FEMS Microbiology Ecology 50 (2004) 231–243

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches

Ramon Massana *, Vanessa Balagué, Laure Guillou, Carlos Pedrós-Alió

Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CMIMA, CSIC. Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain

Received 12 March 2004; received in revised form 25 June 2004; accepted 5 July 2004

First published online 9 August 2004

Abstract

Planktonic picoeukaryotes are important players in coastal marine food webs but little is known about their diversity. Here we report the picoeukaryotic diversity in Blanes Bay (NW Mediterranean) by retrieving environmental 18S rDNA sequences and by obtaining stable cultures. Four genetic libraries (one per season) were constructed and 383 clones analyzed, yielding 176 distinct sequences. The diversity of picoeukaryotes was very large, both at higher and lower phylogenetic levels. Novel alveolates-I (36% of clones), dinoflagellates (17%), novel stramenopiles (10%), prasinophytes (5%) novel alveolates-II (5%), and cryptophytes (4%) were the better represented phylogenetic groups. Nineteen additional groups were found at <3% clonal abundance. The four genetic libraries were dominated by the above-mentioned groups, implying a relative stability at high taxonomic level, but identical sequences were seldom found in consecutive dates, suggesting fast temporal changes of picoeukaryotic populations. Coastal and open sea picoeukaryotes were similar, but the representation of groups varied between habitats. The culturing effort revealed that some groups were well represented in clone libraries and in cultures (prasinophytes), others were found by both approaches but often with different sequences (cryptophytes), and others were found only in cultures (bicosoecids) or in clone libraries (novel alveolates and stramenopiles). Our data confirm that molecular approaches, such as cloning and sequencing 18S rRNA genes, are a necessary first step to study picoeukaryotic diversity. These results will aid to focus future research, most likely based on new and imaginative culturing efforts and the design and application of specific molecular probes.
© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: 18S rDNA genetic libraries; Marine picoeukaryotes; Coastal plankton; Picoeukaryotic cultures

1. Introduction

Marine picoeukaryotes, unicellular eukaryotes less than 3 μm in size, are found throughout the marine photic zone worldwide at concentrations between 10^2 and 10^4 cells ml^{-1} and play fundamental roles in marine ecosystems [1]. This assemblage is composed of chlorophyll-containing cells that are part of the phytoplankton [2,3], and colorless heterotrophic cells, mostly flagellates, that are the main grazers of marine prokaryotes and have a pivotal role in the microbial loop [4,5]. Despite the ecological importance of marine picoeukaryotes, and the general lack of morphological distinct features of these small cells, they have been studied with a molecular perspective only recently. Perhaps they were too large for bacteriologists, who started environmental molecular studies [6], and too small for phycologists and protozoologists. The first studies that analyzed the genetic diversity of natural assemblages of planktonic picoeukaryotes were based on 18S rDNA genetic libraries from

* Corresponding author. Tel.: +34-93-2309500/2216416; fax: +34-93-2309555/2217340.
E-mail address: ramonm@icm.csic.es (R. Massana).

0168-6496/$22.00 © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.femsec.2004.07.001

a handful of open sea samples from the Equatorial Pacific, Mediterranean Sea, North Atlantic and Antarctica [7–9]. Later, some studies applied the same approach to several marine anoxic samples [10–12]. These studies revealed a surprisingly high diversity of marine picoeukaryotes and the existence of novel lineages, which had important evolutionary and ecological implications [13,14].

It is clear that an overall description of marine picoeukaryotic diversity still requires the investigation of more habitats. Coastal systems deserve particular attention since due to the terrestrial influence from shore or sediments and a general higher trophic level [15] they could harbor particular picoeukaryotic assemblages, different from those of the open sea. Coastal sites are also prone to a larger temporal variability induced from episodic events. Further, picoeukaryotes are known to be ecologically important in the coastal plankton [16], and they can even produce blooms that affect the whole ecosystem [17]. So far there is only one study from oxic surface waters in a coastal site of the English Channel [18], a relatively eutrophic system strongly influenced by tidal and wind action. It is expected that the analysis of contrasted coastal environments could offer new views of marine picoeukaryotic diversity.

