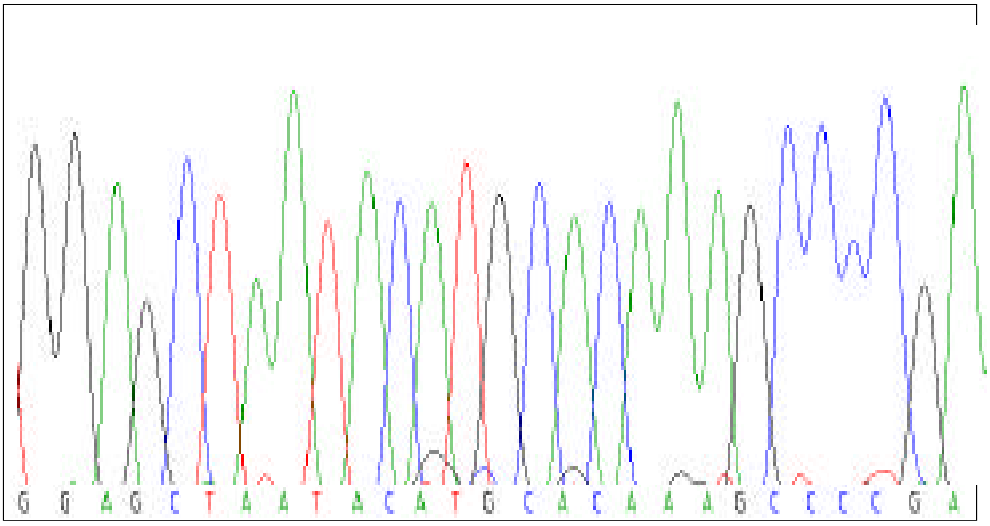

Capítulo 2

STUDY OF GENETIC DIVERSITY OF EUKARYOTIC PICOPLANKTON IN DIFFERENT OCEANIC REGIONS BY SMALL-SUBUNIT rRNA GENE CLONING AND SEQUENCING

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Abstract

Very small eukaryotic organisms (picoeukaryotes) are fundamental components of marine planktonic systems, often accounting for a significant fraction of the biomass and activity in a system. Their identity, however, has remained elusive, since the small cells lack morphological features for identification. We determined the diversity of marine picoeukaryotes by sequencing cloned 18S rRNA genes in five genetic libraries from North Atlantic, Southern Ocean, and Mediterranean Sea surface waters. Picoplankton were obtained by filter size fractionation, a step that excluded most large eukaryotes and recovered most picoeukaryotes. Genetic libraries of eukaryotic ribosomal DNA were screened by restriction fragment length polymorphism analysis, and at least one clone of each operational taxonomic unit (OTU) was partially sequenced. In general, the phylogenetic diversity in each library was rather great, and library included many different OTUs and members of very distantly related phylogenetic groups. Of 225 eukaryotic clones, 126 were affiliated with algal classes, especially the Prasinophyceae, the Prymnesiophyceae, the Bacillariophyceae, and the Dinophyceae. A minor fraction (27 clones) was affiliated with clearly heterotrophic organisms, such as ciliates, the chrysomonad ***Paraphysomonas***, cercomonads, and fungi. There were two relatively abundant novel lineages, novel stramenopiles (53 clones) and novel alveolates (19 clones). These lineages are very different from any organism, that has been isolated, suggesting that there are previously unknown picoeukaryotes. Prasinophytes and novel stramenopile clones were very abundant in all of the libraries analyzed. These findings underscore the importance of attempts to grow the small eukaryotic plankton in pure culture.

Introduction

Marine picoeukaryotes (which are between 0.2 and 2 to 3 µm in diameter) (Sieburth et al. 1978) are probably the most abundant eukaryotes on Earth. They are found throughout the world's at concentrations between 10^2 and 10^4 cells ml^{-1} in the photic zone, and they constitute an essential component of microbial food webs, playing significant roles in global mineral cycles (Fuhrman et al. 1992; Li 1994). Marine picoeukaryotes seem to belong to very different phylogenetic groups. In fact, nearly every algal phylum has picoplanktonic representatives (Thomsen 1986), and in the last 10 years three novel algal classes have been described for picoeukaryotic isolates (Moestrup 1991; Andersen et al. 1993; Guillou et al. 1999a). However, the extent of the diversity and the distribution and abundance of the different taxa in situ remain unknown (Partensky et al. 1997). In the open oceans most picoeukaryotes are coccoid or flagellated forms with chloroplasts (phototrophic) or without chloroplasts (heterotrophic) and with few morphologically distinct features (Thomsen 1986; Simon et al. 1994; Caron et al. 1999). They can hardly be discriminated, even at the class level, by conventional optical microscopy (Murphy et al. 1985). Electron microscopy generally allows assignment to taxonomic classes (Andersen et al. 1996), but most cells do not have enough ultrastructural features for identification at lower taxonomic levels (Potter et al. 1997). Cultivation is the best possible way to characterize a natural organism, and isolation of small picoeukaryotic strains is thus an important task. However, there is no guarantee that organisms grow in culture are dominant or important in the natural community (Guillou et al. 1999c; Lim et al. 1999). Organisms belonging to different algal classes have different diagnostic marker pigments that can be identified and quantified by high performance liquid chromatography (HPLC) (Hooks et al. 1988). HPLC pigment analysis is very useful for characterize new isolates, but it has some technical constraints when it is applied to natural assemblages, since interpretation of the complex pigments pattern of samples requires the application of algorithms (Letelier et al. 1993) which generally involve untestable assumptions. At best, many of the conventional characterization techniques have limited phylogenetic capacity and are cumbersome or time-consuming.

An alternative approach for characterizing the phylogenetic diversity of marine picoeukaryotes is analysis of small-subunit (SSU) rRNA genes (Amann et al. 1995; Partensky et al. 1997). During the last decade, cloning of environmental rRNA genes has provided insight into the diversity of the marine prokaryotic picoplankton and has revealed that this assemblage is dominated by novel lineages of bacteria (Giovannoni et al. 1990) and archaea (DeLong 1992; Fuhrman et al. 1992). Similar studies focusing on marine picoeukaryotes are just beginning. Two very recent papers described the diversity of picoeukaryotes as determined by gene

cloning and sequencing of ribosomal DNA (rDNA) in one surface sample from the equatorial Pacific Ocean (Moon-van der Staay et al. 2001) and several deep-sea samples from the Southern Ocean (López-García et al. 2001). Both studies showed that the phylogenetic diversity of the assemblages was great and that novel lineages were present. Another study analyzed the algal assemblages at two coastal sites by using the plastidic genes found in bacterial SSU rRNA libraries (Rappé et al. 1998). Other molecular studies focused on particular taxonomic groups and have used a similar gene-cloning approach (Guillou et al. 1999c, Moon-van der Staay et al. 2001) or taxon-specific rRNA probes in fluorescent in situ hybridization experiments (Lim et al. 1993; Simon et al. 1995). Finally, fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), have been used to quickly compare the compositions of planktonic eukaryotic assemblages (Van Hannen et al. 1998; Díez et al. 2001b).

The objective of this work was the study of the diversity of marine picoeukaryotes in different marine areas by gene cloning and sequencing of eukaryotic rRNA genes. Clones derived from five genetic libraries were analyzed by restriction fragment length polymorphism (RFLP) analysis, and selected clones were partially sequenced. With this approach we determined whether different picoeukaryotic groups were present in different areas of oceans and estimated their relative abundance.

Materials and methods

Sampling. Samples from different marine areas (Table 1) were collected with Niskin bottles attached to a rosette and a conductivity, temperature and depth probe. Seawater was transferred to 25-liter plastic containers that previously had been rinsed three times with the same water. Microbial biomass was collected on 0.2-µm-pore-size Sterivex units (Durapore; Millipore) by filtering 10 to 20 liters of seawater through a prefilter and a Sterivex unit in succession with a peristaltic pump at rates of 50 -100 ml min^{-1}. Different prefilters were used; these prefilters included 5-µm-pore-size polycarbonate filters in the Mediterranean sample, 2-µm-pore-size polycarbonate filters for the North Atlantic samples and 1.6-µm-pore-size GF/A glass fiber filters for the Antarctic samples. The prefilters and the Sterivex units were covered with lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose) and frozen at -70°C until nucleic acid was extracted. An aliquot of seawater was fixed with glutaraldehyde to obtain epifluorescence counts of heterotrophic flagellates (Porter and Feig 1980). Subsamples of the whole water and the filtrate after passage through the prefilter were used for chlorophyll (Chl ***a***) and the cytometry determinations. Aproximately 100 ml of sample were filtered through GF/F

filters, and Chl ***a*** concentration was determined by measuring the fluorescence in acetone extracts with a Turner Designs fluorometer (Parsons et al. 1984). Subsamples used for flow cytometry counting were collected by fixing 1.2 ml of seawater with glutaraldehyde-paraformaldehyde (final concentration, 0.05 and 1% respectively). Populations of ***Synechococcus***, ***Prochlorococcus*** and photosynthetic picoeukaryotes were distinguished by their distinct size and pigment properties by using a FACScalibur flow cytometer (Becton Dickinson) as explained by Olson et al. (Olson et al. 1993). Strictly speaking, the picoeukaryotes comprise organisms that are between 0.2 and 2 µm in diameter, but here we use the term loosely to include the larger organisms analyzed in the Mediterranean sample (diameter, 0.2 to 5 µm). Moreover, flow cytometry detects the most abundant photosynthetic eukaryotes, often including organisms that are more than 2 µm in diameter.

TABLE 1. Characteristics of the samples used to generate the five libraries of eukaryotic 18S rRNA genes.

	ME1	ANT37	ANT12	NA11	NA37
System	Mediterranean Alborán Sea	Antarctica Weddell Sea	Antarctica Scotia Sea	North Atlantic	North Atlantic
Characteristics	Upwelling	Ice-edge	Open ocean	Within eddy	Outside eddy
Coordinates	36°14'N 04°15W	60°32'S 44°12'W	58°16'S 44°27'W	59°30'N 21°08'W	59°34'N 21°03'W
Date	9/11/97	26/01/98	23/01/98	14/06/98	21/06/98
Temperature	18.0°C	-1.8°C	1.9°C	11.2°C	10.5°C
Fraction analyzed	0.2–5 µm	0.2–1.6 µm	0.2–1.6 µm	0.2–2 µm	0.2–2 µm
Chl ***a*** ($\mu g\ l^{-1}$)					
total	0.87	0.35	0.29	0.87	0.59
in the fraction	0.72	0.01	0.02	0.22	0.19

Nucleic acid extraction. Nucleic acid extraction started with addition of lysozyme (final concentration, 1 mg ml^{-1}) and incubating of the Sterivex units at 37°C for 45 min. Then, sodium dodecyl sulfate (final concentration, 1%) and proteinase K (final concentration, 0.2 mg m^{-1}) were added, and the Sterivex units were incubated at 55°C for 60 min. Lysates were recovered from the Sterivex units with a syringe. Nucleic acids were extracted with phenol-

chloroform-isoamyl alcohol (25:24:1), and the residual phenol was removed with chloroform-isoamyl alcohol (24:1). Nucleic acid extracts were purified further, desalted, and concentrated with a Centricon-100 concentrator (Millipore). DNA integrity was checked by agarose gel electrophoresis, and DNA yield was quantified by a Hoechst dye fluorescence assay (Paul and Myers 1982). Nucleic acid extracts were stored at –70°C until analysis.

Eukaryotic rDNA genetic libraries. Eukaryotic 18S rRNA genes were amplified by PCR with eukaryote-specific primers EukA, EukB (Medlin et al. 1988) and 326f (Lim et al. 1993). Most libraries were constructed with the 326f-EukB primer combination (1.420-bp insert); the only exception was the ME1 library, which was constructed with primers EukA and EukB (1.780-bp insert). The PCR mixtures (100 µl) each contained 10 to 100 ng of environmental DNA as the template, each deoxynucleoside triphosphate at a concentrationh of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U of ***Taq*** DNA polymerase (Gibco BRL) and the PCR buffer supplied with the enzyme. Reactions were carried out in an automated thermocycler (Genius; Techne) with the following cycle: an initial denaturationh at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. Amplified rRNA gene products from several individual PCRs were pooled (four 50-µl samples or two 100-µl samples), ethanol precipitated, and resuspended in 20 µl of sterile water. An aliquot of each concentrated PCR product preparation was ligated into the prepared vector (pCR 2.1) supplied with the TA cloning kit (Invitrogen) by following the manufacturer's recommendations. Putative positive colonies were picked, transferred to a multiwell plate containing Luria-Bertani medium and 7% glycerol, and stored at -70°C.

RFLP analysis. The presence of the 18S rDNA insert in colonies was checked by PCR reamplification with primers 326f and EukB using a small aliquot of a culture as the template. PCR amplifications products containing the right size of insert were digested with 1 U of restriction enzyme ***Hae***III (Gibco BRL) μl^{-1} for 6 to 12 h at 37°C. The digested products were separated by electrophoresis at 80 V for 2 to 3 h in a 2.5% low-melting-point agarose gel. A 50-bp DNA ladder (Gibco BRL) was included in each gel to aid in visual comparison of the RFLP patterns of clones appearing in different gels. When ambiguities appeared, clones were electrophoresed simultaneously in the same agarose gel. Clones that produced the same RFLP pattern (DNA fragments of the same size) were grouped together and were considered members of the same operational taxonomic unit (OTU). Coverage values were calculated for each library by using the relative distribution of OTUs and the equation described by Good (Good 1953).

rDNA sequencing. Double-stranded plasmid DNAs from selected clones were extracted with a QIAprep miniprep Kit (QIAGEN). Sequencing reactions were performed with a Thermo SEQUENASE v.2 kit (Amersham, US Biochemical) and an ABI PRISM model 377 (v. 3.3) automated sequencer. A single reaction with primer 326f was performed for each clone, which resulted in a 550 to 750-bp sequence. Sequences were subjected to a BLAST search (Altchul et al. 1997) to determine the first phylogenetic affiliation and to the CHECK-CHIMERA command (Maidak et al. 2000) to determine potential chimeric artefacts. Sequences were aligned with about 3,200 homologous eukaryotic 18S rRNA primary structures by using the automatic alignment tool of the ARB program package (http://www.mikro.biologie.tu-muenchen.de). Then partial sequences were inserted into the optimized tree derived from complete sequence data by using the Quick add using parsimony tool, which did not affect the initial tree topology. The resulting tree was pruned to save space; only the closest relatives of our clones were retained. Since the similarity value obtained in a BLAST analysis often is for only a fraction of the sequence submitted, similarity values for new and database sequences were calculated by using the aligned ARB file.

