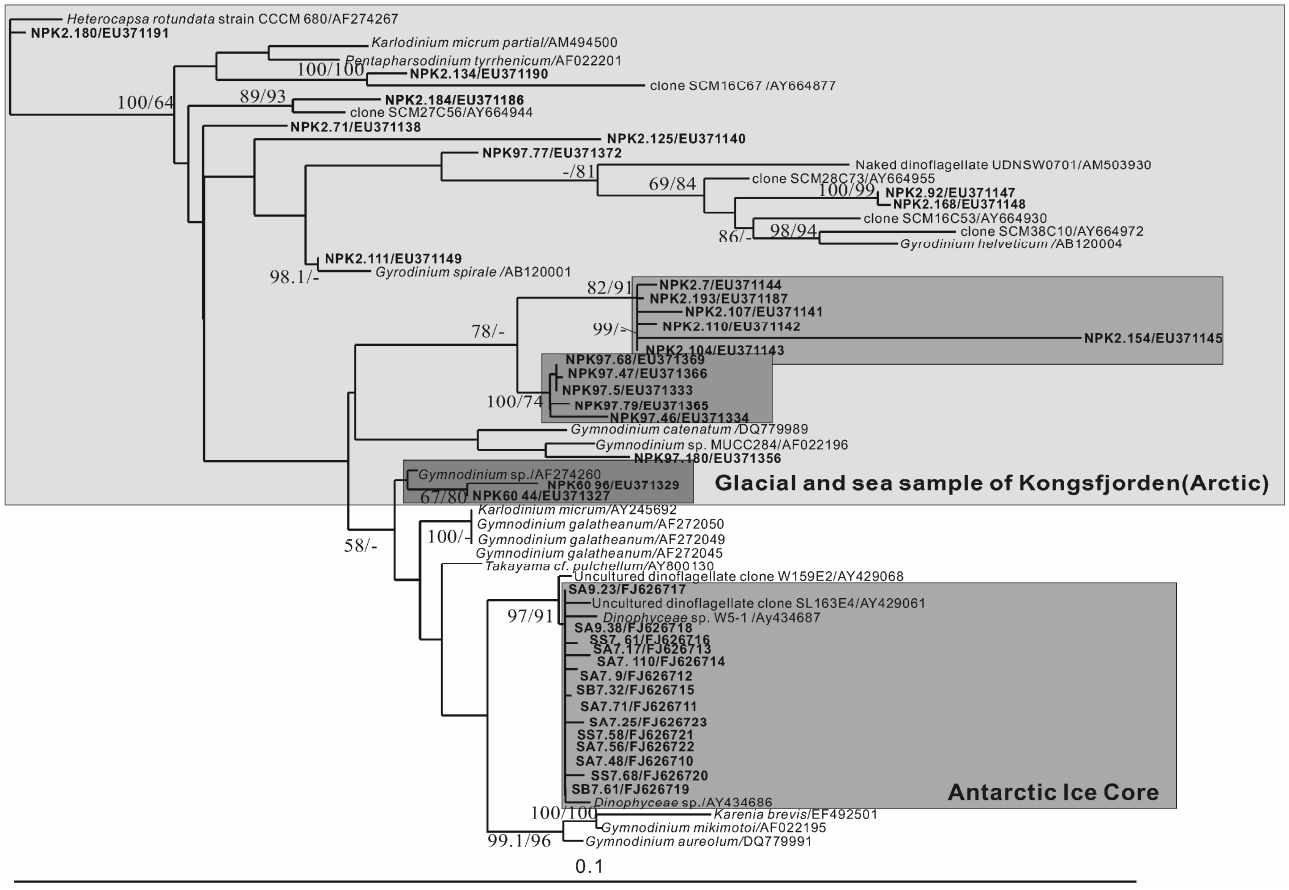

Figure 2 Phylogentic tree inferred using a maximum likelihood method based on a data set of 1796 aligned positions of 63 taxa. Bootstrap values (> 50%) of the maximum likelihood (n=100), neighbor–joining (n=1 000) were calculated and marked in the tree. The scale bars represent nucleotide substitution per site; the actual value depends on the branch lengths in the tree. The positive clones with deposited GenBank accession number (Bold) were sequenced in this study.