Here we studied the diversity of picoeukaryotes from surface waters of Blanes Bay (NW Mediterranean). This site is relatively poor in nutrients and has low plankton biomass (seasonal average chlorophyll *a* of 0.5 $\mu g\, l^{-1}$, [19]), typical of Mediterranean coastal waters. It has been investigated for several years [20], and a reasonable knowledge of seasonal changes in physico-chemical variables, such as temperature, nutrients and solar radiation, and plankton organisms, from bacteria [19] to zooplankton [21], is available. Thus, the background information makes Blanes Bay an ideal system to investigate the composition and variability of eukaryotic picoplankton. First, a molecular study was carried out in which 18S rDNA genes were amplified from the microbial DNA of samples collected throughout a year. The clone libraries generated served to assess the genetic variability of coastal picoeukaryotes along the year, and to compare the composition from coastal and open sea environments. Second, a culturing approach using standard protocols was carried out with organisms smaller than 3 μm as inoculum. Picoeukaryotic cultures can be used as a reference to assess the ecological role of environmental sequences (trophic mode, growth rate, pigment composition). Further, they have been essential to describe new algal classes [22]. Both approaches have well known biases, PCR in one case [23] and culturing selection in the other [24,25], yet a combined study with the same samples has been seldom carried out. While it is obvious that culturing is more selective than the molecular approach, this study will help to identify which groups are more readily cultivable than others.

2. Materials and methods

2.1. Sampling and nucleic acid extraction

Surface seawater was collected in Blanes Bay (Catalan coast, NW Mediterranean, 41°40′N, 2°48′E) 800 m offshore at four different dates: 21 September 2000 (seawater temperature 22 °C), 12 December 2000 (14 °C), 20 March 2001 (14 °C) and 25 June 2001 (22 °C). Seawater was first filtered by a 200 μm nylon-mesh and kept in 25-liter plastic carboys for less than 2 h during transport until processing in the laboratory for microbial collection and DNA extraction (next) and isolation attempts (Section 2.3).

Microbial biomass was collected on 0.2-μm Sterivex units (Millipore, Durapore) by filtering approximately 10 liters of seawater through a 3 μm pore-size polycarbonate filter and the Sterivex unit in succession with a peristaltic pump, at filtration rates of 50–100 ml min^{-1}. 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. Nucleic acid extraction started with the addition of lysozyme (1 mg ml^{-1} final concentration) and incubating the Sterivex units at 37 °C for 45 min. Then, SDS (sodium dodecyl sulfate, 1% final concentration) and proteinase K (0.2 mg ml^{-1} final concentration) were added and the units were incubated at 55 °C for 60 min. The lysate was recovered from the Sterivex units with a syringe. Nucleic acids were extracted with phenol–chloroform–isoamyl alcohol (25:24:1), the residual phenol being removed with chloroform–isoamyl alcohol (24:1). Nucleic acid extracts were further purified, desalted and concentrated in a Centricon-100 concentrator (Millipore) and recovered in MilliQ water. DNA integrity was checked by agarose gel electrophoresis and DNA yield was quantified by a Hoechst dye fluorescence assay [26]. Nucleic acid extracts were stored at −70 °C until analysis.

2.2. Eukaryotic rDNA genetic libraries

The construction and analysis of eukaryotic rDNA genetic libraries was performed following Díez et al. [7]. Almost complete eukaryotic 18S rRNA genes were amplified by polymerase chain reaction (PCR) with the eukaryotic specific primers EukA and EukB [27]. The PCR mixtures (50 μl) contained 10 ng of environmental DNA as template, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 2.5 units of *Taq* DNA polymerase (Invitrogen) and the PCR buffer supplied with the enzyme. Reactions were carried out in an automated thermocycler (Genius, Techne) with the following cycle: an initial denaturing step at 94 °C for 3 min, 30 cycles of denaturing 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 step at 72 °C for 10 min. Amplified rDNA

products from 3 to 6 individual PCR reactions were pooled, ethanol precipitated, resuspended in 20 μl of sterile water and cleaned with the Qiagen PCR purification kit. An aliquot of this purified PCR product was cloned using the TOPO-TA cloning kit (Invitrogen) following manufacturer's recommendations. Putative positive colonies were picked and transferred to a multiwell plate with LB medium and 7% glycerol and stored at −70 °C.

