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ABUNDANCE OF A NOVEL DINOFLAGELLATE PHYLOTYPE IN THE ROSS SEA, ANTARCTICA[1]

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The biodiversity of protistan assemblages present in microhabitats of the Ross Sea, Antarctica, was examined using molecular biological methods to obtain a greater understanding of the genetic diversity present. Sequencing of 18S clone libraries indicated genetically diverse collections of organisms in the water column, ice, and meltwater layer (slush), but a single small subunit ribosomal DNA (srDNA) sequence type dominated clone libraries (>30%) from seawater and slush samples taken within the ice pack of this ecosystem. The BLAST searches indicated that this dominant clone was derived from a dinoflagellate, and that it shared sequence similarity (97.6%–98.3%) with both *Karenia* and *Karlodinium* species. Phylogenetic analyses based on small subunit ribosomal gene sequences supported its placement as a sister group to these taxa, and suggested that it represented a novel genus. The dinoflagellate was successfully recovered in culture, and morphological analyses have shown that it contains chloroplasts, is gymnodinoid, appears not to have thecal plates, and has an apical groove and sulcal structure that confirm its placement as a relative of the *Karenia/Karlodinium* group. The abundance of this phylotype in natural samples was confirmed by quantitative PCR analyses of water and slush communities, and suggests that this dinoflagellate can be a major constituent of the protistan assemblages of some Antarctic microhabitats of the Ross Sea.

Key index words: **Antarctica; molecular phylogeny; novel dinoflagellate; quantitative PCR; ribosomal DNA**

Abbreviations: **DCM, deep chl maximum; DGGE, denaturing gradient gel electrophoresis; EPCR, quantitative PCR; RDP, Ribosomal Database Project**

Understanding the structure and diversity of microbial communities in cold environments, and the adaptations that allow these assemblages to flourish near the lower limit of temperature tolerance for biological systems, is of fundamental importance to biological oceanography and to understanding the adaptation of life on our planet to extremely low temperature. Perennially cold ecosystems such as the Arctic, Antarctic and the deep-sea are often envisioned as harsh environments inhabited by relatively few species, occurring at very low abundances. This perception is not correct for marine microbial communities of coastal Antarctica (Garrison et al. 1986). Diverse and abundant assemblages of protists in the water surrounding the Antarctic continent have been described for more than two decades based on traditional microbiological approaches and microscopical analyses (Stoecker et al. 1992, 1993, 1995, Garrison and Gowing 1993, Palmisano and Garrison 1993, Dennett et al. 2001, Kang et al. 2001). Microalgae (phototrophic protists) are virtually the only primary producers in pelagic ecosystems of extremely cold environments including sea-ice communities (chroococcoid cyanobacteria are very rare; Garrison et al. 1986), and these assemblages often reach exceptionally high abundances during austral summer (Smith and Gordon 1997, Dieckmann et al. 1998). Protozoa (heterotrophic protists) are also plentiful in cold-water environments and appear to be active consumers of bacteria, eukaryotic algae and small protozoa (Garrison and Gowing 1993, Caron 1994, Sherr and Sherr 1994), as they are at lower latitudes. It has been documented that 33% to >80% of the biomass of protozooplankton during austral summer in the Weddell and Scotia Seas consisted of heterotrophic flagellates, and most of this biomass was heterotrophic dinoflagellates (Garrison and Gowing 1993).

Traditional microscopical approaches for documenting the diversity and abundances of protistan assemblages in the coastal regions around Antarctica have contributed greatly to our current understanding of Antarctic microbial food web structure and biogeochemical processes (El-Sayed and Fryxell 1993, Garrison and Gowing 1993). This work has been most informative for species possessing unambiguous morphological features, such as frustules or skeletons

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(e.g. diatoms, radiolaria, choanoflagellates). Yet, these approaches have not succeeded in fully characterizing the diversity or abundance of species that are present, or in identifying morphologically nondescript organisms. These shortcomings are due to the tremendous size range and morphological diversity among protists that necessitates the use of a variety of disparate approaches to identify them in natural communities (Gifford and Caron 1999). As a consequence, genetic and immunological approaches for the identification and quantification of protists have become common in recent years (Caron et al. 1999, 2004). Several recent manuscripts have described the diversity of small marine eukaryotes through srDNA cloning and sequencing (Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-Van der Staay et al. 2001). These studies have revealed a large diversity of ribosomal types, have identified novel ribosomal lineages within the microbial eukaryotes, and indicate that important components of the microbial diversity in environmental samples have remained undetected using traditional methods.

Most of these molecular studies have not yet linked DNA sequence information with the corresponding morphological descriptions of phylotypes, and thus the function of many of these entities remains unknown. We approached the characterization of Antarctic marine microbial eukaryotic communities with this in mind, applying molecular techniques to differentiate taxa based upon their small subunit ribosomal gene sequences, along with traditional enrichment culture techniques to link morphologic and physiologic features with genetic identification.

Our study focused on examining the genetic diversity (i.e. sequencing of small subunit ribosomal RNA genes; 18S) of microbial eukaryotes less than 200 μm in size in Antarctic seawater, slush (snow melt and seawater that percolates up through fissures in the sea-ice and pools at the interface of ice and snow), and ice cores. In addition to a range of diverse phylotypes, we found that one sequence type in particular occurred in high numbers in the slush and water clone libraries, but not in the ice libraries. This observation suggested that the organism might be abundant in the former environments, and we subsequently developed and applied a quantitative PCR (qPCR) method to substantiate our results obtained from clone libraries. We were also successful in establishing several cultures that had the same ribosomal sequence type, permitting a preliminary morphological identification of the dinoflagellate. Despite the apparent prevalence and relatively wide spatial and temporal distribution of this organism, it has not been formally described in the literature. It is likely represented by the prevalent 'gymnodinoid dinoflagellate' that has been noted by other researchers (Kang et al. 2001; P. Assmy and J. Henjes, AWI Bremerhaven, personal communication). Our ribosomal sequence information and preliminary morphological analysis indicate that this dinoflagellate represents a new genus and is a close relative of the toxic genera, *Karenia* and *Karlodinium*.