DGGE. Eukaryotic 18S rRNA genes were amplified with eukaryotic-specific primers Euk1A and Euk516r-GC, which amplify an approximately 560-bp fragment (Díez et al. 2001b). The PCR program included an initial denaturing at 94°C for 130 s and 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 130 s. The PCR products were quantified with a Low DNA MASS Ladder (Gibco BRL) by performing agarose gel electrophoresis with a DGGE-2000 system (CBS Scientific Company). A 0.75 mm-thick 6% polyacrylamide gel was cast by mixing two stock solutions containing 45 and 65% DNA denaturant agents (100% was defined as 7 M urea and 40% deionized formamide). Approximately 800 ng of PCR product was applied to each lane in the gel. Electrophoresis was performed at 100 V and 60°C for 16 h in 1X TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA; pH 7.4). The DGGE gel was stained with GelStar (FMC BioProducts) for 30 min, rinsed with 1X TAE buffer, and visualized with UV radiation in a Fluor-S MultiImager and the MultiAnalyst imaging software (Bio-Rad*)*. The presence and intensity of DGGE bands were estimated by image analysis as previously described (Schauer et al. 2000).

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study have been deposited in the GeBank database under accession numbers AF363153 to AF363228.

Results and discussion

The aim of this study was to analyze the phylogenetic composition of marine planktonic picoeukaryotes, an ubiquitous, heterogeneous, poorly identified assemblage. We present the results obtained with a molecular approach, gene cloning and sequencing of SSU rRNA genes, that has been used very successfully to identify marine bacteria and archaea (Giovannoni et al. 1990; DeLong 1992) and has only recently been applied to marine eukaryotes (López-García et al. 2001; Moon-van der Staay et al. 2001). We analyzed clone libraries from five surface samples taken in three distant marine regions, the Mediterranean Sea (library ME1), the Southern Ocean (libraries ANT37 and ANT12) and the North Atlantic Ocean (libraries NA11 and NA37). These samples exhibited a wide range of in situ temperatures, from 18°C in the Mediterranean Sea to –1.8°C in the Weddell Sea (Table 1). They also differed in terms of the composition of the phototrophic picoplankton; picoeukaryotes were present in all three systems, together with ***Synechococcus*** in the Mediterranean and Atlantic samples and ***Prochlorococcus*** in the Mediterranean sample (Table 2). Therefore, the physical and biological parameters of the five samples analyzed were very different.

In contrast to marine bacteria and archaea, the planktonic eukaryotes cover a broad size spectrum; they vary from microns to millimeters in diameter. Therefore, the approach used to collect picoeukaryotes is very important. Picoplanktonic biomass was obtained by prefiltering a sample and collecting the organisms that passed though the prefilter. The performance of this size fractionation for the five samples used to construct clone libraries was assessed by carrying out Chl ***a*** (Table 1), flow cytometry (Table 2), and molecular fingerprinting (Fig. 1) analyses. For the Mediterranean sample, filtration through a 5-µm-pore-size filter resulted in a slight reduction in the level of Chl ***a*** (Table 1) but no reduction in the level of phototrophic picoeukaryotes (Table 2). For the North Atlantic samples filtration through a 2-µm-pore-size filter resulted in a significant reduction in the level of Chl ***a***, but it had no effect on phototrophic picoeukaryote abundance. For the Antarctic samples, filtration through a 1.6-µm-pore-size filter resulted in a dramatic decrease in the level of Chl ***a*** (only 3 to 7% passed though the filter) and in the level of phototrophic picoeukaryotes (5 to 31% passed though the filter). When possible, distinct picoeukaryotic populations were distinguished on the cytometry graph and analyzed separately (Table 2).

TABLE 2. Concentrations (10^3 cells ml^{-1}) of heterotrophic and phototrophic picoeukaryotes, ***Prochlorococcus*** and ***Synechococcus*** in whole water and in the fractions passing through the prefilters for the samples used to generate eukaryotic genetic libraries. Heterotrophic picoeukaryotes were counted by the epifluorescence method, and phototrophic populations were counted by the flow cytometry method. P1, P2 and P3 are distinct phototrophic picoeukaryote populations (P1 cells are smaller than P3 cells). nd: not determined.

Sample		Picoeukaryotes					***Prochlorococcus***	***Synechococcus***
		Heterotrophic	Phototrophic	P1	P2	P3		
ME1	ww	1.5	13.9				12.7	12.9
	<5µm	nd	14.1				14.8	12.7
ANT37	ww	0.4	9.9	2.5	5.2	2.2	0	0
	<1.6µm	nd	0.5	0.4	0.1	0	0	0
ANT12	ww	0.7	3.2	1.3	1.5	0.4	0	0
	<1.6µm	nd	1.0	0.8	0.2	0.0	0	0
NA11	ww	nd	6.6	4.8	1.8		0	4.6
	<2µm	nd	6.6	4.6	2.0		0	2.6
NA37	ww	nd	3.2				0	7.3
	<2µm	nd	4.6				0	6.8

The two populations detected in the NA11 sample were not affected by prefiltration, whereas the abundance of the three populations detected in Antarctic samples decreased after filtration and there was a more pronounced effect on the largest of the three populations (P3). Therefore, the fractions analyzed appeared to contain all of the phototrophic picoeukaryotes for the Mediterranean and Atlantic samples and only a fraction of the phototrophic picoeukaryotes for the Antarctic samples.

We then checked whether the eukaryotes that passed though the prefilter (and thus were analyzed in the clone library) were phylogenetically different from the eukaryotes that were retained in the prefilter. It is well known that filters allow passage of cells larger than their nominal pore sizes and that filters can clog, which results in retention of smaller cells. The DGGE fingerprints with eukaryotic-specific primers for both size fractions were very different for the five samples analyzed (Fig. 1).

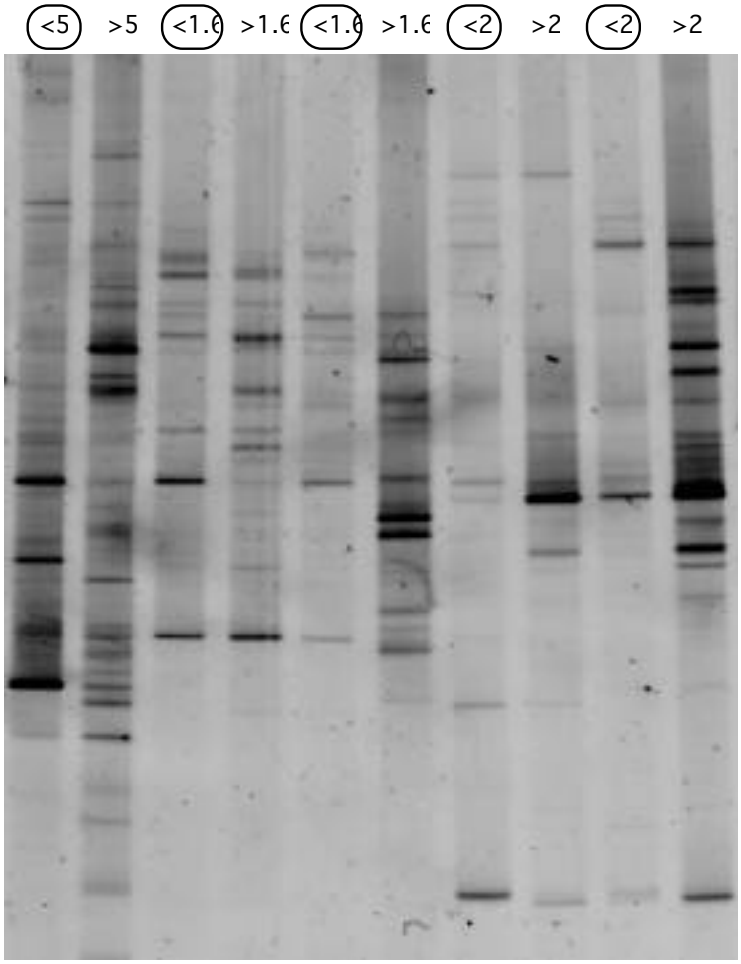


FIG. 1. DGGE gel separating eukaryotic 18S rDNA fragments from the populations retained on prefilters and from the populations appearing in filtrates from the five samples used to generate genetic libraries. The filtrate samples analyzed by using genetic libraries are circled.

On average, 66% of the bands that appearing in the larger-fraction fingerprint (accounting for 45% of the total band intensity) were not found in the smaller-fraction fingerprint, indicating that many populations were totally retained in the prefilter (Fig. 2). Conversely, on average, one-half of the bands that appeared in the smaller-fraction fingerprint (accounting for 32% of the band intensity) were unique to this fraction, indicating that many populations completely passed though the prefilters used. Although the prefiltration method was not perfect and some other populations appeared in both size fractions, this method appeared to enrich the smallest cells and exclude most large eukaryotes. Therefore, we were confident that we were analyzing mostly picoeukaryotes.

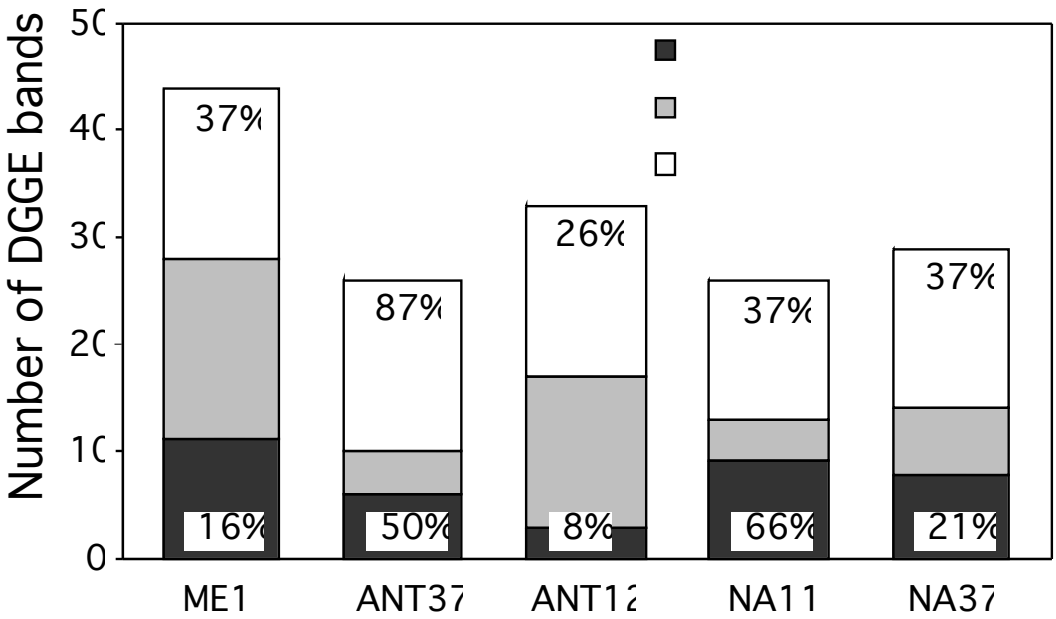


FIG. 2. Numbers of bands that were unique to the filtrates, that were shared, and that were unique to the prefilters for the five samples used to generate genetic libraries after a quantitative analysis of the DGGE gel shown in Fig.1. The values above the bars for unique bands are the percentage of band intensity accounted for by these bands in the DGGE profile.

The five libraries of picoeukaryotic rRNA genes were first screened by RFLP analysis, which grouped clones into discrete OTUs (Table 3). An OTU comprising 33 clones in library ME1 was identical to the appendicularian ***Oikopleura***, and an OTU comprising 43 clones in library NA11 was similar to a copepod. These metazoan OTUs were clearly artifacts of the prefiltration step and were thus excluded from further analyses. Apart from these two OTUs, we analyzed a total of 225 clones, which yielded 76 different OTUs. The coverage values, calculated from the relative distribution of OTUs in each library, were relatively high, ranging from 47% in library NA11, in which few clones were obtained, to 82% in library ANT12 (Table 3). These high values indicated that most the diversity at the level examined had been sampled; only a few more OTUs would be recovered by analyzing more clones. About one-half of the OTUs that appeared in each library were unique to that library (Table 3), whereas the remaining OTUs appeared in two or more libraries, indicating that there was potential overlap of picoeukaryotic phylotypes among samples.

TABLE 3. Results of RFLP analysis of the five genetic libraries. The total number of OTUs in each library has been separated into OTUs unique to the library, OTUs shared with libraries in the same system (between ANT or NA libraries), and OTUs appearing in more than one system. na: not applicable.