The presence of the 18S rDNA insert in the colonies was checked by PCR reamplification with the same primers using a small aliquot of the culture as template. PCR amplifications showing the right insert size were digested with 10 U μl^{-1} of the restriction enzyme *Hae*III (Invitrogen) for 6–12 h at 37 °C. Digested products were separated by electrophoresis at 80 V for 3 h in a 2.5% low melting point agarose gel. A 50-bp DNA ladder (Invitrogen) was included in each gel to aid in the comparison of RFLP patterns of clones from different gels. Clones showing the same RFLP pattern (DNA fragments of equal size) were grouped together and considered to belong to the same operational taxonomic unit (OTU). The average similarity (using partial sequences) among clones from the same OTU was 98.7% (range: 96.3–99.9%; data from 18 different OTUs). This similarity increases when considering the whole gene (data not shown), since the fragment sequenced here encompasses one of the most variable 18S rDNA regions. Therefore, although seldom identical, clones from the same OTU were highly related phylogenetically. The relative distribution of OTUs in each library was used to calculate coverage values [28] and two independent estimators of the total number of OTUs. The first was the non-parametric S_{Chao1} estimator [29]. The second (S_T) was calculated assuming a log-normal species abundance curve, a total number of individuals of 5×10^7 (10 liters of sample at 5×10^3 cells ml^{-1}), and an abundance of 1 for the least abundant species [30].

The 18S rDNA insert from selected clones was partially sequenced by the Qiagen Genomics Sequencing Services using the internal primer Euk528f [31]. This single reaction yielded a sequence of more than 700 bp. Sequences were submitted to the BLAST search [32] for a first phylogenetic affiliation and to the CHECK-CHIMERA command [33] for detecting potential chimeric artifacts. Only one chimeric sequence was identified and it was excluded of the analyses. Sequences were aligned with about 4500 homologous eukaryotic 18S rRNA primary structures by using the automatic alignment tool of the ARB program package (http://www.mikro.biologie.tu-muenchen.de). The resulting alignment was checked and corrected manually. 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 retaining only the closest relatives of our clones. Some sequences presented here have been submitted to GenBank in separate papers, 4 prasinophyte sequences in Guillou et al. [34] and 30 novel stramenopile sequences in Massana et al. [35]. The remaining 116 sequences have been deposited under the Accession Nos. AY426829 to AY426945.

2.3. Culturing marine picoeukaryotes

Isolation attempts with Blanes Bay samples were performed with the same samples as the genetic libraries (Table 2). We also integrated in this study another isolation trial using a sample from the Barcelona harbor, a site 70 km south of Blanes Bay (Table 2). Large cells were removed from the seed sample by prefiltering through 0.6, 1, or 3 μm polycarbonate filters. These filtered samples were placed in 50 ml flasks without any amendment. They were used to inoculate (10 ml of sample in 50 ml final volume) three different media: autoclaved 0.2-μm filtered seawater (SW), mineral media for phytoplankton (F/2, [36]), and 0.2-μm filtered seawater autoclaved twice with 40 rice grains by liter (HET). Initial filtered samples and flasks with SW and F/2 media were placed at light (12:12 h light:dark regime, at 100 $\mu E\ m^{-2}\ s^{-1}$ light intensity provided by Sylvania Daylight fluorescent bulbs) whereas flasks with HET medium were placed at dark. Incubation temperature was 20 °C. Besides the initial inoculation (W1), additional ones were performed two (W2), six (W3) or eight (W4) weeks later using the initial filtered sample as explained before. Consequently, up to 36 pre-cultures (not all combinations were always performed) were obtained for each date: 3 filtered sizes (<0.6, <1 and <3 μm) × 3 media (SW, F/2 and HET) × 4 inoculations (W1, W2, W3 and W4). Each of these pre-cultures received a different code (BLA following by a number).