MATERIALS AND METHODS

Sample collection. Seawater, sea-ice, and slush samples were collected in the Ross Sea, Antarctica, during the austral summer of 1999 (1 January–2 February 1999) onboard the RVIB Nathaniel B. Palmer (cruise NBP 99-01). Samples analyzed in this study were collected at eight stations within the pack-ice along a north-south transect at 150° W between 73° and 65° S, and also at one station near the Antarctic continent (Fig. 1), for both molecular analyses and establishment of enrichment cultures. Sampling stations within the pack-ice were separated by approximately 1° latitude (60 nautical miles). Water samples at each location were collected in Niskin bottles from several depths between the surface and the deep chl maximum (DCM; usually between 30 and 60 m) and combined for analysis. Slush was collected from areas discolored by microbial biomass (red/brown or green) by scooping the material with sterile containers. Ice cores were collected using motorized Sipre corers, and sections showing color were recovered for analysis. Slush and ice samples were brought back to the ship and allowed to melt slowly at approximately 4° C. All samples were then prefiltered through 200 μm mesh Nitex® (Wildlife Supply Company, Buffalo, NY, USA) to remove most metazoan plankton. Protists were collected by filtration onto 0.8 μm polycarbonate filters. Typically, 12 L of seawater, 200–500 mL of slush or melted ice (depending upon the sample volume and the biomass), or 10 mL of cell culture were collected for nucleic acid extraction. All filtration was carried out at 2° C in a walk-in cold room. The DNA was extracted from cells immediately after filtration.

Enrichment culture. Enrichment cultures were established by inoculating *f*/2 + silica (Guillard 1975) for phytoplankton or 0.01% yeast extract (in sterile seawater) for phagotrophic protists with 1 mL of Antarctic water, slush or ice sample, and incubating at 0–1° C in lighted incubators (12:12 L:D). Samples were collected and enriched from two separate cruises, one in 1997 and the other in 1999. The cultures subsequently identified as the dinoflagellate observed as the dominant phylotype in clone libraries (see Results) are currently growing as mixed enrichment cultures in lighted incubators (12:12 L:D) kept at 0–1° C. The mixed enrichments are maintained in inorganic medium (f/2 + silica; Guillard 1975) at salinities ranging from 30 to 36 psu. Micropipetting individual cells into fresh media generated clonal cell cultures for DNA analysis.

DNA extraction. Nucleic acids were extracted by a modified combination of hot detergent lysis and mechanical disruption (Kuske et al. 1998, Gast et al. 2004). The 2× lysis buffer (100 mM Tris pH 8.0, 40 mM ethylenediaminetetraacetic acid, 100 mM NaCl, 1% sodium dodecyl sulfate) was preheated to 70° C and 200 μL was added to the filter in a 5 mL Nunc™ tube. Approximately 200 μL of zircon beads (0.55 mm; BioSpec Products Inc, Bartlesville, OK, USA) were added, and the tube was vortexed for 1 min. The sample was incubated at 70° C for 5 min, then vortexed for 1 min. The incubation/vortexing was repeated twice. Sodium chloride was added to bring the solution to 0.7 M, and 10% hexadecyltrimethyammonium bromide (CTAB; Sigma, St. Louis, MO, USA) was added to a final concentration of 1%. The solution was then incubated at 70° C for 15 min. An equal volume of chloroform was added, the sample was vortexed and centrifuged, and the aqueous layer removed to a sterile 1.5 mL microcentrifuge tube. Nucleic acids were precipitated by the addition of 0.6 volumes of isopropanol and recovered by centrifugation. The nucleic acid pellets were allowed to air dry briefly and then resuspended in 50–100 μL of sterile Milli-Q water.

PCR amplification. One microliter of nucleic acid extract (25–400 ng) was used as the template in PCR amplification of the small subunit ribosomal gene (Saiki et al. 1988). Standard