Genetic library	ME1	ANT37	ANT12	NA11	NA37
Number of clones	63	58	67	17	20
Number of OTUs	28	29	23	12	10
Coverage (%)	81	66	82	47	75
OTUs:					
Unique to library	17	20	8	7	5
Libraries same system	na	4	4	1	1
Libraries different systems	11	5	11	4	4

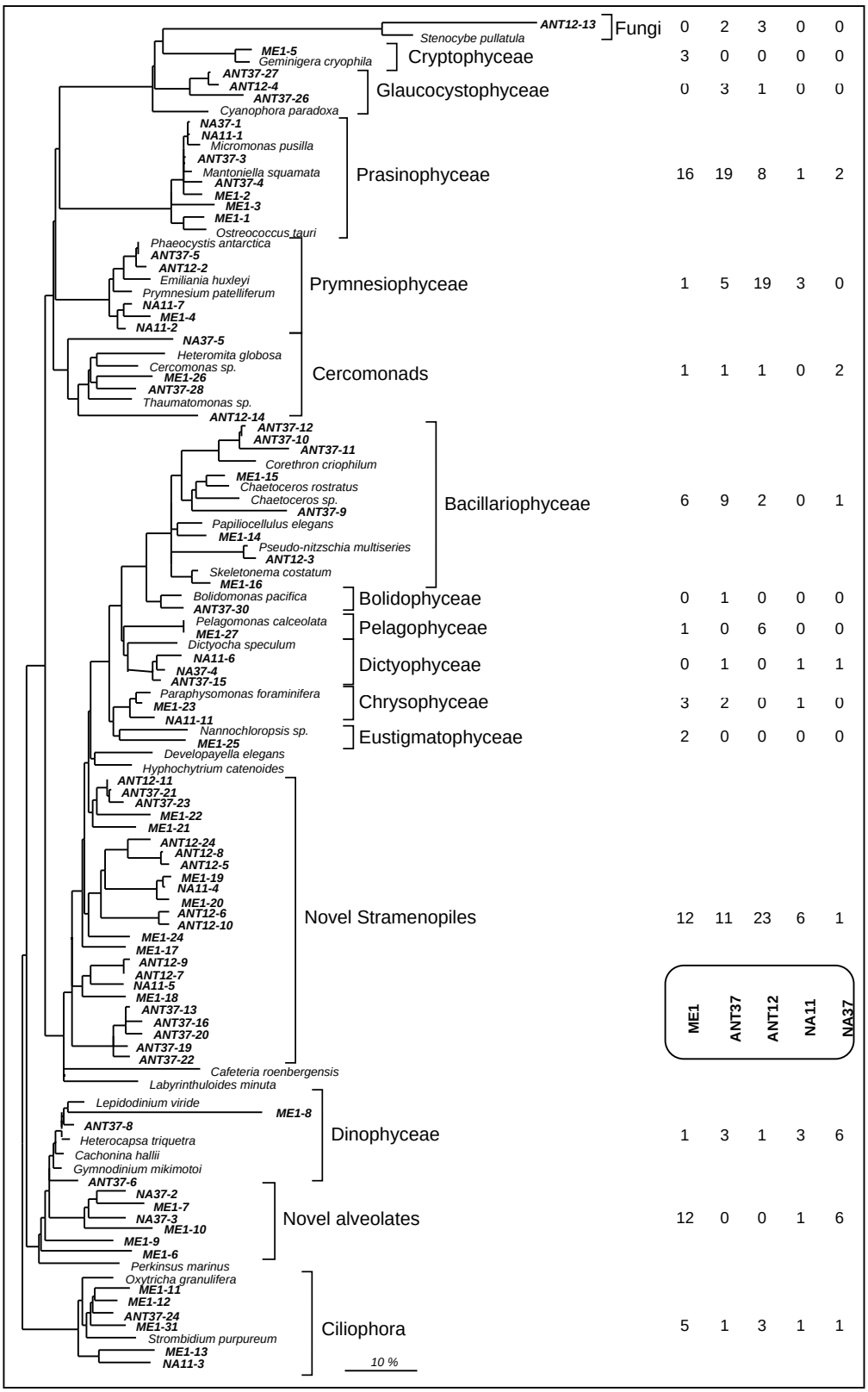
In the ME1 and ANT37 libraries one clone of each OTU was partially sequenced. When clones from different libraries belonging to the same OTU were compared they were found to be very similar (average similarity for 10 cases examined, 98.6% (data not shown)). Thus, clones from the remaining three libraries were affiliated with an OTU found in ME1 or ANT37, and only clones representing new OTUs were sequenced. The affiliation of clones from each library with the 76 defined OTUs and the closest match in the database for the clone representing each OTU are shown in Table 4. These clones and database sequences are compared in a phylogenetic tree in Fig. 3. All of the clones were affiliated with eukaryotes, demonstrating the specificity of the eukaryotic primers used to construct clone libraries. The clones are also widely distributed in the eukaryotic tree, showing the ability of the primers to recover distantly related phylogenetic groups. Some clones were very similar to previously isolated organisms, mainly organisms affiliated with groups having known picoeukaryotic representatives, such as prasinophytes, prymnesiophytes and pelagophytes. Other clones seemed to represent new phylotypes in well-defined phylogenetic groups or even novel phylogenetic lineages.

TABLE 4. Number of clones belonging to each OTU in genetic libraries and phylogenetic affiliation of the representative clone sequenced (closest match and percentage similarity).

		Number of clones in libraries						
	OTU	ME1	ANT37	ANT12	NA11	NA37	Clone	Closest match
Prasinophytes	1	6					ME1-1	***Ostreococcus tauri*** (98.1)
	2	8	16	5			ME1-2	***Mantoniella squamata*** (96.8)
	3	2	1	3			ME1-3	***Ostreococcus tauri*** (94.1)
	4		1				ANT37-3	***Mantoniella squamata*** (99.9)
	5		1				ANT37-4	***Mantoniella squamata*** (96.1)
	6				1		NA11-1	***Micromonas pusilla*** (99.0)
	7					2	NA37-1	***Micromonas pusilla*** (99.1)
Prymnesiophytes	8	1		2			ME1-4	***Prymnesium patelliferum (94.3)***
	9		5	16	1		ANT37-5	***Phaeocystis antarctica*** (99.8)
	10			1			ANT12-2	***Phaeocystis antarctica*** (97.6)
	11				1		NA11-2	***Emiliania huxleyi*** (96.0)
	12				1		NA11-7	***Emiliania huxleyi*** (95.6)
Diatoms	13	3				1	ME1-14	***Papiliocellulus elegans*** (95.5)
	14	1					ME1-15	***Chaetoceros rostratus*** (96.7)
	15	2		1			ME1-16	***Skeletonema costatum*** (96.8)
	16		1				ANT37-10	***Corethron criophilum*** (95.9)
	17		5				ANT37-11	***Corethron criophilum*** (89.0)
	18		1				ANT37-12	***Corethron criophilum*** (95.3)
	19		2				ANT37-9	***Chaetoceros*** sp. (86.7)
	20			1			ANT12-3	***Pseudo-nitzschia multiseries*** (98.6)
Dinophytes	21	1					ME1-8	***Lepidodinium viride*** (76.0)
	22		2	1	3	6	ANT37-6	***Gymnodinium mikimotoi*** (96.7)
	23		1				ANT37-8	***Lepidodinium viride*** (98.5)
Pelagophytes	24	1		6			ME1-27	***Pelagomonas calceolata*** (100)
Glaucocystophytes	25			1			ANT12-4	***Cyanophora paradoxa*** (84.9)
	26		2				ANT37-26	***Cyanophora paradoxa*** (80.0)
	27		1				ANT37-27	***Cyanophora paradoxa*** (85.8)
Dictyochales	28		1				ANT37-15	***Dictyocha speculum*** (90.3)
	29				1		NA11-6	***Dictyocha speculum*** (91.0)
	30					1	NA37-4	***Dictyocha speculum*** (91.5)
Cryptophytes	31	3					ME1-5	***Geminigera cryophila*** (98.5)
Eustigmatophytes	32	2					ME1-25	***Nannochloropsis*** sp. (89.0)
Bolidophytes	33		1				ANT37-30	***Bolidomonas pacifica*** (94.6)
Novel stramenopiles	34	1					ME1-17	***Hyphochytrium catenoides*** (90.8)
	35	3					ME1-18	***Hyphochytrium catenoides*** (87.6)
	36	2					ME1-19	***Hyphochytrium catenoides*** (87.8)
	37	2					ME1-20	***Hyphochytrium catenoides*** (88.3)
	38	1					ME1-21	***Hyphochytrium catenoides*** (90.9)
	39	1					ME1-22	***Hyphochytrium catenoides*** (90.3)
	40		1	5			ANT12-6	***Hyphochytrium catenoides*** (89.2)
	41			6			ANT12-7	***Hyphochytrium catenoides*** (88.8)
	42		1	5			ANT12-24	***Hyphochytrium catenoides*** (89.4)
	43			1			ANT12-9	***Hyphochytrium catenoides*** (88.5)
	44			2	2		ANT12-10	***Hyphochytrium catenoides*** (88.0)
	45		1	1			ANT12-11	***Hyphochytrium catenoides*** (93.7)
	46		1				ANT37-19	***Hyphochytrium catenoides*** (90.0)
	47		1				ANT37-20	***Hyphochytrium catenoides*** (85.5)
	48		1				ANT37-21	***Hyphochytrium catenoides*** (92.9)
	48		1				ANT37-22	***Hyphochytrium catenoides*** (85.4)
	50		1				ANT37-23	***Hyphochytrium catenoides*** (90.3)
	51				1		NA11-4	***Hyphochytrium catenoides*** (86.4)
	52				3	1	NA11-5	***Hyphochytrium catenoides*** (87.7)
	53		2				ANT37-13	***Hyphochytrium catenoides*** (87.7)
	54		1				ANT37-16	***Hyphochytrium catenoides*** (87.5)
	55	2		1			ME1-24	***Hyphochytrium catenoides*** (90.2)
	56			1			ANT12-8	***Hyphochytrium catenoides*** (88.9)

	57			1			ANT12-5	***Hyphochytrium catenoides*** (87.4)
Novel alveolates	58	6			1	3	ME1-6	***Pentapharsodinium tyrrhenicum*** (87.2)
	59	2					ME1-7	***Heterocapsa triquetra*** (87.8)
	60	1					ME1-9	***Heterocapsa triquetra*** (89.3)
	61	3					ME1-10	***Heterocapsa triquetra*** (85.8)
	62					2	NA37-2	***Gymnodinium catenatum*** (89.2)
	63					1	NA37-3	***Lepidodinium viride*** (89.3)
Ciliates	64	2		1		1	ME1-11	***Strombidium purpureum*** (93.3)
	65	1					ME1-12	***Oxytricha granulifera*** (92.2)
	66	1		2			ME1-13	***Oxytricha granulifera*** (87.5)
	67	1					ME1-31	***Oxytricha granulifera*** (91.3)
	68				1		NA11-3	***Oxytricha granulifera*** (88.1)
	69		1				ANT37-24	***Oxytricha granulifera*** (93.5)
Chrysophytes	70	3	2				ME1-23	***Paraphysomonas foraminifera*** (98.7)
	71				1		NA11-11	***Paraphysomonas foraminifera*** (95.3)
Cercomonads	72	1					ME1-26	***Heteromita globosa*** (88.1)
	73			1			ANT12-14	***Thaumatomonas*** sp. (82.9)
	74		1				ANT37-28	***Thaumatomonas*** sp. (89.6)
	75					2	NA37-5	***Cercomonas*** ATCC50318 (83.0)
Fungi	76		2	3			ANT12-13	***Stenocybe pullatula*** (80.0)
Metazoans	77	36						***Oikopleura*** sp. (99.2)
	78				43			***Cancrincola plumipes*** (94.0)

The fact that clones belonging to the same OTU were seldom identical indicated that we were underestimating the true diversity by sequencing only one clone of each OTU. However, this was the approach chosen since we were more interested in broad identification of the picoeukaryotic phylotypes present in different marine environments than in a detailed list of species. Moreover, only partial sequences were obtained (at least one-third of the 18S rRNA gene), and the phylogenetic affiliation of the clones and the percentages of similarity calculated were not as precise as they would have been if we had sequenced the whole gene. It is clear, however, that partial sequences are sufficient to infer the position of clones in a given line of descent (Stackebrandt and Rainey 1995). Finally, the clonal representation of a group does not necessarily reflect its precise abundance in nature, given the potential biases inherent with PCR-based methods (Von Wintzingerode et al. 1997). For this reason we refer here to the percentage of clones in the libraries, which are useful values for comparing the distributions of groups among libraries.



	ME1	ANT37	ANT12	NA11	NA37
Fungi	0	2	3	0	0
Cryptophyceae	3	0	0	0	0
Glaucocystophyceae	0	3	1	0	0
Prasinophyceae	16	19	8	1	2
Prymnesiophyceae	1	5	19	3	0
Cercomonads	1	1	1	0	2
Bacillariophyceae	6	9	2	0	1
Bolidophyceae	0	1	0	0	0
Pelagophyceae	1	0	6	0	0
Dictyophyceae	0	1	0	1	1
Chrysophyceae	3	2	0	1	0
Eustigmatophyceae	2	0	0	0	0
Novel Stramenopiles	12	11	23	6	1
Dinophyceae	1	3	1	3	6
Novel alveolates	12	0	0	1	6
Ciliophora	5	1	3	1	1

FIG. 3. Phylogenetic tree for partial sequences of environmental clones and the most closely related cultured organisms. Environmental clones are indicated by boldface tipe and each code is designated by the library designation followed by a number. One clone representing each different OTU detected by the RFLP analysis of the five genetic libraries is included. The bar indicates 10% estimated sequence divergence. The number of clones in each genetic library belonging to each phylogenetic group is indicated on the right.

←

Phototrophic picoeukaryotic isolates generally belong to the classes Prasinophyceae, Chlorophyceae, Prymnesiophyceae and Pelagophyceae (Thomsen 1986; Hooks et al. 1988; Simon et al. 1994;Potter et al. 1997). The relevance of these isolates in natural assemblages is uncertain, given the biases that occur when microorganisms are cultured (Amann et al. 1995; Guillou et al. 1999c; Lim et al. 1999). A significant number of clones in our libraries affiliated with these classes; however, there was a conspicuous absence of chlorophytes. Our clones exhibited rather high similarity with picoeukaryotic isolates (between 94.1 and 100%; average, 97.4%), suggesting that the cultures available are fair representatives of natural phototrophic picoeukaryotes. The prasinophyte group was the most abundant and widespread algal group in our libraries and was represented by 46 clones and seven OTUs. These clones were present in all libraries and were dominat in ME1 and ANT37. The two Atlantic libraries contained clones very similar to ***Micromonas pusilla***. In the other three libraries all clones were most similar to ***Mantoniella squamata*** or ***Ostreococcus tauri***. In particular, one OTU that exhibited 96.8% similarity to ***M. squamata*** was represented 8 times in the Mediterranean library and 16 and 5 times in the two Antarctic libraries. Clones belonging to this OTU obtained from systems separated by thousands of kilometers were very similar (98.8%), indicating that very similar phylotypes are widely distributed. The second most abundant OTU had a phylogenetic position between ***Ostreococcus*** and ***Mantoniella*** and was also widely distributed. Finally, an OTU very similar to ***O. tauri*** appeared six times, but only in the ME1 library. It is perhaps not coincidental that ***O. tauri*** was described from a Mediterranean lagoon (Courties et al. 1994). Our culture-independent data confirm the importance of prasinophytes in marine picoplankton, in which their marker pigment prasinoxanthin is found widely (Peeken 1997; Latasa and Bidigare 1998), and indicate that their diversity is relatively high since several phylotypes coexisting in the same sample.