Diverse genotypes of dinoflagellates from Arctic samples were clustered, while only one typical Antarctic novel dinoflagellate clade of *Dinophyceae* sp. was traced (Figure 2). 23 gymnodinioid dinoflagellate related sequences were recovered from the Kongsfjorden libraries (Arctic). They were closely related to characterized dinoflagellates *Gymnodinium* sp., *Pentapharsodinium*, *Gyrodinium,* and *Karlodinium* (Table 2).

Moreover, the diverse phylotypes in Arctic samples were clearly confirmed by in-situ microscopic observation. Different morphotypes of the gymnodinioid dinoflagellates were shown using microscopic photography (Figure 3 A−F). Unidentified *Gymnodinium*, *Gyrodinium* and *Protoperidinium* species were often observed in this fjord. However, the single *Dinophyceae* sp. from Antarctic ice core samples, screened from two clone libraries, was observed as a cyst in the extreme low temperature of winter (Figure3 G−H). Unfortunately these dinoflagellates were not successfully cultured and thus further details such as pigment, toxicity and other descriptors of this isolate require further study.

3 Discussion

Dinoflagellates are dynamic components of plankton in some aquatic ecosystems[18], even in polar regions they can form blooms in suitable seasons. Dinoflagellates have very large genomes and there are potentially many more copies of their ribosomal genes than other microbial eukaryotes, which could skew clone library results toward predominance of dinoflagellate phylotypes. Nonetheless, due to lack of successful culturing, study of dinoflagellate biodiversity and biogeography using molecular tools can potentially give basic insight into processes like speciation, local adaptation and biogeography for these microorganisms.