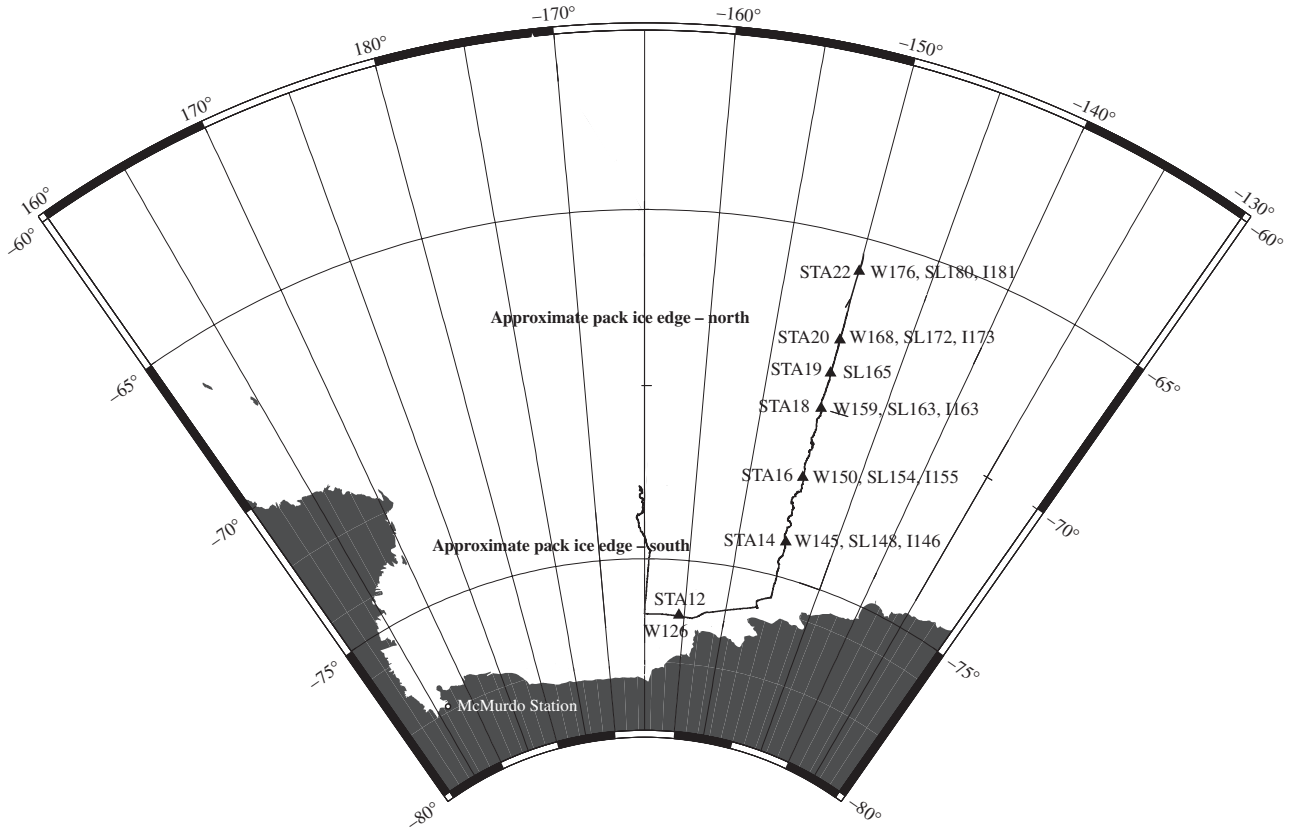

FIG. 1. Cruise track from a portion of NBP 99-01 ice cruise, January–February 1999. Triangles indicate the stations where samples discussed in this manuscript were collected. Letters indicate the type of sample (W, water; I, ice; SL, slush); numbers are individual sample indicators. The map was generated by Antarctic Support Associates as part of cruise data.

50 µL reactions were assembled using eukaryotic-specific srDNA primers A and B (Medlin et al. 1988) and amplified with 30–35 cycles of 45 s at 95° C, 45 s at 42° C, and 3 min at 72° C, followed by a final extension at 72° C for 7 min. Products were visualized on 1% agarose gels stained with ethidium bromide.

Clone library generation. The PCR-amplified srRNA genes (~1.8 to ~2.0 kb) were excised from crystal violet-stained agarose gels following the manufacturers instructions in the TOPO® XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). Purified amplification products were ligated into the linearized plasmid vector pCR®-XL-TOPO® (Invitrogen) and used to transform competent One Shot® TOP10 *Escherichia coli* cells (Invitrogen). Transformants were grown overnight at 37° C, and positive transformants were selected and grown as miniprep cultures in 96-well blocks. Libraries of approximately 96 clones each were picked for each water, slush, and ice sample (except SL165) collected from the indicated sites (Fig. 1). Plasmid DNA was recovered from bacterial cells using the R.E.A.L.™ Prep 96 Kit (Qiagen, Valencia, CA, USA) and a QIAGEN BioRobot™ 9600. Ribosomal gene clones for dinoflagellate cultures were generated following the same amplification protocol, but purified products were ligated into pGEM®-T Easy Vector System (Promega, Madison, WI, USA) following instructions and colonies were picked for miniprep screening (Wizard® MiniPrep Kit, Promega).

Sequence Analysis. Clones from seawater (six libraries, 482 clones total), slush (five libraries, 430 clones total) and sea-ice (five libraries, 466 clones total) libraries were screened initially by sequencing near the 5′ end of each clone using the eukaryote rRNA-specific primer 373 (Weekers et al. 1994). Sequencing reactions were run in a total volume of 10 µL consisting of 1–2 µL of plasmid, 2 µL ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), 1 µL primer (100 ng/µL) and dH_2O. Cycle sequencing parameters were 5 min at 95° C, followed by 25 cycles of 96° C for 30 s, 50° C for 15 s, and 60° C for 4 min. Completed reactions were run on an ABI 377 automated sequencer (Applied Biosystems) resulting in read lengths of ~500 basepairs (bp). All sequences were assembled and edited using the Sequencher™ 4.1 editing program (Gene Codes Corporation, Ann Arbor, MI, USA). The BLAST analysis (Altschul et al. 1997) indicated the prevalence of a narrow range of phylotypes among the water and slush clone libraries that grouped within the dinoflagellates, prompting us to obtain more information on this phylotype. Full-length sequences of the novel dinoflagellate ribosomal gene were obtained for 20 selected clones, 10 each from the water and slush libraries at station 18, using internal eukaryote-specific primers (Gast et al. 1996). Additional full-length sequences were obtained from two clonal dinoflagellate cultures from our Antarctic protistan culture collection (http://www.whoi.edu/science/B/protists). The BLAST (Altschul et al. 1997) searches were conducted on full-length sequences to refine potential taxonomic relationships, and Chimera Check (Ribosomal Database Project (RDP) II; http://rdp.cme.msu.edu) was used to check for possible chimeric sequences.

Phylogenetic analysis. Full-length sequences were aligned with other dinoflagellate ribosomal genes from GenBank

(Bilofsky and Burks 1988) using the GCG Wisconsin Package™ SeqLab® v.10 (Genetics Computer Group). The reference sequences used in the analysis were *Pentapharsodinium tyrrhenicum* (AF022201), *Pentapharsodinium* sp. (AF274270), *Gyrodinium galatheanum* (AF274262), *Karlodinium galatheanum* GE-2 (AF272050), *K. galatheanum* GE (AF272046), *K. galatheanum* (AF272049), *K. galatheanum* (AF272045), *Gymnodinium mikimotoi* (AF022195), *G. fuscum* (AF022194), *Gymnodinium* sp. (AF022196), *Heterocapsa triquetra* (AF022198), *Heterocapsa niei* (AF274265), *Heterocapsa pygmaea* (AF274266), *Heterocapsa rotunda* (AF274267), *Karenia brevis* (AF274259), and *Gymnodinium breve* (AF172714). Note that the sequences for *K. brevis* and *G. breve* were obtained from different clones of the same species (Tengs et al. 2000, Saldarriaga et al. 2001). The data set for 33 taxa contained a total of 1650 characters, of which 1454 were constant, 114 were variable, but parsimony uninformative, and 82 were parsimony informative. Modeltest 3.6 (Posada and Crandall 1998) was used to determine that the optimal parameters for maximum likelihood analysis were TrN + I + G (Tamura Nei model of evolution with a proportion of invariant sites and γ distribution). Aligned sequences were analyzed using PAUP* 4.0b10 (Swofford 1999) with the parameters Nst = 6, $R(AC) = 1$, $R(AG) = 2.7323$, $R(AT) = 1$, $R(CG) = 1$, $R(CT) = 5.1207$, $R(GT) = 1$, base frequency $A = 0.2611$, $C = 0.2053$, $G = 0.2584$, $\gamma = 0.7207$, and pinvar = 0.3902. One thousand bootstrap replicates were run using the heuristic search in parsimony, where gaps were treated as missing data, the starting trees were obtained with simple stepwise addition, and tree-bisection-reconnection was used in branch swapping. All resulting trees were unrooted, but were drawn with respect to *Gymnodinium fuscum* as the outgroup.