The prymnesiophytes were represented by 28 clones belonging to five OTUs. They were abundant in the two Antarctic libraries but rare in the other three libraries. The most abundant OTU, with 5 clones in ANT37 and 16 clones in ANT12, was almost identical to ***Phaeocystis antarctica***. The presence of this organism is expected in Antarctic waters, where it frequently

forms large blooms, many times consisting of the colonial form. Unicellular flagellated forms of ***P. antarctica*** were probably responsible for the sequences detected. It must be noted that prefiltration of the Antarctic samples removed many phototrophic picoeukaryotes, and thus, a large fraction of the natural assemblage remained undescribed. In the Atlantic libraries two clones were moderately related to ***Emiliania huxleyi***. This prymnesiophyte accounted for up to 60% of the phytoplankton biomass in the Atlantic samples (unpublished results) but was obviously effectively removed by the prefiltration step. Finally, a few clones in the ME1 and ANT12 libraries were moderately related to ***Prymnesium patelliferum***.

Members of the Bacillariophyceae (diatoms) were represented by 18 clones belonging to eight OTUs. Most OTUs were found only in one library, indicating a different diatom assemblage occurred in each marine region. In ANT37 most diatom clones were affiliated with ***Corethron criophilum,*** and one clone showed a very low level of similarity to ***Chaetoceros***. The three OTUs in ME1 exhibited relatively high levels of similarity to ***Papiliocellulus elegans***, ***Chaetoceros rostratus*** and ***Skeletonema costatum***, and one clone in ANT12 was very similar to ***Pseudo-nitzschia multiseries***. Although most known diatoms tend to be larger than the size fraction analyzed here, very small diatoms have been described (Thomsen 1986). In an HPLC study of the distribution of size-fractionated pigments in the Arabian Sea, Latasa and Bidigare (Latasa and Bidigare 1998) found that between 70 and 92% of the marker pigment fucoxanthin occurred in the <2-µm fraction during the Spring Intermonsoon and between 26 and 85% of this pigment occurred in this fraction during the Southwest Monsoon in the two most open sea stations. The retrieval of diatom genes in our study is consistent with the presence of the marker pigment in the small-size fraction. Another possible explanation is cell breakage during prefiltration or squeezing of cells through the filter.

Dinoflagellates were represented by 14 clones in three different OTUs and accounted for a significant fraction of the eukaryotic clones in the North Atlantic libraries. One OTU that was most similar to ***Gymnodinium mikimotoi*** appeared in all Antarctic and North Atlantic libraries. A single clone recovered from ANT37 was very similar to ***Lepidodinium viride***, whereas another clone in ME1 was very different from any known dinoflagellate. Dinoflagellates tend to be large and conspicuous organisms, and there is not any known form of picoplanktonic size. Dinoflagellates might be overrepresented in genetic libraries because they have larger genomes that members of others phytoplankton groups (Rizzo 1985) and therefore potentially higher rRNA gene copy numbers (Cavalier-Smith 1985). Like diatoms, their presence in genetic libraries might be due to inefficient prefiltration or the existence of unknown picodinoflagellates. In the above mentioned study, Latasa and Bidigare (Latasa and Bidigare 1998) found that often more than 50% (and up to 75%) of peridinin, the marker pigment of dinoflagellates, appeared in the <2-µm fraction.

The remaining phytoplankton groups were minor components of our libraries (Table 4). One OTU represented by one clone in ME1 and six clones in ANT12 was affiliated with the Pelagophyceae, and the sequenced clone was 100% similar to ***Pelagomonas calceolata***. Three ME1 clones were very similar to the cryptophyte ***Geminigera criophila***, and a single clone in ANT37 was moderately affiliated with the recently described picoeukaryote ***Bolidomonas pacifica***. The closest relative of four clones in the Antarctic libraries was the glaucystophyte ***Cyanophora paradoxa***, but the similarity was so low (80.0 to 85.8%) that even an affiliation with this algal class is uncertain. The same is true for three clones in the ANT37 and Atlantic libraries affiliated with ***Dictyocha speculum*** (similarities, around 90%) and two ME1 clones distantly related to the eustigmatophyte ***Nannochloropsis*** (89.0%).

Among the clearly heterotrophic groups we found clones belonging to the Ciliophora, the cercomonads and the Fungi (Fig. 3). Clones clustering with the ciliates were present in all libraries (11 clones and six OTUs); one-half of them from were in the ME1 library, which was constructed by using the prefilter with larger pores. These sequences were rather distantly related to database sequences (the levels of similarity were between 87.5 and 93.5%) and thus belong to new organisms. Five clones representing four different OTUs were distantly related to cercomonads (the levels of similarity were between 83.0 and 89.6%), and low amounts of these clones were detected in the three systems. Five Antarctic clones were affiliated with the fungi and exhibited rather low levels of similarity to any known organism (80.0%). Six clones found in the three systems affiliated with the class Chrysophyceae, which is known to contain mostly phototrophic organisms but also some heterotrophs. These clones were closely related to ***Paraphysomonas foraminifera*** and thus likely are heterotrophic flagellates and not phototrophic organisms.

A significant number of clones in the libraries did not show a close affiliation with any known class of organisms and formed two novel phylogenetic lineages. Novel stramenopiles were the most abundant of these two lineages (Fig. 3). These sequences, representing 53 clones and 24 different OTUs, accounted for a significant fraction of the clones in each library: 19% in ME1, 19% in ANT37, 34% in ANT12, 36% in NA11 and 5% in NA37. They clustered in the basal branches of the stramenopile line of descent; the sequence of the fungus-like organisms ***Hyphochytrium catenoides*** was the most similar sequence in the database, but the levels of similarity were always very low. The novel stramenopile clones were more similar to each other than to any other sequence and showed a relatively high degree of genetic diversity; separate clusters were apparent (Fig. 3). These clusters did not necessarily correspond to different phylotypes found in different samples. Instead, some phylotypes had a wide geographic distribution: for instance, a clone from the Mediterranean Sea (ME1-19) was almost identical (99.5% similarity) to a clone from the North Atlantic (NA11-4).

The stramenopiles (Patterson 1989) form a monophyletic group that is extremely diverse in terms of metabolism and cell type and includes algal cells, fungi-like cells, and heterotrophic flagellates. Phylogenetic relationships determined by using 18S rDNA sequences suggest that stramenopiles were initially heterotrophic and acquired a chloroplast at a certain point in evolution (Leipe et al. 1996). Although the exact position of the novel stramenopile sequences within the different heterotrophic branches could not be unambiguously resolved, our phylogenetic analyses indicated that these organisms appeared before the chloroplast was acquired. Thus, these new sequences probably belong to heterotrophic organisms, and we hypothesize that they account for the bulk of the heterotrophic flagellates in the oceans (Fenchel 1986). In open ocean waters, heterotrophic flagellates are mainly cells less than 2 or 3 µm in diameter (Sherr et al. 1997;Caron et al. 1999) and might be as abundant as phototrophic picoeukaryotes. Therefore, we expected these organisms to be included in our clone libraries, but we detected very few clones related to known heterotrophic flagellates (Fenchel 1986; Tong 1997); only five clones were distantly related to cercomonads and six clones were affiliated with the chrysomonad ***Paraphysomonas***. This is not surprising, since in a previous study it was shown that ***Paraphysomonas imperforata*** systematically dominated enrichment cultures from coastal samples but accounted for less than 1% of the heterotrophic flagellates in the natural system (Lim et al. 1999). The novel stramenopile sequences are also distantly related to known heterotrophic flagellates, such as ***Developayella elegans*** or the bicosoecids. In addition, there is reasonable agreement between the percentage of heterotrophic flagellate cells (based on the total number of picoeukaryotic cells) and the percentage of novel stramenopile clones (based on the total number of clones); these values are 10 and 19% in ME1, 11 and 34% in ANT12, and 4 and 19% in ANT37, respectively.

Novel alveolates formed the second novel lineage that was abundant in our libraries. They were represented by 19 clones and six OTUs and were recovered only from the Mediterranean and North Atlantic samples. Perhaps they were excluded from Antarctic samples by the more drastic prefiltration technique used. The Mediterranean library contained the greatest diversity of marine alveolates, with 12 clones and four OTUs, whereas the Atlantic libraries (especially NA37) contained large percentage of marine alveolates, given the low number of clones analyzed. The novel alveolate sequences clustered in the basal part of the dinoflagellate clade and exhibited very low levels of similarity (76.0 to 89.3%) to dinoflagellate sequences. Their intermediate position between dinoflagellates and the newly established phylum Perkinsozoa, which contains marine parasites, do not allow us to hypothesize about their role in planktonic systems. Similar sequences have also been found in other picoeukaryotic genetic libraries from a surface sample (Moon-van der Staay et al. 2001) and deep samples (López-García et al. 2001).

Our results uncovered several patterns related to the diversity of the smallest eukaryotic plankton in the ocean. First, the diversity of picoeukaryotes in a single sample was great, and the organisms belonged to very different phylogenetic groups. Second, prasinophytes were very important in all the libraries, and this group may be the most widespread and abundant group of small phytoplankton in the ocean. Although quantitative data from PCR-based methods should be regarded with caution (Von Wintzingerode et al. 1997), two additional PCR-based methods (involving the use of different primers) applied to the Mediterranean sample also showed dominance of prasinophytes (Díez et al. 2001b). Moreover, HPLC data also showed that there was a high proportion of chlorophyll ***b***-containing algae (including prasinophytes) in the same sample (Díez et al. 2001b). Thus, the conclusion that prasinophytes are abundant in the oceans seems to be robust. Third, a large number of novel alveolates sequences (unrelated to known sequences) were relatively abundant in all libraries. And, fourth, clones belonging to novel lineages of stramenopiles were present at very high frequencies in all libraries; these lineages appear to branch among the basal heterotrophic groups of stramenopiles and may have important roles in the dynamics of the plankton.

Acknowledgments

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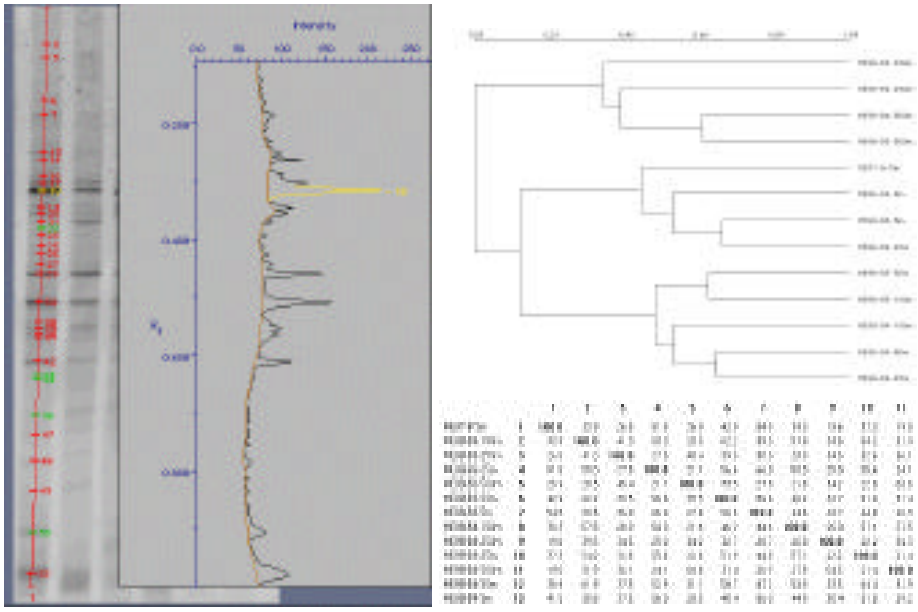

Capítulo 3

APPLICATION OF DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) TO STUDY THE DIVERSITY OF MARINE PICOEUKARYOTIC ASSEMBLAGES AND COMPARISON OF DGGE WITH OTHER MOLECULAR TECHNIQUES

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APPLICATION OF DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) TO STUDY THE DIVERSITY OF MARINE PICOEUKARYOTIC ASSEMBLAGES AND COMPARISON OF DGGE WITH OTHER MOLECULAR TECHNIQUES

Abstract

We used denaturing gradient gel electrophoresis (DGGE) to study the diversity of picoeukaryotes in natural marine assemblages. Two eukaryotic-specific primer sets targeting different regions of the 18S rRNA gene were tested. Both primer sets gave a single band when used with algal cultures and complex fingerprints when used with natural assemblages. The reproducibility of the fingerprints was estimated by quantifying the intensities of the same bands obtained in independent PCR and DGGE analyses, and the standard error of these estimates was less than 2% on average. DGGE fingerprints were then used to compare the picoeukaryotic diversity in samples obtained at different depths and on different dates from a station in the southwest Mediterranean Sea. Both primer sets revealed significant differences along the vertical profile, whereas temporal differences at the same depths were less marked. The phylogenetic composition of picoeukaryotes from one surface sample was investigated by excising and sequencing DGGE bands. These results were compared with an analysis of a clone library and a terminal restriction fragment length polymorphism fingerprint obtained from the same sample. The three PCR-based methods, performed with three different primer sets, revealed very similar assemblage composition; the same main phylogenetic groups were present at similar relative levels. Thus, prasinophyte group appeared to be the most abundant group in this surface Mediterranean samples as determined by our molecular analyses. DGGE bands corresponding to prasinophytes were always found in surface samples but were not present in deep samples. Other groups detected were prymnesiophytes, novel stramenopiles (distantly related to hyphochytrids or labyrinthulids), cryptophytes, dinophytes, and pelagophytes. In conclusion, the DGGE method presented here provided a reasonably detailed view of marine picoeukaryotic assemblages and allowed tentative phylogenetic identification of the dominant members.