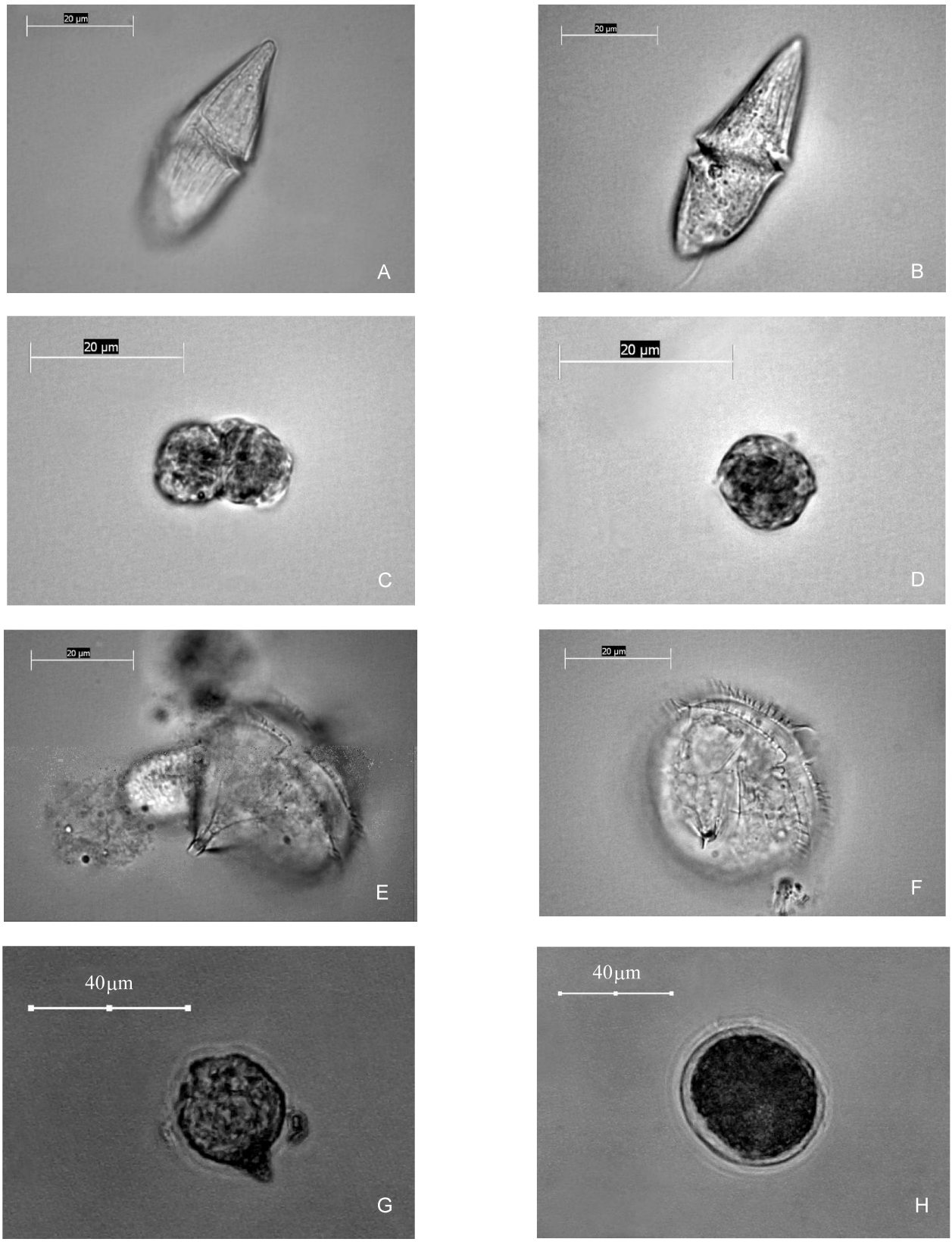

Figure 3 Three different morphotypes of gymnodinioid dinoflagellates were observed using microscopy from the sea water sample of Arctic Kongsfjorden (A–F) and Antarctic sea ice cruise (G–H). A–B: unidentified *Gyrodinium* species; C–D: unidentified *Gymnodinium* species; E–F: unidentified *Protoperidinium* species; G–H: novel *Dinophyceae* sp. in the clone library of SS7 from Antarctic sea ice cruise, Cyst in the extreme low temperature of winter.

Table 2 Clones used in this study

Clones of different genotype [a]	Closest match (accession no.)	Query/subject [b] (Maximum Identity)
SS7.58	*Dinophyceae* sp. RS–24 (AY434686)	1792/1798 (99%)
SA7.110	*Dinophyceae* sp. RS–24 (AY434686)	1792/1798 (99%)
SB7.32	*Dinophyceae* sp. RS–24 (AY434686)	1792/1798 (99%)
SA9.38	*Dinophyceae* sp. RS–24 (AY434686)	1790/1798 (99%)
NPK97.68	*Pentapharsodinium tyrrhenicum* (AF022201)	1757/1798 (97%)
NPK2.7	*Pentapharsodinium tyrrhenicum* (AF022201)	1756/1798 (97%)
NPK2.134	*Pentapharsodinium tyrrhenicum* (AF022201)	1761/1803 (97%)
NPK2.92	*Gyrodinium rubrum* (AB120003)	1706/1726 (98%)
NPK2.111	*Gyrodinium spirale* (AB120001)	1724/1725 (99%)
NPK97.77	*Gyrodinium spirale* (AB120001)	1720/1725 (99%)
NPK2.180	*Heterocapsa rotundata* CCCM 680 (AF274267)	1751/1752 (99%)
NPK2.71	*Karlodinium micrum* (AM494500)	1772/1806 (98%)
NPK2.125	*Gymnodinium* sp. MUCC284 (AF022196)	1743/1802 (96%)
NPK60.44	*Gymnodinium* sp. (AF274260)	1750/1755 (99%)

a Clone designations are from Table 1. *b* Ratio of sequence match length to length of the nearest matc.