qPCR. A primer specific for the srDNA of the novel dinoflagellate (RSdino1B; 5′ CTCCCGGGCAAGGTCGTA) was designed based upon 17 full-length sequences that were less than 1% different from each other in base composition. The GenBank and RDP comparisons of this sequence show no fully homologous matches in the database (closest match was 15 out of 18 bases). The specific primer RSdino1B was utilized for qPCR amplification in conjunction with the eukaryotic ribosomal gene primer 1055F (5′ GGTGGTGCATGGCCG). The size of the amplified fragment was 227 bp and the optimal annealing temperature for this primer pair was determined to be 60° C using the gradient function on an Eppendorf® Mastercycler® PCR machine (Brinkmann Instruments, Eppendorf, Westbury, NY, USA). Samples for standard curves of cell number equivalents were generated using 10-fold serial dilutions of DNA extracted from a known number of cells collected from novel dinoflagellate cultures. We made serial dilutions of DNA after a single extraction rather than of diluted cells to generate a consistent standard of cell number. Amplification products were quantified using the IQ™ SYBR Green Supermix system (BioRad Laboratories Inc, Hercules, CA, USA). The PCR amplification reactions were run in triplicate on an iCycler Thermal Cycler with the Optical System for real-time PCR (Bio-Rad Laboratories Inc.) detection and were accomplished using 50 cycles of 95° C for 30 s, 60° C for 30 s, and 72° C for 1 min, followed by melt curve analysis and then a hold at 4° C. Cell numbers per liter were calculated by determining the number of cells per reaction (1 μL of template DNA), multiplying that by the total extraction volume and dilution factors to determine the number of cells in the whole extraction, then dividing by the original volume of sample extracted. Some of the original samples used for library construction were not available for the qPCR as they had been used up in previous analyses (denaturing gradient gel electrophoresis (DGGE), cloning), and we limited our study to analysis of the subset that was available. Triplicate reactions were set up using DNA from the same extracts, which should allow assessment of the error associated with the PCR setup and amplification process. Samples were not available to test the variability associated with different extractions of the same sample.

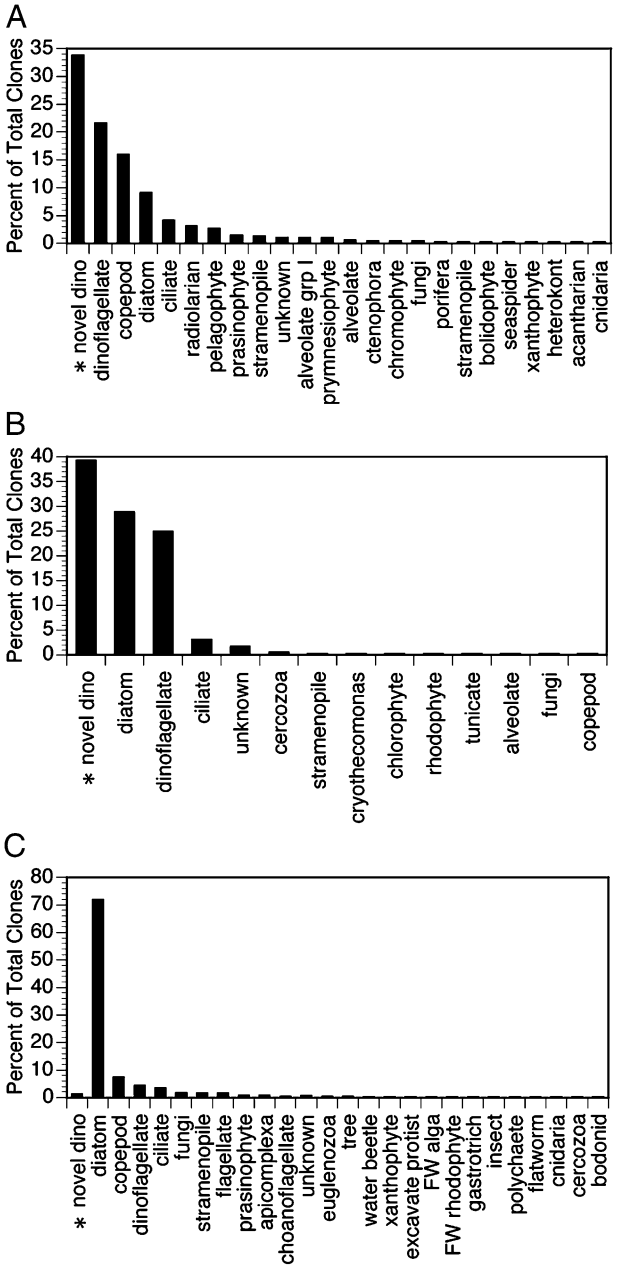

FIG. 2. Clone prevalence in ribosomal libraries. (A) Combined water libraries. Six libraries were sequenced for a total of 482 partial ribosomal sequences. BLAST results indicated that 34% of the total number of clones in the library was represented by the novel dinoflagellate. (B) Combined slush libraries. Five libraries yielded a total of 430 partial sequences, with the novel dinoflagellate representing 39% of those clones. (C) Combined ice libraries. Five libraries resulted in 466 partial sequences, of which the novel dinoflagellate represented about 1% of the total clones. Asterisks indicate the novel dinoflagellate group.

RESULTS AND DISCUSSION

Clone library analysis. We analyzed clone libraries generated for water, slush, and ice along the transect

TABLE 1. Calculated percent differences between small subunit ribosomal gene sequences from novel dinoflagellate clones and dinoflagellates from the database.

	Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*Gymnodinium mikimotoi*	0.00																				
2	*Pentapharsodinium tyhrrenicum*	1.51	0.00																			
3	*Karenia brevis*	0.24	1.63	0.00																		
4	*Gyrodinium galatheanum* (*Karlodinium micrum*)	1.99	2.05	1.99	0.00																	
5	W159A10	2.17	1.69	2.11	2.41	0.00																
6	W159B4	2.13	1.69	2.17	2.35	0.97	0.00															
7	W159C1	2.11	1.63	2.11	2.35	0.90	0.90	0.00														
8	W159C7	2.13	1.69	2.13	2.35	0.97	0.97	0.90	0.00													
9	W159D10	2.11	1.63	2.11	2.35	0.90	0.90	0.84	0.90	0.00												
10	W159E2	1.93	1.33	1.93	2.05	0.84	0.84	0.78	0.84	0.78	0.00											
11	W159F8	1.75	1.27	1.75	1.99	0.54	0.54	0.48	0.54	0.48	0.42	0.00										
12	W159G6	1.75	1.27	1.75	1.99	0.54	0.54	0.48	0.54	0.48	0.42	0.12	0.00									
13	W159H8	1.93	1.45	1.93	2.17	0.72	0.72	0.66	0.72	0.66	0.60	0.30	0.30	0.00								
14	SL163A10	1.99	1.39	1.99	2.11	0.78	0.78	0.72	0.78	0.72	0.54	0.36	0.36	0.54	0.00							
15	SL163B6	1.99	1.39	1.99	2.11	0.78	0.78	0.72	0.78	0.72	0.54	0.36	0.36	0.54	0.48	0.00						
16	SL163B11	1.99	1.39	1.99	2.11	0.78	0.78	0.72	0.78	0.72	0.54	0.36	0.36	0.54	0.48	0.48	0.00					
17	SL163C8	2.11	1.63	2.11	2.35	0.91	0.91	0.84	0.91	0.84	0.78	0.48	0.48	0.66	0.72	0.72	0.72	0.00				
18	SL163E4	1.99	1.39	1.99	2.11	0.78	0.78	0.72	0.78	0.72	0.54	0.36	0.36	0.54	0.48	0.48	0.36	0.72	0.00			
19	SL163H12	1.87	1.39	1.87	1.99	0.78	0.78	0.72	0.78	0.72	0.54	0.36	0.36	0.54	0.48	0.48	0.48	0.72	0.48	0.00		
20	RS-24 culture	1.76	1.15	1.76	1.88	0.55	0.55	0.48	0.55	0.48	0.30	0.12	0.12	0.30	0.24	0.18	0.24	0.55	0.24	0.24	0.00	
21	W5-1 culture	1.82	1.21	1.82	1.94	0.61	0/61	0.54	0.61	0.54	0.30	0.18	0.18	0.36	0.30	0.30	0.30	0.55	0.30	0.30	0.06	0.00

The number of base differences between two sequences were divided by the length of the shorter one, without gaps.

shown in Figure 1 by 5′ end sequence analysis. Greater than 34% of the total clones from the water clone libraries were represented by a single phylotype that had greater than 99% sequence similarity, while 39% of total slush clones were also represented by this phylotype (Fig. 2). This sequence type occurred very rarely in the ice libraries. The lack of this sequence type in the ice libraries could be due to the melting strategy used. The cells could have suffered lysis because of osmotic shock by not having a large volume (1 L) of sterile seawater added to the melting core sections. In previous work, we did not see a significant difference in DGGE patterns from ice cores melted in both ways (Gast et al. 2004), so it seems reasonable to conclude that the novel dinoflagellate is probably not a large component of the ice community. Although this novel phylotype was abundant in combined analyses, it actually had a gradient of distribution in the individual libraries. The BLAST (Altschul et al. 1997) searches with consensus sequences derived from these alignments identified the sequence as being from a dinoflagellate.

TABLE 2. Quantitative PCR results for the novel dinoflagellate abundance in Ross Sea environmental samples.

Sample	Original sample volume (L)	Resuspension volume (μL)[a]	Amplification dilution[b]	qPCR cell number[c] (cells $\cdot L^{-1}$)	% total clones in individual library that are novel dinoflagellate
W145 (Sta 14)	9 L	100	1:1	90 (± 25)	28
W150 (Sta 16)	12 L	100	1:100	29,000 (± 1900)	60
W168 (Sta 20)	6 L	50	1:1	240 (± 11)	36
W176 (Sta 22)	4.5 L	50	1:1	1 (± 0.3)	6.4
SL148 (Sta 14)	1.5 mL	100	1:1	890,000 (± 100,000)	22
SL154 (Sta 16)	60 mL	50	1:100	390,000 (± 220,000)	26
SL165 (Sta 19)	250 mL	50	1:1	7500 (± 420)	60
SL172 (Sta 20)	400 mL	50	1:1	2100 (± 550)	15
SL180 (Sta 22)	250 mL	50	1:1	10,000 (± 660)	25

[a]The resuspension volume for extracted DNA was based upon the general size of the nucleic acid pellet, to provide a concentration generally favorable for amplification.

[b]Some environmental nucleic acid extracts were diluted to bring their values within the range of other samples in the same qPCR experiment.

[c]The cell number value is the arithmetic mean from triplicate reactions. The cell number per reaction was multiplied by the extraction volume and by the dilution factor (if used) to determine cell number per extraction. This number was then divided by the original sample volume to determine cell number per liter. Numbers in parentheses represent the standard deviation for the triplicate reactions.

qPCR, quantitative PCR.