Introduction

Small phototrophic and heterotrophic eukaryotes between 0.2 and 5 µm in diameter are found throughout the world's oceans at concentrations between 10^2 and 10^4 cells per ml in the upper photic zone (Li 1994; Caron et al. 1999). They constitute an essential component of microbial food webs and play significant roles in global carbon and mineral cycles, especially in oligotrophic parts of the oceans (Li 1994; Fogg 1995). However, the identities of the eukaryotic picoplankton have remained elusive due to their small size and the lack of distinctive taxonomic characters (Simon et al. 1994; Potter et al. 1997). Conventional approaches based on morphological criteria, such as optical, epifluorescence, or electron microscopy (Murphy and Haugen 1985; Andersen et al. 1996), can barely discriminate between these organisms, even at the class level. Although informative, analysis of diagnostic marker pigments by high-performance liquid chromatography (HPLC) provides information about the composition of photosynthetic picoplankton populations only at the class level (Hooks et al. 1988) and appears to be a complementary method that is useful for gross characterization of populations. Culturing efforts have revealed the presence of novel lineages of heterotrophic (Tong 1995; Guillou et al. 1999b) and phototrophic (Andersen et al. 1993; Guillou et al. 1999a) picoeukaryotic organisms, but only a small percentage of marine picoeukaryotes have been grown in culture, and often the cultured organisms are not dominant in the plankton community (Guillou et al. 1999c; Lim et al. 1999).

Molecular techniques based on ribosomal RNA genes from natural assemblages have provided new insights into the diversity of marine microbial plankton (Amann et al. 1990; Pace 1997). In particular, cloning and sequencing of rRNA genes have been very useful for describing the compositions of marine bacterial (Giovannoni et al. 1990) and archaeal (DeLong 1992; Fuhrman et al. 1992) assemblages. Similar studies have been performed recently with 18S rRNA eukaryotic genes (Díez et al. 2001a; López-García etal. 2001; Moon-van der Staay et al. 2001), and the results suggest that the picoeukaryotic assemblage is very diverse and that there are many undiscovered taxa. However, analysis of clone libraries is time-consuming and not suitable when many different samples are analyzed. This is the case, for example, in studies focusing on changes of microbial assemblages exposed to a perturbation or on how the microbial composition changes along environmental gradients, such as depth in the water column, longitudinal gradients across oceanographic features, or temporal changes at different time scales. A technique that allows processing of many samples simultaneously is necessary for such studies. Fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1997; Muyzer 1999) or terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997; Marsh 1999; Moeseneder et al. 1999),

offer the best compromise between the number of samples processed and the information obtained. DGGE, in particular, provides both rapid comparison data for many communities and specific phylogenetic information derived from excised bands (Muyzer et al. 1997).

DGGE has been widely used to investigate several patterns of distribution of marine bacterial assemblages (Murray et al. 1996; Rappé et al. 1998; Moeseneder et al. 1999; Riemann et al. 1999), but this technique has not been applied previously to the marine picoeukaryotic component. The first application of DGGE for detection of eukaryotic 18S rRNA genes was a study of fungal pathogens in coastal plants in which fungus-specific primers were used (Kowalchuk et al. 1997). A few recent studies have focused on the whole eukaryotic assemblage by using eukaryotic-specific primers. These studies involved an analysis of temporal changes in an activated sludge bioreactor (Marsh et al. 1998), a comparison of natural assemblages in different freshwater lagoons (Van Hannen et al. 1998), and a description of the development of eukaryotic populations in a mesocosm experiment (Van Hannen et al. 1999). As noted before, marine picoeukaryotic assemblages have not been investigated by DGGE previously.

In this study we examined the diversity of marine picoeukaryotes with DGGE by using two sets of primers specific for eukaryotic 18S rRNA genes. We optimized the conditions for both primer sets and tested their performance with cultures and environmental samples. The relative effectiveness of each set was evaluated by comparing of community profiles obtained from different samples from the Mediterranean Sea. The most intense DGGE bands from a surface sample were sequenced, and tentative phylogenetic affiliations of organisms derived from eukaryotic picoplankton were determined. Finally, the DGGE results were compared with the results obtained using two other molecular, PCR–based methods, gene cloning and T-RFLP analysis.

Material and methods

Eukaryotic cultures. ***Thalassiosira pseudonana*** (Bacillariophyceae), ***Heterosigma akashiwo*** (Raphydophyceae), ***Heterocapsa*** sp. (Dinophyceae), ***Platymonas suecica*** (Prasinophyceae), and ***Dunaliella primolecta*** (Chlorophyceae) were obtained from the culture collection at the Institut de Ciències del Mar, Barcelona, Spain. ***Pelagomonas calceolata*** (Pelagophyceae) and ***Nannochloropsis oculata*** (Eustigmatophyceae) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. Cultures were grown in f/2 medium (16) under continuous light conditions or under a daily regime consisting of 12 h of ligth and 12 h of

darkness. When cultures reached sufficient biomass (after 7 to 10 days of growth), cells were harvested by filtration on 0.2-µm-pore-size Durapore filters. The filters were submerged in 2 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose), and nucleic acid extraction was performed immediately.

Marine samples. Samples were collected during two MATER cruises on board ***B/O García del Cid*** and ***B.I.O. Hespérides***. Samples were obtained from a frontal upwelling area (station ME-B) located at 36º14'N/ 4º15'W, across the Western Alborán Sea Gyre (southwest Mediterranean Sea) on 11 November 1997 (sample ME-B0) and four times in May 1998 (in this study we only used samples collected on May 9 (ME-B3) and May 12 (ME-B4)). Seawater from different depths was collected with Niskin bottles attached to a rosette. Microbial biomass was collected on a 0.2-µm-pore-size Sterivex unit (Durapore; Millipore) by filtering between 8 and 18 liters of seawater through a 5-µm-pore-size Durapore prefilter (diameter, 47 mm; Millipore) and the Sterivex unit in succession with a peristaltic pump, using filtration rates of 50 to 100 ml min^{-1}. Each Sterivex units was filled with lysis buffer and frozen at -70°C until nucleic acid extractions was performed in the laboratory.

Nucleic acid extraction. Nucleic acid extraction was performed essentially as described in Massana et al. (1997). For samples from cultures, 0.5-mm-diameter sterile glass beads were added to tubes containing the filters, and the tubes were vortexed in order to physically disrupt the cells. For all samples, nucleic acid extraction started with the addition of lysozyme (final concentration, 1 mg ml^{-1}) and incubation of the filters at 37°C for 45 min. Then, sodium dodecyl sulfate (final concentration, 1%) and proteinase K (final concentration, 0.2 mg ml^{-1}) were added, and the filters were incubated at 55°C for 60 min. The lysates were purified twice by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the residual phenol was removed by extracting with an equal volume of chloroform-isoamyl alcohol (24:1). Finally, nucleic acid extracts were further purified, desalted, and concentrated in a Centricon-100 concentrator (Millipore). The integrity of the total DNA was checked by agarose gel electrophoresis. DNA yield was quantified by a Hoechst dye fluorescence assay (Paul and Myers 1982). Nucleic acid extracts were stored at –70°C until analysis.

PCR. About 10 ng of extracted DNA was used as template in a PCR in which eukaryotic 18S ribosomal DNA (rDNA)-specific primers were used. For DGGE we tested two sets of primers (Table 1): set A (Euk1A and Euk516r-GC), which amplifies a fragment approximately 560 bp long, and set B (Euk1209f-GC and Uni1392r), which amplifies a fragment approximately 210 bp long. The PCR mixtures (50 µl) contained each deoxynucluotide triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U of ***Taq*** DNA Polymerase (Gibco BRL), and the PCR buffer supplied with the enzyme. The PCR program for

primer set A included an initial denaturation at 94°C for 130 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 130s. The PCR program for primer set B included an initial denaturation at 94°C for 1 min and 10 touchdown cycles of denaturation at 94°C for 1 min, annealing at 65°C (with the temperature decreasing 1°C each cycle) for 1 min, and extension at 72°C for 3 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. During the last cycle of both programs, the extension step was increased to 7 min. An aliquot of the PCR product was electrophoresed in a 0.8% agarose gel, stained with ethidium bromide, and quantified by using a standard (Low DNA MASS Ladder, Gibco BRL).

TABLE 1. Oligonucleotide sequences used in this study.

Primer set	Sequence (5´ to 3´)	***S. cerevisiae*** positions	Specificity	Reference
Set A[a]				
Euk1A	CTGGTTGATCCTGCCAG	4 to 20	Eukarya	(Sogin and Gunderson 1987)
Euk516r-GC[b]	ACCAGACTTGCCCTCC	563 to 548	Eukarya	(Amann et al. 1990)
Set B				
Euk1209f- GC[c]	CAGGTCTGTGATGCCC	1423 to 1438	Eukarya	(Giovannoni et al. 1988)
Uni1392r	ACGGGCGGTGTGTRC	1641 to 1627	Universal	(Lane te al. 1985)
Set C				
EukA	AACCTGGTTGATCCTGCCAGT	1 to 21	Eukarya	(Medlin et al. 1988)
EukB	TGATCCTTCTGCAGGTTCACCTAC	1795 to 1772	Eukarya	(Medlin et al. 1988)

[a] Set A was also used for T-RFLP analysis, using HEX-labeled Euk1A and Euk516r without the GC clamp

[b] GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG

[c] GC clamp: CGCGCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

DGGE. DGGE was performed with the DGGE-2000 system (CBS Scientific Company) as described previously (Murray et al. 1996; Muyzer et al. 1997; Schauer et al. 2000). Electrophoresis was performed with 0.75-mm-thick 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) submerged in 1X TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA; pH 7.4) at 60°C. Around 600 to 800 ng of PCR product from environmental samples and 100 ng of PCR product from cultures were applied to individual lanes in the gel. The following electrophoresis conditions were selected based on the results of perpendicular DGGE and time travel experiments (Fig. 1): 16 h at 100 V in a linear 40 to 65% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide) for primer set A; and 6 h at 200 V in a 40 to 80% denaturant agent gradient for primer set B. The gels were stained for 30 min in 1X TAE buffer with SybrGold nucleic acid stain (Molecular Probes) and visualized with UV radiation in a Fluor-S MultiImager and the MultiAnalyst imaging software (Bio-Rad). The number of operational taxonomic units (OTUs) in each sample was defined as the number of DGGE bands.

DGGE bands were sequenced after excision from the gel and reamplification. Briefly, bands were excised, resuspended in 20 µl of MilliQ water, and stored at 4º C overnight. An aliquot of supernatant was used for PCR reamplification with the original primer set. Between 30 and 50 ng of the reamplified PCR product was used for a sequencing reaction (with the corresponding forward primer) with the Thermo Sequenase v.2 kit (Amersham, US Biochemical) and an ABI PRISM model 377 (v. 3.3) automated sequencer. The sequences obtained (300 to 400 bases for primer set A and 100 to 200 bases for primer set B) were compared with with public DNA database sequences by using BLAST (Altschul et al. 1997).

Quantitative analysis of DGGE fingerprints. Digitized DGGE images were analyzed with the Diversity Database software (Bio-Rad) as previously described (Schauer et al. 2000). The software carries out a density profile analysis for each lane, detects the bands, and calculates the relative contribution of each band to the total band intensity in the lane after subtracting a rolling disk background value. Then the software identifies the bands occupying the same position in the different lanes of the gel. Two matrices were constructed; the first took into account the presence or absence of individual bands in all lanes (binary matrix), and the second incorporated the percentage of the intensity for each band based on the total intensity in the lane (intensity matrix). The binary matrix was used to calculate a similarity matrix with the Jaccard coefficient of similarity, and the intensity matrix was used to calculate a distance matrix with Euclidean distances. Both matrices were then used to construct a nonmetric multidimensional scaling (NMDS) diagram with the software SYSTAT 5.2.1. Such a diagram places each sample at a point in a plane (with dimensions of no special significance) so that very similar samples are plotted close together. By connecting consecutive data points

(for instance, consecutive samples from a vertical profile), relative changes in the community structure can be visualized and interpreted (Van Hannen et al. 1999).

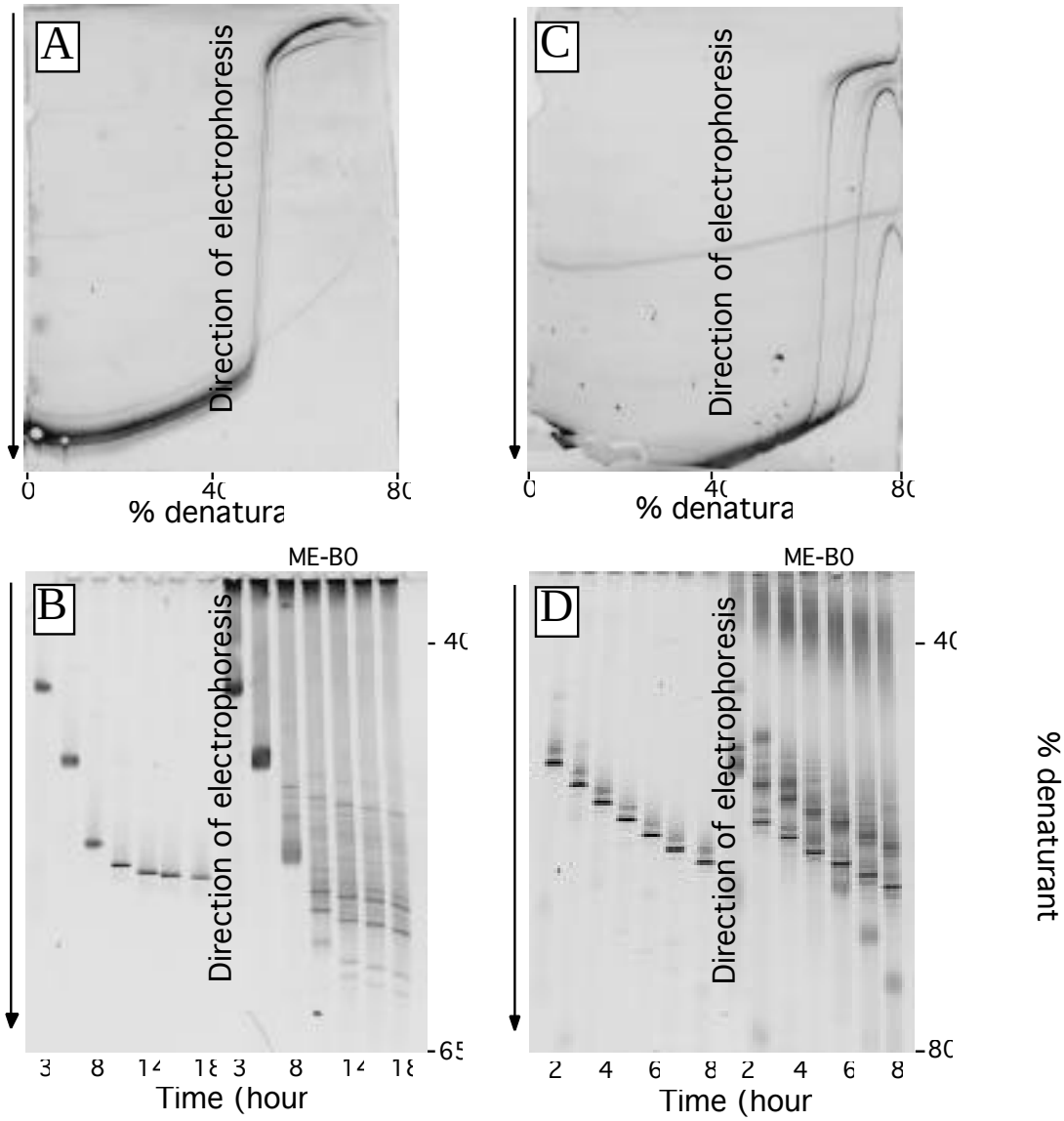

FIG. 1. (A) Negative image of a perpendicular DGGE gel with PCR products obtained with primer set A from different algal cultures (***P. calceolata***, ***T. pseudonana*** and ***P. suecica***) and electrophoresed at 100 V for 16 h. (B) Time course separation of PCR products obtained with primer set A from an algal culture (***P. suecica***) and marine sample ME-B0. Samples were electrophoresed for 3, 5, 8, 11, 14, 16 and 18 h at 100 V. (C) Same as panel A but with PCR products amplified with primer set B. The electrophoresis conditions were 200 V for 6 h. (D) Same as panel B but with PCR products amplified with primer set B. Samples were electrophoresed for 2, 3, 4, 5, 6, 7 and 8 h at 200 V.