A single dinoflagellate sequence type was prevalent in analyses of full–length nuclear small subunit ribosomal gene clone libraries from sea water and slush samples (snow melt–seawater mix from annual pack ice) collected during austral summer of 1999 from Ross sea, Antarctica. Phylogenetic analysis of the sequences indicated that they belonged to the novel dinoflagellate genus *Dinophyceae*, a sister taxon to the dinoflagellate genera *Karenia* and *Karlodinium* [14]. Libraries constructed from samples in the pack–ice had higher numbers of novel dinoflagellate phylotypes, comprising more than half of the total clones analyzed and sometimes almost all of the dinoflagellate clones [14]. Our libraries showed similar trends between the two stations in the winter season, all 14 of the positive clones were related to the novel dinoflagellate genotype. Occasionally traces occurred even in small size class filter samples (SS7, 0.2–3 μm).

Since *Dinophyceae* sp. is known to be larger than 3μm their presence in the fraction analyzed could be due to cell breakage during the filtering process. Microscopic observations suggested that they existed as cyst stage to survive the extreme low temperature (Figure 3 G and H).

However, this novel genotype has not been reported in Arctic libraries yet. It is thought to be an extreme low temperature genotype which can survive in freezing temperatures of < 0℃. The potential for toxin production of this novel dinoflagellate requires further research. *Polarella glacialis*, another small gymnodinioid dinoflagellate, has been found to be very abundant in the upper land–fast sea ice of Antarctica during the austral spring[7], where it could both grow and overwinter as a spiny encysted stage. However, this genotype was not found in this investigation of winter samples.

The identification of some borderline species of the dinoflagellate genera *Gyrodinium* and *Gymnodinium* has generated argument and confusion for decades[19]. The separation of *Gymnodinium* and *Gyrodinium* was artificially made based solely on the singular displacement, which should be more than 20% of the body length in the genus *Gyrodinium* and less than 20% for the genus *Gymnodinium* [20]. The genotype of *Gymnodinium* was distinguished into a seperate clade in this study (Figure 2). Taxa assigned to the genus *Gymnodinium* are known to be toxin producers of extreme importance to biomedicine. Around 14 clones from the Kongsfjorden (Arctic) clone libraries were highly *Gymnodinium* related, the culture and toxin detection for these Arctic genotypes has been requested. Others, such as NPK2.111 showed close affinity to *Gyrodinium* strains (Table 2).

The problem of bipolarity has been poorly investigated for planktonic algae and data for dinoflagellate distribution in the polar regions is similarly limited. The abundance of dinoflagellates in polar waters could be substantial and seasonal but polar expeditions and sampling are not consistent year to year. Genotype diversity based on limited samples from each polar region were used in this study, which traced genotypes of dinoflagellates using clone libraries. Further studies integrating approaches are required combining culturing and morphology, molecular phylogeny and quantitative ecology. This could help to investigate abundance of dinoflagellates in the extreme cold, polar ecosystems, especially during bloom seasons.

4 Conclusion

Dinoflagellates are an important component of polar microbial–ecosystems. From clone library analysis

dinoflagellates genotypes varied between and within poles in this study. Microscopic and genetic analysis suggested that significant biodiversity occurred during the summer-time in Kongsfjorden (Arctic) samples. Meanwhile, one single genotype of dinoflagellate adapted to extreme cold temperature was isolated from ice core samples, from the Antarctic cruise. Occasionally these specimens would be the only eukaryotic microbial genotype existing in the winter ice cores from the libraries of two stations of the Antarctic cruise. To obtain better understanding of their roles, quantitative measurement of free swimming and mixotrophic populations are required using molecular techniques combined with traditional protocols. This should increase our understanding of their ecological role in this perennially cold microbial ecosystem.

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