We also extracted DNA from clonal dinoflagellate cultures established from water and slush samples, and the small subunit ribosomal gene were amplified, cloned, and sequenced. Three of these cultures yielded sequences with greater than 99% similarity to the novel dinoflagellate clones from the libraries, indicating that we had successfully established this organism in culture. The small subunit ribosomal genes from two of these cultures were completely sequenced (RS-24 and W5-1) and found to be identical. The identical nature of the ribosomal sequences from the two clonal cultures is noteworthy because one was recovered from enrichment cultures collected in the Ross Sea as part of a 1997 Joint Global Ocean Flux Study program, and the other from water collected in association with the 1999 cruise described in this study. We also found that one of the partial sequences recovered by Lopez-Garcia et al. (2001;DH148-5-EKD46/AF290062.1) from the Antarctic Polar Front (59° 19′ 48″ S, 55° 45′ 11″ W) was very similar (96.7%) to our dinoflagellate sequence, but we did not use it further in our analysis because it was not complete.

Full-length srDNA sequences were obtained for 20 of the novel phylotype clones (10 each from the station 18 water and slush libraries) to examine the extent of variation within this dinoflagellate clone group. Four clones from the slush library and one from the water library were determined to be potentially chimeric by analyzing the sequences with Chimera Check at the RDP II (http://rdp.cme.msu.edu). The occurrence of chimeric sequences in clone libraries generated from PCR-amplified fragments has been documented previously (Wang and Wang 1997, Judo et al. 1998). Factors implicated in chimera formation during PCR amplification include short elongation time, slow annealing time, and too many cycles. Our amplification protocol had a long elongation time and a short annealing step, but used 30–35 cycles, which is within the range where increased likelihood of chimera formation has been noted. Therefore, the identification of several potentially chimeric clones was not unexpected. These clones were eliminated from further analyses and have not been submitted to GenBank. The non-chimeric nature of our other clones was established by Chimera Check and by comparison with a full-length sequence from the cultured dinoflagellate. A total of 17 full-length sequences belonging to the novel dinoflagellate clone group (15 sequences from our clone libraries and two sequences obtained from cultures) have been deposited in GenBank (Bilofsky and Burks 1988) under the accession numbers AY429057–AY429071, AY434686 and AY434687.

The percent differences between aligned clone sequences of the novel dinoflagellate are listed in Table 1, along with a comparison to their "closest" relatives, *P. tyrrhenicum* (AF022201), *Karenia brevis* (AF274259), *Gymnodinium breve* (AF172714), *G. mikimotoi* (AF022195), and *Gyrodinium galatheanum* (AF274262). The full-length sequences from the novel dinoflagellate clones (~1800 bp) were generally greater than 99.5% similar to each other, although there were instances where the similarities between two sequences were as low as approximately 99%. Overall, the clones were more similar to each other than they were to any of the other dinoflagellate sequences. Differences between novel dinoflagellate clone sequences ranged from 0 to 5 bases between compared pairs of sequences, which is within the number likely to occur between strains of the same species (Haywood et al. 2004). Because we are examining environmental sequences in these libraries, it is also possible that some of the minor sequence variation arose from multiple copies of the ribosomal gene within a single cell. Although the novel dinoflagellate clone sequences appeared to have greater similarity to *Pentapharsodium* (98.3%–98.7%) than to *Karenia* or *Karlodinium* (97.6%–98.3%), the nucleotide differences with *Pentapharsodium* occurred in phylogenetically significant positions resulting in the placement of the novel dinoflagellate phylotype as a sister taxon to *Karenia* and *Karlodinium* rather than to *Pentapharsodinium*.

Phylogenetic reconstructions. The taxonomic affinities of the novel dinoflagellate were further examined using complete srDNA sequences to perform phylogenetic reconstructions. Initially, reconstructions were accomplished using most of the dinoflagellate sequences available in the GenBank database (Bilofsky and Burks 1988), but resolution of the branch order within the region of the tree where the novel dinoflagellate occurred was difficult to determine with confidence. This is a region of the tree that has previously been shown to be difficult to resolve in dinoflagellate srDNA phylogenies (Saldarriaga et al. 2001).

Subsequent reconstructions were then limited to representatives of the closest relatives to the novel dinoflagellate and the novel dinoflagellate clones in an attempt to more robustly determine the placement of these sequences (Fig. 3). Maximum likelihood reconstructions indicate that the novel dinoflagellate clones form a separate lineage from any previously sequenced dinoflagellates, and that the group clusters most closely with the *Karenia/Karlodinium* genera.

General morphology of the novel dinoflagellate. The novel dinoflagellate contains chloroplasts and coloration typical of phototrophic dinoflagellates, although we have information that indicates this is actually the result of kleptoplasty (unpublished data). The organism exhibits a solitary motile vegetative stage (Fig. 4A), a temporary cyst (Fig. 4B), a resting stage (Fig. 4C), and a dormant cyst stage (Fig. 4D). All non-motile cell stages can be covered by a heavy mucilaginous layer, which can be seen in Fig. 4B, C. Vegetative cell size ranges from 8 to 20 μm width and from 14 to 28 μm length in both mixed enrichment and clonal cultures. The dinokont cell has a conical epicone, a hemispherical hypocone, and appears to be unarmored. Cells have a displaced cingulum that is about two times its width, a sulcal extension that appears to invade the epitheca, and an

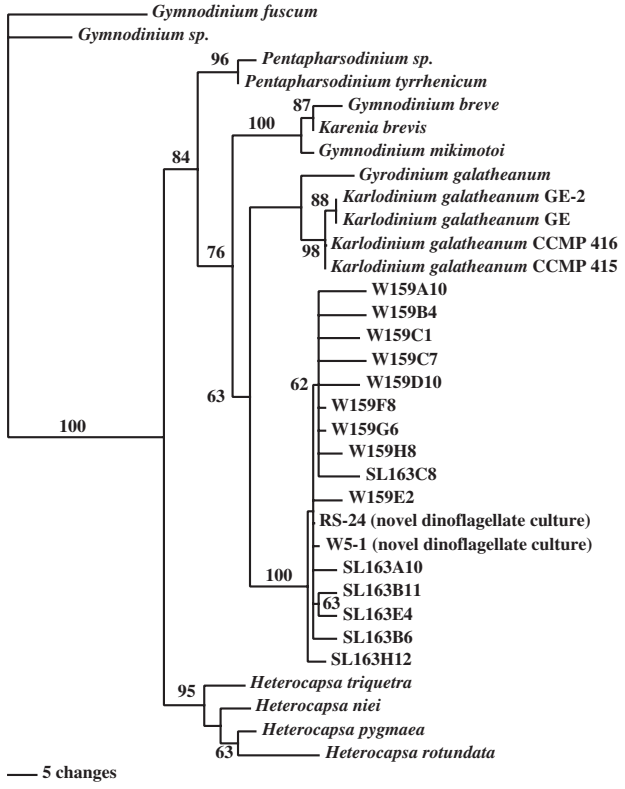

FIG. 3. Maximum likelihood phylogenetic reconstruction of novel dinoflagellate sequence taxonomic affiliations. Numbers are percentages that represent the support of each node based upon 1000 maximum parsimony bootstrap replicates. The tree is unrooted, but was drawn with respect to the outgroup containing *Gymnodinium fuscum* and *Gymnodinium* sp.