T-RFLP analysis. The PCR for T-RFLP analysis was performed with primer set A (Table 1) and the corresponding PCR program, except that primer Euk1A was 5´ labeled with hexachlorofluorescein (Operon technologies) and primer Euk516r did not have the GC-clamp. Fluorescently labelled PCR products were purified by using Wizard PCR purification columns (Promega). Purified PCR products were digested separately with restriction enzymes ***Hha***I, ***Msp***I, and ***Rsa***I (Boehringer Mannheim Biochemicals). Terminal Restriction Fragments (TRFs) were resolved by electrophoresis at 3,000 V for 14 h in a denaturing 6% acrylamide gel (ratio of acrylamide to N,N-methylenebisacrylamide, 19:1) with an ABI PRISM model 373 Automated Sequencer. The sizes of TRFs were determined with the software GeneScan 2.1 at 1-bp resolution by using the size standard TAMRA-2500 (ABI), and the intensity of the TRF was measured using the peak area. The number of TRFs corresponded to the number of OTUs in each sample. TRF length predictions were determined for most of the eukaryotic organisms by using complete sequences extracted from the Ribosomal Database Project (Maidak et al. 2000) and the pattern-searching algorithm PatScan (Dsouza et al. 1997). These values obtained were used to identify the putative phylogenetic affiliation of the measured peaks. In cases in which the experimental fragment corresponded to several possible organisms, a most likely candidate was indicated when there were other supporting data.

Cloning and sequencing of 18S rDNA. PCR was performed with primer set C (Table 1), which amplify almost the entire 18S rRNA gene. The PCR program involved an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. The PCR product was used to construct a clone library with a TA cloning kit (Invitrogen). The presence of an 18S rDNA insert was confirmed by PCR reamplification with the same primers. Positive amplification products of the right size were digested with restriction enzyme ***Hae***III (Gibco BRL). The resulting restriction fragment length polymoephism (RFLP) products were separated by electrophoresis in a 2.5% low- melting-point agarose gel at 80 V for 2 to 3 h. Clones with the same RFLP pattern (same bands at the same positions) were considered members of the same OTU. At least one clone representative of each OTU was partially sequenced with an ABI PRISM model 377 (v. 3.3) automated sequencer.

Results

DGGE optimization. We optimized the use of two sets of eukaryotic 18S rDNA-specific primers. First, the four primers were checked against a database of about 4,000 eukaryotic sequences containing the whole 18S rDNA gene (more than 1,649 bases), and they gave very good results. The percentages of sequences having no mismatch and one mismatch with the primers were 79 and 93% for Euk1A, 87 and 96% for Euk516r, 90 and 99% for Euk1209f, and 96 and 98% for Uni1392r, respectively. Moreover, in most cases no consistent bias against any phylogenetic eukaryotic entity was identified; the only exception were the ***Cercomonas*** group with primer Euk1A and the ***Tetrahymena*** group with primer Euk516r. Second, the specificity of the primers was investigated by PCR using as the templates the DNA extracts of several marine algal cultures of organisms that belonged to the classes Pelagophyceae, Eustigmatophyceae, Bacillariophyceae, Chlorophyceae, Prasinophyceae, Raphydophyceae, and Dinophyceae. These cultures always yielded positive PCR amplification results with both sets of primers, whereas several bacterial and archaeal DNA extracts did not (data not shown). Third, a perpendicular DGGE analysis of a mixture of PCR products from three cultures (***P. calceolata***, ***T. pseudonana*** and ***P. suecica***) was performed to determine an appropriate gradient of denaturant concentration for each primer set. At a denaturant concentration range of 50 to 55%, the fragments obtained with primer set A displayed reduced mobility (Fig. 1A), whereas with primer set B the three PCR products melted at 65 to 70% (Fig. 1C). We thus determined that the optimal denaturant gradient was 40 to 65% for primer set A and 40 to 80% for primer set B. Forth, we performed time travel experiments with PCR products from a culture (***P. suecica***) and a natural sample (ME-B0) to determine the optimal electrophoresis time. PCR products obtained with primer set A were loaded into a gel every 2 to 3 h for 18 h and electrophoresed at 100 V (Fig. 1B). After 11 hours bands were clearly defined and showed reduced mobility. PCR products obtained with primer set B were loaded into a gel every 1 to 2 h for 8 h and electrophoresed at 200 V (Fig. 1D). After 3 h the bands were clearly defined, but in this case the bands migrated continuously and did not show reduced mobility. Thus, the electrophoresis conditions used were 100 V for 16 h with primer set A and 200 V for 6 h with primer set B.

Once the optimal conditions for electrophoresis were defined for both primer sets, the performance of DGGE was tested further with the collection of algal cultures available (Fig. 2). The DGGE gel obtained indicated that each culture presented a single dominant band that appeared at a different position in the gel, indicating the potential of the primers to resolve different phylotypes. Some cultures produced additional bands, but the intensities of these bands was always much lower than the intensity of the dominant band.

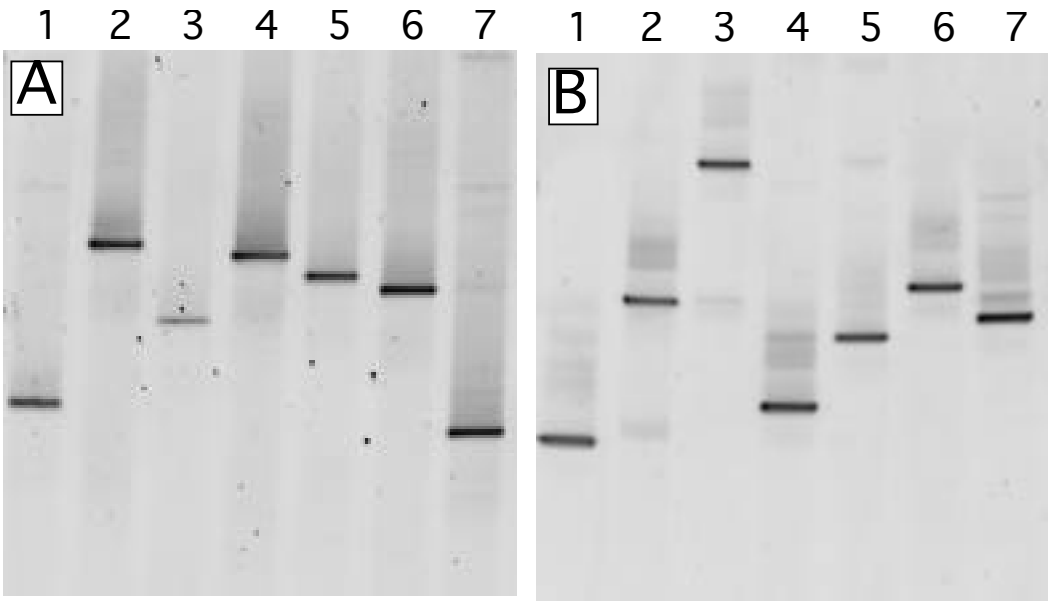

FIG. 2. DGGE fingerprints of algal cultures amplified with primer set A (panel A) and primer set B (panel B). The following cultures were tested: lane 1, ***P. Calceolata***; lane 2, ***N. Oculata***; lane 3, ***T. pseudonana***; lane 4, ***D. primolecta***; lane 5, ***P. suecica***; lane 6, ***H. akashiwo***; lane 7, ***Heterocapsa*** sp.

Fingerprinting of natural assemblages. We used DGGE with primer set A (Fig. 3A) and primer set B (Fig. 3B) to compare the picoeukaryotic assemblages from 13 sothwest Mediterranean Sea samples taken at the same station at different depths and on different dates. Each sample presented a complex fingerprint composed of a large number of bands; 33 to 45 bands at the surface (0 to 100 m) and 20 to 28 bands at depth (250 and 500 m) were obtained with primer set A, and 10 to 17 bands at the surface and approximately 20 bands at depth (250 to 500 m) were obtained with primer set B. Some bands were unique to surface samples, whereas other bands were obtained only with deep samples. With both sets of primers the fingerprints obtained for the surface samples were similar and the fingerprints obtained for the deep samples were similar, and there were clear differences when surface and deep fingerprints were compared.

The data in the DGGE gels shown in Fig. 3 were extracted by image analysis, which resulted in a binary matrix (presence or absence of bands in different samples) and an intensity matrix (binary matrix information plus band intensity information). Using intensity matrices for comparative purposes requires reproducibility of band patterns.

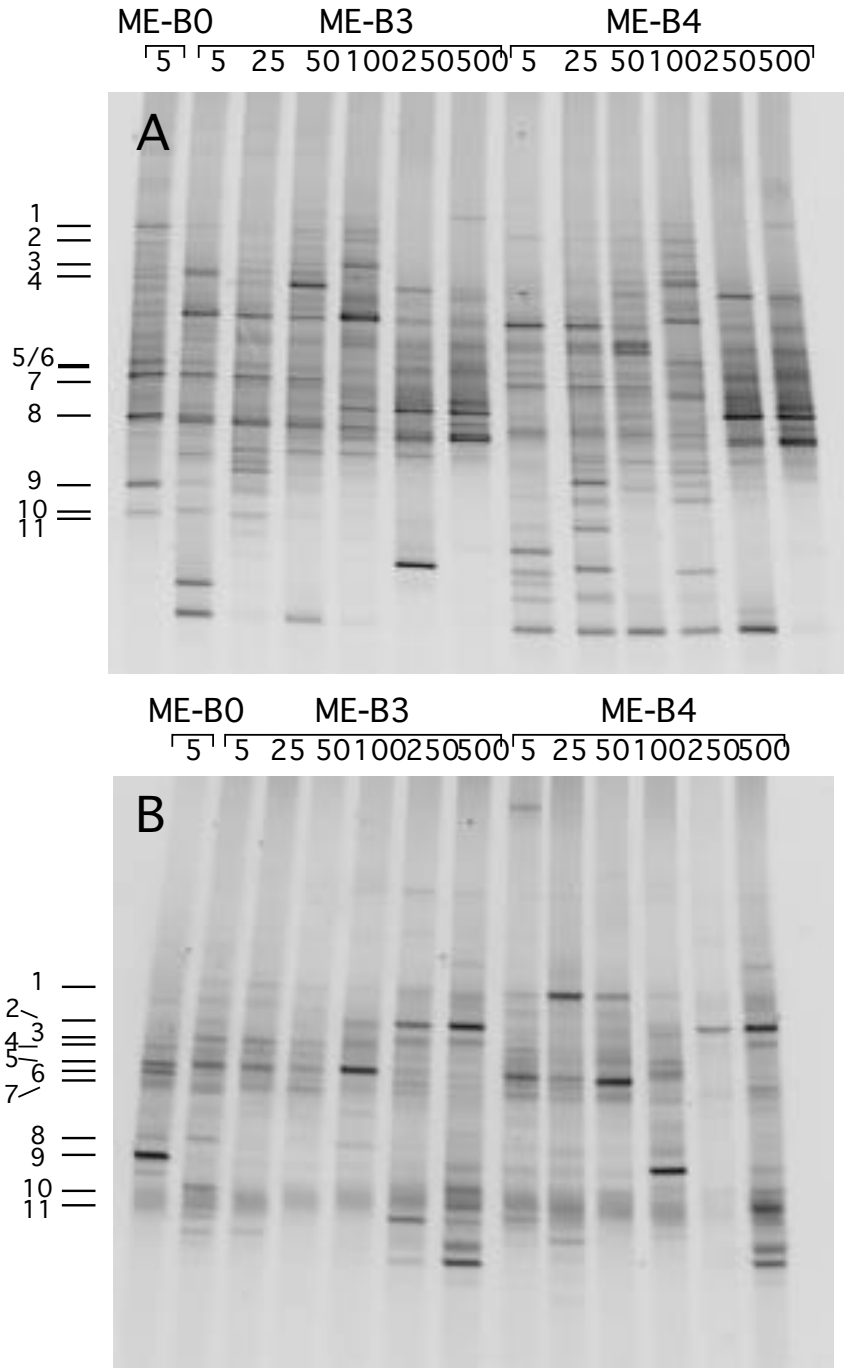

FIG. 3. DGGE fingerprints of picoeukaryotic assemblages obtained at station ME-B (southwest Mediterranean Sea) at different times (ME-B0, Nov. 11 November 1997; ME-B3, 9 May 1998; ME-B4, 12 May 1998) and at different depths (5 to 500 m). The fingerprints were obtained with primer set A (A) and primer set B (B). Bands from sample ME-B0 that were sequenced are indicated on the left side of each gel.

To test reproducibility, we selected the most intense bands from sample ME-B0 and determined the relative intensities of these bands in several DGGE fingerprints obtained after different PCRs and from different DGGE gels. As Fig. 4 shows, the intensities of these bands were always very reproducible; the average standard errors were 0.9% (n=5) and 1.3% (n=4) for bands obtained with primer sets A and B, respectively. The binary and intensity matrices were then used in an NMDS analysis for statistical comparison of the different samples (Fig. 5). The NMDS analysis showed that the samples grouped together primarily according to their positions in the water column. Thus, surface samples obtained on different dates appeared to be similar, and a cluster that included samples obtained in November 1997 and May 1998 was formed. Similarly, deep samples that were obtained at depths of 250 and 500 m formed another cluster that was clearly separated from surface samples. This distribution pattern was observed when we analyzed the data obtained with both primer sets and considered the two types of matrices. However, the results obtained when we used the binary matrix with both primer sets appeared to be more consistent with expectations of gradual change along a vertical profile; the differences between consecutive depths in the vertical profiles were more gradual, and both vertical profiles changed more in parallel.

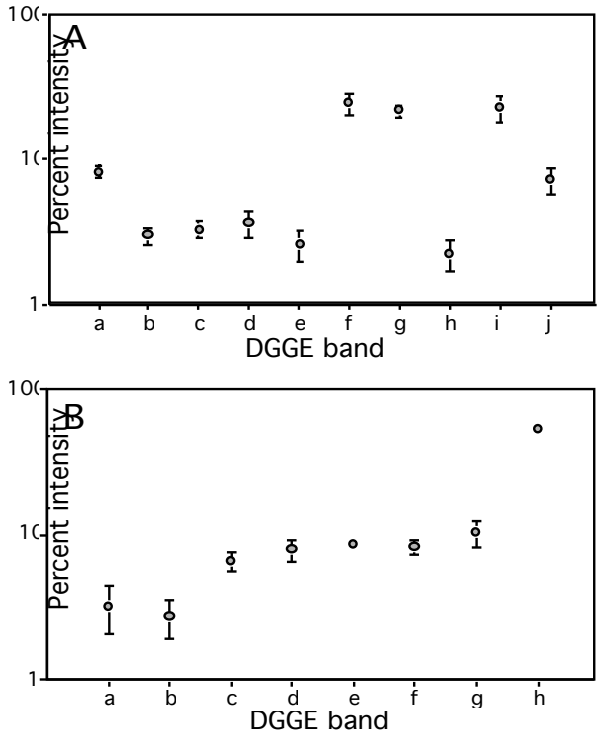

FIG. 4. Average and standard errors of intensity values for DGGE bands of sample ME-B0 as quantified from separate PCR and DGGE analyses with primer set A (A) (n=5) and primer set B (B) (n=4). When error bars are not visible, the error was smaller than the symbol.

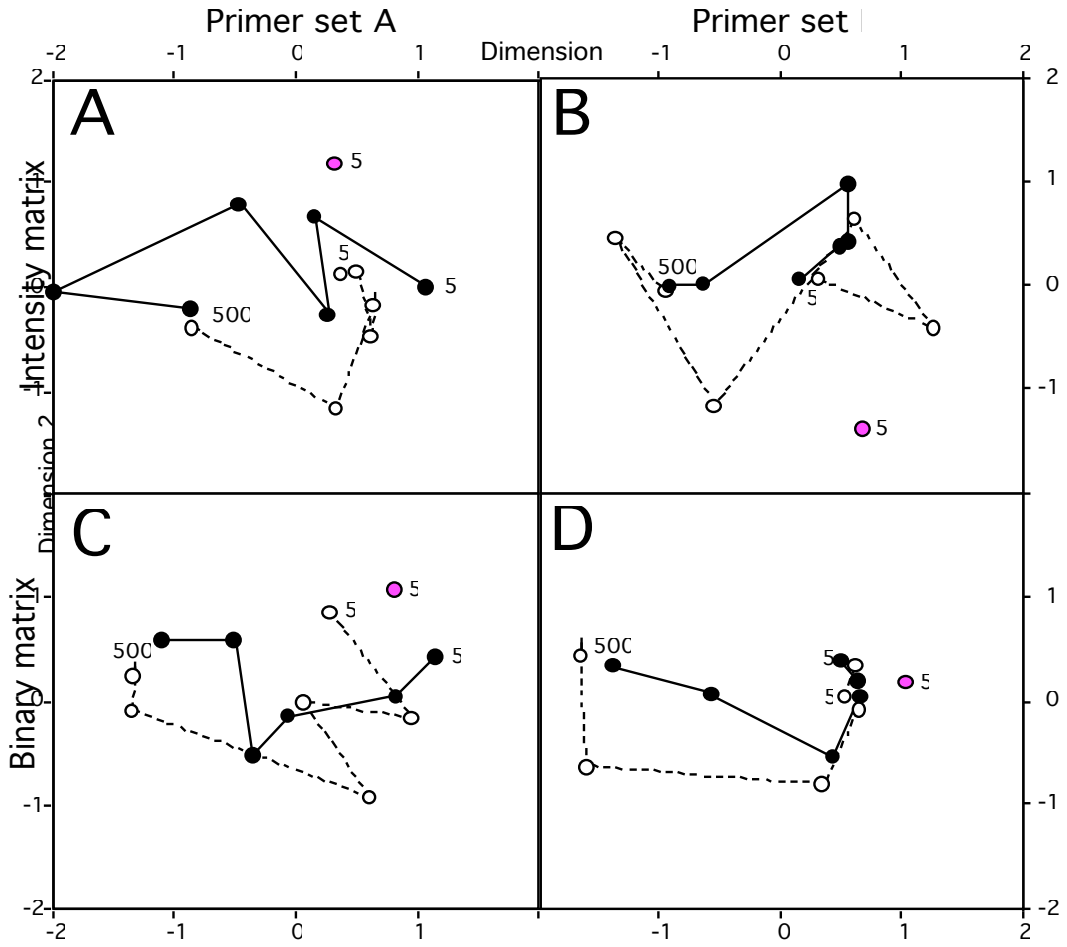

FIG. 5. NMDS diagrams relating the picoeukaryotic assemblages in ME-B samples on the basis of the DGGE gels shown in Fig. 3. NMDS diagrams were calculated from fingerprints obtained with primer set A using the intensity (A) and binary (C) matrices and from fingerprints obtained with primer set B using the intensity (B) and binary (D) matrices. On each diagram the grey circle corresponds to sample ME-B0, the solid circles correspond to ME-B3 samples, and the open circles correspond to ME-B4 samples. Solid and dashed lines join the data for the ME-B3 and ME-B4 samples, respectively, obtained from the surface (5 m) to a depth of 500 m. Only bands that accounted for at least 1% of the intensity in a lane were used in this analysis.

Identification of picoeukaryotic populations. The potential of DGGE for identifying picoeukaryotic populations was addressed by sequencing DGGE bands of the ME-B0 sample. We sequenced 11 bands obtained with primer set A (Fig. 3A) that accounted for 70% of the total band intensity and 11 bands obtained with primer set B (Fig. 3B) that accounted for 84% of the band intensity. The closest matches (and percentages of similarity) for the sequences retrieved were determined by a BLAST search (Table 2). The number of bases used to calculate each similarity value is also shown in Table 2 as an indication of the quality of the sequence. The most intense bands in the profiles obtained with both primer sets corresponded to the prasinophytes ***Mantoniella squamata*** (bands A7, B1 and B6 (Fig. 3 and Table 2)) and ***Ostreococcus tauri*** (bands A8, B3, B5 and B7) and to the appendicularian ***Oikopleura*** sp. (bands A9 and B9). Several other groups, such as prymnesiophytes (bands A5 and B4), cryptophytes (band A11), ciliates (bands A6, B10 and B11), dinophytes (band B2), and novel stramenopile groups closely to labyrinthulids and hyphochytrids (bands A1 to A4) were also represented. Many of these groups are known to include organisms which are very small, and thus the sequences obtained probably belong to true picoeukaryotes. In other cases, such as ciliates and especially an appendicularian and a copepod, the presence of the organisms in the sample analyzed was obviously the result of inefficient prefiltration. Finally, only eukaryotic sequences were recovered, indicating that the two primer sets were very specific.

TABLE 2. Sequence similarities of excised eukaryotic bands that appear in Fig. 3. The numbers in parentheses are the numbers of bases used to calculate the levels of sequence similarity.

Band - Intensity (%)	Most closely related organisms	% sequence similarity (no. of bases)	Taxonomic group
Set A			
A1 – 6%	***Thraustochytrium multirudimentale***	91% (484)	Stramenopiles
A2 – 3%	***Thraustochytrium multirudimentale***	89% (424)	Stramenopiles
A3 – 1%	***Thraustochytrium multirudimentale***	92% (270)	Stramenopiles
A4 – 3%	***Thraustochytrium multirudimentale***	93% (432)	Stramenopiles
A5 – 3%	Unidentified ***prymnesiophyte***	89% (88)	Prymnesiophyte
A6 – 3%	***Oxytricha*** sp.	90% (67)	Ciliophora
A7 – 16%	***Mantoniella squamata***	96% (269)	Prasinophyte
A8 – 14%	***Ostreococcus tauri***	90% (407)	Prasinophyte
A9 – 19%	***Oikopleura*** sp.	98% (422)	Appendicularian
A10 – 9%	***Calanus*** sp.	95% (489)	Copepoda
A11 – 2%	***Geminigera cryophila***	90% (285)	Cryptophyte

Set B			
B1 – 1%	***Mantoniella squamata***	93% (90)	Prasinophyte
B2 – 3%	***Prorocentrum*** sp.	94% (114)	Dinophyte
B3 – 3%	***Ostreococcus tauri***	86% (122)	Prasinophyte
B4 – 5%	Unidentified ***prymnesiophyte***	91% (33)	Prymnesiophyte
B5 – 11%	***Ostreococcus tauri***	95% (172)	Prasinophyte
B6 – 11%	***Mantoniella squamata***	97% (187)	Prasinophyte
B7 – 5%	***Ostreococcus tauri***	89% (193)	Prasinophyte
B8 – 7%	***Pelagomonas calceolata***	89% (169)	Pelagophyte
B9 – 34%	***Oikopleura*** sp.	96% (191)	Appendicularian
B10 – 2%	***Oxytricha*** sp.	91% (65)	Ciliophora
B11 – 2%	***Strombidium*** sp.	81% (141)	Ciliophora

These results provided the identities of the most intense bands in the ME-B0 sample. This in turn permitted these phylotypes to be monitored along the vertical profiles in the Mediterranean Sea study (Fig. 3). Thus, bands corresponding to prasinophytes (especially bands A7, A8, B5 and B6) were detected at depth from the surface down to 50 m (sometimes down to 100 m) in both years, but they were absent at depth of 250 and 500 m. Some other bands, such as those corresponding to ciliates (A6, B10 and B11), seemed to be present at practically all depths. Something similar occurred with the band associated with a dinophyte (B2), which was present at practically all depths. The intensity of this band increased with depth, and it was very intense at 250 to 500 m. Finally, the intense band corresponding to ***Oikopleura*** sp. (bands A9 and B9) was not found in the other samples, confirming that its presence was a prefiltration artifact that occurred only with the ME-B0 sample.

Comparison with other molecular techniques. The phylogenetic composition of the picoeukaryotic assemblage in sample ME-B0 was also investigated by two other molecular techniques. The first technique was analysis of a clone library constructed using primer set C (Table 1), and the results are described in the accompanying paper (Díez et al. 2001a). Ninety-nine eukaryotic clones in this library were analyzed by the RFLP method with ***Hae***III, and at least one representative of each of the 29 OTUs obtained was sequenced. The second technique was analysis of a T-RFLP fingerprint obtained with modified primer set A (Table 1) and assignment of the TRFs to phylogenetic entities by comparison with a computer-simulated restriction analysis of sequences in the database. Analyses of the results of three restriction digestions were also preformed, but only results obtained with ***Hha***I are presented here since this enzyme gave more TRFs (19 different fragments) and the phylogenetic assignment were the least ambiguous. Whereas cloning and sequencing are very time-consuming but very informative, analysis of TRFs is fast but the phylogenetic assignments are only tentative.

Similar numbers of OTUs (between 14 and 29) were detected with the three techniques (Table 3), which is remarkable since the definition of OTU was different for each technique. Moreover, the three techniques identified the same phylogenetic groups, and when the relative abundance of each group in the PCR pool was quantified (by estimating the percentage of total DGGE band intensity, the percentage of clonal representation, and the percentage of the total peak area for each TRF), the results were reasonably consistent (Table 3).

All three techniques showed that the appendicularian ***Oikopleura*** sp. sequence was one of the most abundant 18S rDNA sequences in the sample; this sequence accounted for 19 and 34% of the total as estimated by DGGE with two primers sets, 36% of the total as estimated by clonal representation, and 27% of the total as estimated by the peak area percentage of the 195-bp fragment (Table 3). Two DGGE bands obtained with primer set A (accounting for 30% of the band intensity) and up to five DGGE bands obtained with primer set B (31% of the band intensity) were affiliated with the prasinophytes ***M. squamata*** and ***O. tauri***. In the genetic library, 16% of the clones (distributed in three OTUs) were affiliated with the same two organisms. The T-RFLP analysis detected TRFs of 418- and 420-bp TRFs, each presenting 12% of the total fluorescence. The size of these TRFs were the sizes expected for ***O. tauri*** and ***M. squamata***. Another important group in the clone library was a set of sequences (12% of the total) that formed novel lineages in the stramenopile group distantly related to the labyrinthulids and hyphochytrids (Díez et al. 2001a). Similar sequences were detected by DGGE with primer set A (bands A1 to A4; 13% of the intensity) but were not detected by the other techniques. However, it must be noted that a significant fraction of the PCR product analyzed by DGGE and T-RFLP techniques could not be identified (Table 3), and part of this fraction could account for these sequences. Several other groups, such as the dinophytes, prymnesiophytes, cryptophytes, ciliates, eustigmatophytes, diatoms and pelagophytes, were detected by two or three of the techniques, and the level of these groups was always minor (Table 3). As an example of the conclusion that T-RFLP analysis results cannot be used for phylogenetic identification, the 430-bp TRF (8% of the intensity) was produced by the ciliate ***Oxytricha granulifera*** and the diatom ***Skeletonema costatum***, and the 433-bp TRF (4% of the intensity) was produced by the eustigmatophyte ***Nannochloropsis sp.*** and the diatom ***Papiliocellulus elegans***. This has been pointed out previously for prokaryotes (Marsh 1999; Marsh et al. 2000).