apical groove that is present at the front and back of the cell (SEM; Fig. 4E, F). We have not observed an apical pore. The temporary cysts were found to contain from 1 to 19 cells that range from 18 to 22 μm width and from 20 to 37 μm length (Fig. 4B). These clusters appear to be a normal condition of the organism and have been observed in natural water samples from the Antarctic with similar numbers of cells per colony (Rebecca J. Gast Dawn M. Moran, David J. Beaudoin, Jonathan N. Blythe, Mark R. Dennett, and David A. Caron personal observations; P. Assmy and J. Henjes, AWI Bremerhaven, personal communication). Despite this conspicuous clustered life stage, the organism is generally difficult to identify in natural samples because of its unremarkable gymnodinoid shape, and in prior morphological studies it has likely been grouped with other phototrophic gymnodinoid dinoflagellates with unresolved morphologies.

Distribution and prevalence of the novel dinoflagellate sequence type. The combined results from the analysis of the libraries along the entire cruise transect illustrated both the conspicuous contribution of dinoflagellates to the clone libraries throughout the study area, as well as the highly variable contribution of the novel dinoflagellate to the total number of clones obtained in the study (Fig. 2, Table 2). The novel dinoflagellate sequence was present in all the clone libraries generated from both water and slush, but it constituted a highly variable percentage ($<10\%$ to $>70\%$) of the clones obtained among the different stations. The novel dinoflagellate phylotype clones were often more abundant from samples toward the middle of the transect, whereas the contribution of this phylotype was reduced in the open water areas of the transect (Fig. 1, Table 2). The diminished contribution of the dinoflagellate was most evident in the samples collected near the Antarctic continent or the northern edge of the pack-ice, in which the novel dinoflagellate constituted less than 10% of the total clones. Libraries constructed from samples collected further into 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.

We desired to determine whether the overwhelming abundance of novel dinoflagellate clones in our seawater and slush libraries reflected relative abundances of the organism in those environments. Clone libraries are subject to biases introduced through nucleic acid isolation, PCR amplification, and cloning, that could result in the prevalence of a particular sequence type that is not indicative of its abundance in the original sample. Dinoflagellates have very large genomes, and potentially many more copies of their ribosomal genes than other microbial eukaryotes (Cavalier-Smith 1985), which could also skew clone library results toward predominance of dinoflagellate phylotypes. The qPCR analyses of cellular abundance using SYBR green incorporation were carried out using standards generated from a known number of cultured novel dinoflagellates. Thus, our qPCR results were correlated with the cell number of the organism rather than the copy number of the ribosomal repeats.

The error reported in parentheses below the cell number in Table 2 was associated with the qPCR reaction itself and not with extraction variation within a sample. The standard deviation was determined for each set of triplicate reactions set up from the same DNA extract. In most cases the variability between the triplicates was fairly low and less than or equal to 11% of the estimated cell number, but a few samples showed much higher deviation (SL154 at 54%; W176 at 30%; SL172 at 26%; W145 at 27%). This suggests that there might have been problems in setting up the individual reactions, or that there were factors associated with those samples that might cause the PCR to be variably efficient. In the case of the two water samples, overall there appear to be very low numbers of the dinoflagellate, which might result in greater inconsistency in aliquoted reactions. For the slush samples, SL154 was from a dense community while SL172 was not, but both had reasonably significant numbers of the dinoflagellate present. The variation in these reactions