TABLE 3. Relative levels of several eukaryotic groups in sample ME-B0 as estimated by different molecular methods, including DGGE band intensity determined with two primers sets, clonal representation in a genetic library, and TRF peak intensity (between brackets the corresponding TRF size in bp).

	% of total in the following analyses:			
Taxon	DGGE set A	DGGE set B	Library set C	***Hha*I T-RFLP set A**
Oikopleura sp.	19	34	36	27 (195)
Prasinophyceae	30	31	16	24 (418,420)
Prymnesiophyceae	3	5	1	1 (280)
Pelagophyceae		7	1	1 (442)
Ciliophora	3	4	5	0-8 (430*)
Eustigmatophyceae			2	0-4 (433*)
Dinophyceae		3	1	
Cryptophyceae	2		3	
Diatoms			6	0-12 (430*,433*)
Novel stramenopiles	13		12	
% not assigned	30	16	16	35
Number of OTUs	29	14	29	19

* The fragment(s) was produced by more than one phylogenetic group

We performed a final check to directly compare the results obtained for the ME-B0 sample with DGGE and the clone library. Clones belonging to different OTUs and the ME-B0 sample were amplified by using the primer set A and electrophoresed together in a DGGE gel (Fig. 6). The products from the amplified clones loaded to the right of the ME-B0 sample (novel stramenopiles, ***Prymnesium***, ***Strombidium***, ***Mantoniella***, ***Ostreococcus*** and ***Oikopleura***) migrated to the same position in the gel as the community-derived DGGE bands having the same sequence (Fig. 6). This expected coincidence supported the results obtained by sequencing of the DGGE bands and the conclusion that different primers amplify the same sequences. On the other hand, clones loaded to the left of the ME-B0 sample (***Nannochloropsis***, ***Geminigera***, ***Heterocapsa***, ***Skeletonema*** and ***Papiliocellulus***) migrated to positions in the gel where there were several bands in ME-B0 sample that could not be reamplified and/or sequenced.

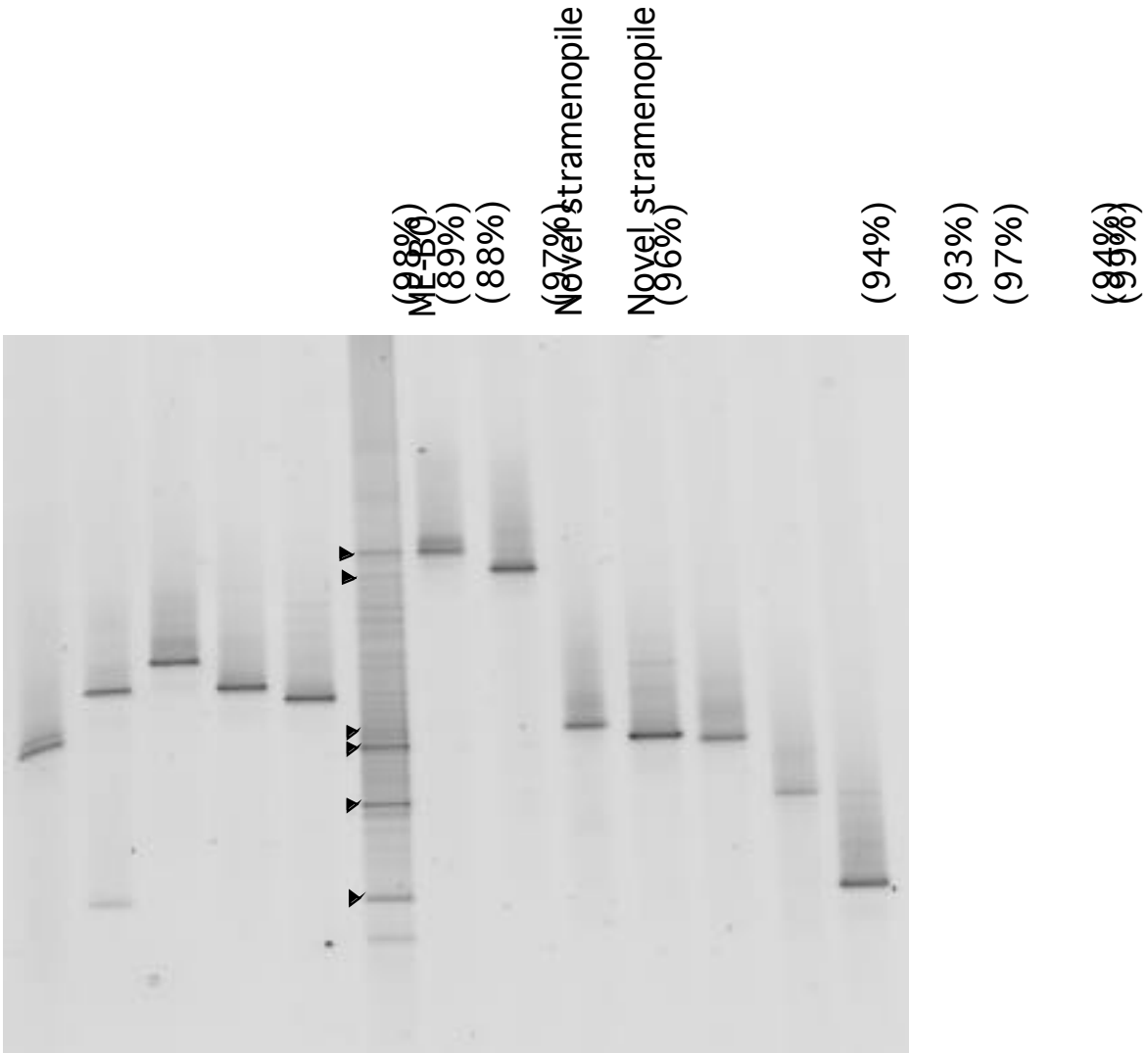

FIG. 6. DGGE fingerprints obtained with primer set A for the ME-B0 sample and several clones from the genetic library obtained from the same sample. The clone names are the names of the most closely related organisms in the database (levels of similarity are given in parentheses) found in a BLAST search. The lanes to the right of the ME-B0 sample contained clones representing phylotypes that have been retrieved by sequencing DGGE bands (indicated by arrowheads).

Discussion

Planktonic picoeukaryotes are widely distributed in the photic zone in marine systems. They form a heterogeneous assemblage composed of small flagellated or coccoid algae and small heterotrophic flagellates. Direct identification of marine picoeukaryotes by microscopy is problematic given their small sizes. Fortunately, diversity studies of picoeukaryotes can take advantage of the approaches used with marine prokaryotes (Amann et al. 1990; Pace 1997) since they can be similarly collected and processed using culture-independent techniques. The resulting environmental DNA extracts can be analyzed by an array of molecular techniques. Recent analysis of marine picoeukaryotes by gene cloning and sequencing (Díez et al. 2001a; López-García et al. 2001; Moon-van der Staay et al. 2001) has indicated that the diversity of this assemblage is rather high and this group includes organisms belonging to very different groups, some of which represent new and undescribed phylogenetic lineages. Here we describe the use of DGGE to compare the structure and composition of different marine picoeukaryotic assemblages.

It is well known that the quantification of organisms by PCR-based methods presents many uncertainties (Von Wintzingerode et al. 1997). Some biases may be due to differences in rRNA gene copy number (Farrelly et al. 1995), and this could be especially important for eukaryotic organisms that may contain up to several thousand copies of the rRNA gene (Long and Dawid 1980). During the PCR some phylotypes can be amplified preferentially due to preferential priming or differences in elongation rates between amplicons. Another bias can occur when the PCR includes many cycles; according to the kinetic model, when the number of cycles is increased, there is a tendency for the different amplicons to reach equimolarity (Suzuki et al. 1998). All of these potential biases can change the relative concentrations of PCR products so that the resulting profile of phylotypes no longer reflects the composition of the native community. In this study we attempted to quantify the relative levels of several picoeukaryote populations in one sample. In order to have a control for PCR biases, we compared the results obtained with three different approaches (DGGE, T-RFLP analysis, and gene cloning) using three different primer sets and different PCR protocols. The accidental presence of rDNA of ***Oikopleura*** in ME-B0 was used to illustrate the relative quantitation of a single population with these different approaches. Thus, the relative levels of this rDNA were 19 and 34% as estimated by DGGE, 36% as estimated by clone library analysis, and 27% as estimated by T-RFLP analysis. These values were reasonably comparable considering the substantial technical differences among the three approaches. Note that the greatest difference occurred with the different primer sets used in the DGGE analysis. Moreover, a more exhaustive

analysis of all the data revealed that the same phylotypes, at similar relative levels, were detected with the different techniques.

Fingerprinting techniques, such as DGGE and T-RFLP analysis, allow easy and quick comparison of profiles from related microbial assemblages and are now used in many ecological studies (Liu et al. 1997; Muyzer et al. 1997; Marsh 1999; Moeseneder et al. 1999). An advantage of DGGE is that selected bands can be sequenced, and thus, the presence of a particular phylotype can be monitored in the environmental samples studied. However, sequences obtained from DGGE bands are short (less than one-third the total length of small-sububit rRNA) and of variable quality. The shorter the sequence derived from DGGE fragments, the less refined the phylogenetic inference. Regarding the quality of the sequences, character ambiguities for directly sequenced PCR amplification products probably arise from amplification of different phylotypes with very similar electrophoretic mobilities. While these ambiguities do not prohibit identification with BLAST, the number of informative characters decreases in proportion to the number of ambiguities. One way to obtain cleaner sequences would be to clone excised bands and analyze several of the clones, but this would be prohibitively laborious when complex communities are analyzed. Therefore, as pointed out previously (Muyzer et al. 1997; Casamayor et al. 2000), sequencing of DGGE bands is sufficient to determine broad phylogenetic affiliations but inadequate to perform a precise phylogenetic analysis.

We used two primer sets for DGGE in order to measure reliability. These primer sets amplify nonoverlapping regions of the 18S rRNA gene; set A amplifies a region between positions 4 and 563, including variable regions V1 to V3 (Neefs et al. 1993), and set B amplifies a region between positions 1423 and 1641, including variable region V8. Primer set B amplifies the same region as a primer set described previously (Van Hannen et al. 1998), but the primers are not the same. When tested with pure cultures, both sets were found to be specific for eukaryotic organisms and gave a single DGGE band, and when applied to environmental samples, they gave complex and reproducible fingerprints. The number of bands obtained with natural samples with set A (20 to 45 bands) was higher than the number of bands obtained with set B (10 to 22 bands). This could have been due to the fact that set B amplifies a smaller fragment with less sequence variability. Based on our results, there are at least two reasons to recommend using primer set A: it amplified a much larger DNA fragment and provided more phylogenetic information, and time travel experiments indicated that this primer set performed better.

The fingerprints obtained by the DGGE method were used to examine the similarity of a group of samples with NMDS diagrams calculated from binary and intensity matrices. The fact that

the intensity of DGGE bands was reproducible argued in favor of using the intensity matrix for such analyses. In fact, this is what we proposed in a previous study in which marine bacterial assemblages were compared (Schauer et al. 2000). However, the results for picoeukaryotes appeared to be better when the binary matrix was used (Fig. 5). This was due to the random presence in some samples of very intense bands corresponding to larger eukaryotic organisms, such as ***Oikopleura*** in sample ME-B0 or a copepod in sample ME-B3 obtained at 250 m (the lower, dominant band in the fingerprint (Fig. 3A)). The presence of these bands, which obviously did not correspond to picoeukaryotes, revealed that prefiltration did not always work perfectly. These bands could dominate the grouping of samples when using the intensity matrix was used but were less influential when the binary matrix was used. This explains why the ME-B0 sample (which produced the intense ***Oikopleura*** band) always grouped better with the other surface samples when the binary matrix was used.

The picoeukaryotic diversity as measured by the different techniques appeared to be great; numerous OTUs and widely separated phylogenetic groups were detected (Table 3). The clone library provided a detailed list of the phylotypes present in sample ME-B0 that could be compared with the sequences obtained from DGGE bands and the database of terminal fragments. The prasinophyte group appeared to be the most abundant group, suggesting that these organisms are important components of marine picoplankton in Mediterranean waters. Significant levels of prasinophytes in other open ocean and coastal environments were detected in libraries of 18S rDNA genes (Díez et al. 2001a; Moon-van der Staay et al. 2001), in an analysis of plastidic clones of a bacterial 16S rDNA library (44), by HPLC pigment analysis (Hooks et al. 1988), and by electron microscopy (Andersen et al. 1996). Other groups detected in the clone library, such as prymnesiophytes, pelagophytes, and novel stramenopiles, were also identified by sequencing DGGE bands. In addition, clones belonging to groups not retrieved from DGGE bands, such as diatoms, cryptophytes, dinophytes and eustigmatophytes, migrated to positions where several DGGE bands were not sequenced (Fig. 6), indicating that these groups could be represented in the unsequenced bands. Finally, we examined the ME-B0 sample by performing an HPLC analysis of pigments, a PCR-independent approach. This analysis attributed a high proportion of the phototrophic fraction to chlorophyll ***b***-containing algae, including prasinophytes (M. Latasa, personal communication). Smaller amounts of pigments found in prymnesiophytes and cryptophytes were also detected by HPLC. Although the HPLC data were preliminary, they agreed with the molecular results.

In conclusion, we demonstrated that the combination of 18S rDNA community libraries sequencing and molecular fingerprinting is as revealing for picoeukaryotic communities as it is for prokaryotic communities. Similar phylogenetic groups at comparable relative levels were recovered by three different molecular approaches. Moreover, differences in community

structure could be easily discerned with both DGGE and T-RFLP analysis. Direct application of these approaches to analysis and comparison of eukaryotic picoplankton assemblages should prove to be profitable.

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