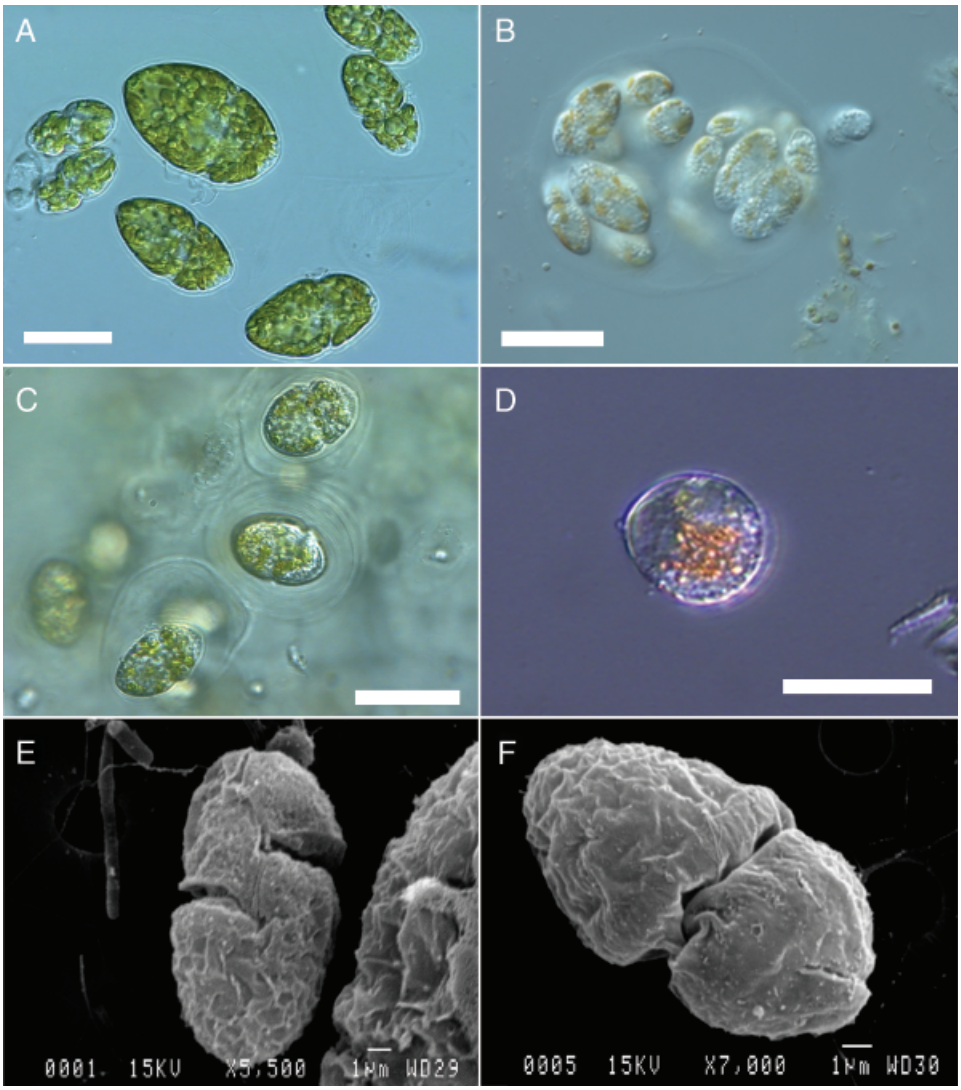

FIG. 4. Light (A–D) and SEM (E and F) images of novel dinoflagellate culture. (A) Motile, vegetative cells of the novel dinoflagellate. Small and large cells are present in the sample. Scale bar, 20 μm. (B) Temporary cyst. Scale bar, 25 μm. (C) Resting state with extensive mucilage that forms around the cells. Scale bar, 30 μm. (D) Cyst. Scale bar, 25 μm. (E) SEM image of the front of a vegetative cell. Scale bar, 1 μm. (F) SEM image of the back of a vegetative cell. Scale bar, 2 μm.

could result from inhibitory substances that were present or even from a very high amount of background DNA (in the case of SL154). Overall, we would like to emphasize that these qPCR values represent the general abundance of the novel dinoflagellate, not absolute numbers.

Our analyses indicated that the novel dinoflagellate was present in some water and slush samples at very high levels (Table 2). In the water samples tested, we obtained cell abundances ranging from 1 to 29,000 cells $\cdot$ L^{-1}. These abundances correlate fairly well with the abundances of unidentified gymnodiniod dinoflagellates in the Bransfield Straight (Kang et al. 2001). In 1995, ''gymnodinoid dinoflagellates'' less than 20 μm in size comprised 30% of the autotrophic flagellate biomass, at numbers ranging from 5000 to 200,000 cells $\cdot$ L^{-1}.

Numbers exceeding 300,000 cells $\cdot$ L^{-1} within the meltwater communities of the pack-ice (samples SL148 and SL154; Table 2) indicate the major contribution that the novel dinoflagellate made to this microhabitat of the pack-ice ecosystem of the Antarctic. Slush, or meltwater, is a very active environment, often containing very high numbers of total microbial cells and often showing dominance by a single taxon of protist. The novel dinoflagellate was found to occur at levels of 2100–890,000 cells $\cdot$ L^{-1} in these samples. This later value is comparable to exceptional bloom conditions for natural abundances of dinoflagellates.

A comparison of the prevalence of the novel dinoflagellate phylotype in clone libraries with abundances obtained using the qPCR method indicated a poor correlation between these measurements. For example, the percent of novel dinoflagellate clones in the

library obtained from SL148 was only 22%, yet the number of cells per liter determined by qPCR was extremely high (890,000). The qPCR analysis, when standardized against cell number, provides an estimate of *absolute* abundance of the phylotype, irrespective of the abundance of other eukaryotes. In contrast, phylotype prevalence in our clone libraries is a measure of the abundance of the phylotype *relative* to other recovered eukaryote phylotypes. The discrepancy observed for sample SL148 may indicate that the novel dinoflagellate phylotype was present at high absolute abundance, yet constituted a minor fraction of the total number of other phylotypes in the sample. The SL148 sample was "dense" with cells, as evidenced by the 1.5 mL volume used for extraction. The opposite situation may explain sample SL165, where the novel dinoflagellate phylotype was abundant relative to the total number of other phylotypes in the sample (60%), yet present at low abundance (7500 cells $\cdot L^{-1}$). Based on the information contained in Table 2, both scenarios occurred in our slush samples.

The qPCR analysis confirmed the general abundance of the novel dinoflagellate phylotype predicted by the number of clones in the libraries. There is always potential bias when amplifying and cloning, and it has been suggested that prevalence of a sequence type is not necessarily indicative of the natural abundance of the organism. Our results indicate that while clone libraries are not quantitative, they can provide a crude approximation of the potential prevalence of a phylotype in a natural sample. Most significantly, both the qPCR and clone libraries were useful in indicating that this dinoflagellate was common and widespread in the Antarctic marine environment.

CONCLUSIONS

We have recovered the sequence of a novel dinoflagellate phylotype in an analysis of full-length ribosomal gene clone libraries from microhabitats of the Ross Sea, Antarctica. We have been successful in demonstrating the predominance of this sequence type in clone libraries from a variety of stations and sample types in the pack-ice and polynya. Further, we have successfully linked this genotype to a cultured organism, a fundamental aspect that has been lacking in many studies of ribosomal gene diversity.

Based on our observations, we hypothesize that this dinoflagellate can be an ecologically significant organism in some Antarctic marine ecosystems. The organism occurred at very high densities, and was present during late austral summer in Antarctic seawater and in the meltwater habitats (slush) at the snow/ice interface. A further interesting aspect of the novel dinoflagellate is that its closest taxonomic relatives are harmful marine dinoflagellates. Species of both *Karenia* and *Karlodinium* have been implicated in marine fish or invertebrate mortalities. No such impacts have been reported in the Ross Sea, but the abundance and widespread distribution of the organism in the water and slush warrant further investigation. We are currently pursuing further morphological analyses in order to produce a formal description of this dinoflagellate, including studies of its pigment composition and potential for toxin production.

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