Contents of each flask were regularly examined by optical microscopy and flow cytometry. When growth was detected, the organisms were transferred into smaller tubes (15 ml) and maintained by subculturing into new medium every two weeks, keeping the same name. Sometimes, serial dilutions (SD) were done to purify flasks containing more than one species. Most of the strains isolated in Blanes Bay are available from the Roscoff Culture Collection (RCC, Roscoff, France, http://www.sb-roscoff.fr/Phyto/collect.html). Strains that do not have an RCC number were lost before their deposition in the collection. In the RCC, photosynthetic strains are maintained in F/2 medium and heterotrophic strains in HET medium. For phylogenetic analyses, cells from 50 ml of culture were collected by centrifugation and the genomic DNA was extracted with 3% cetyltrimethylammonium bromide (CTAB) [37]. The 18S rDNA gene was amplified, cloned (in most cases; 10 clones analyzed by RFLP and selection of distinctive

clones), and sequenced as previously explained. Sequences from Blanes cultures have been deposited in GenBank under Accession Nos. AY665979 to AY665996.

3. Results and discussion

The aim of this study was a description of the phylogenetic diversity of picoeukaryotes from an oligotrophic coastal system and a comparison of these molecular results with cultures obtained from the same samples. The diversity of planktonic picoeukaryotes has been studied mostly in the open sea [7–9] and very seldom in coastal environments [18], and molecular and culturing results from the same samples have never been directly compared. We constructed four genetic libraries of 18S rDNA genes from surface Blanes Bay samples (Table 1) and obtained 15 distinct isolates of picoeukaryotes following standard protocols (Table 2). Seven additional isolates from the nearby Barcelona harbor were also included in the study (Table 2). The reported isolates have been selected after a exhaustive culturing effort of 138 precultures and 57 serial dilutions attempts, choosing cultures that were pure, of small cell size and of different phylogenetic affiliation. The phylogenetic affiliation of clones in genetic libraries and of isolated cultures was assessed by partial sequencing of their 18S rDNA gene.

Approximately 100 clones from each genetic library were analyzed by RFLP, yielding between 36 and 54 different OTUs per library (Table 1). Coverage values in each library were relatively low, between 54 and 76% (Table 1), and accumulation curves showed little signs of stabilization (Fig. 1). Both indices suggested that we were undersampling the picoeukaryotic diversity in these samples, and showed a slightly larger diversity in BL000921 and BL010625 libraries. This was also confirmed by two independent estimators of the total number of OTUs in the libraries: S_{Chao1} estimated the total number of OTUs as 2–4 times the number of OTUs detected, whereas this ratio ranged from 4 to 22 after the S_{T} estimator. Despite the different values, both models indicated similar trends. Nevertheless, the predominant OTUs (those that appear repeatedly in our clone libraries) were clearly detected by our analytical strategy, and the undersampled diversity is likely accounted for by rare (unique) OTUs.

A representative clone of each OTU in each library was partially sequenced (Table 1). A fraction of the libraries (17 OTUs and 32 clones) affiliated with marine metazoans, such as appendicularians, bivalves, copepods, nemertea, polychaetes, siphonophorans and sponges. The mechanism by which the DNA of these large organisms is collected in the 0.2–3 μm fraction remains unexplained, although this seems to be a recurrent phenomenon in similar studies [7,18, unpublished results]. In some cases, breakage of organisms could have occurred resulting in small fragments of the corresponding size. Another explanation would be the existence of free DNA from these organisms, dissolved or attached to particles. Also, most benthic metazoans have a complex life cycle including a phase of small planktonic cells. This could have been the case for the bivalves and polychaetes found in the June library (10 OTUs and 21 clones), since these organisms spawn in early summer in the Mediterranean coast [38]. In any case, metazoan sequences were never dominant (overall they were <10% of clones) and neither were the target of our study, and therefore have been excluded from further analysis.

As expected, most clones from libraries affiliated with planktonic protists (Table 1), and they were analyzed in four steps. First, 18S rDNA sequences from clones (and from cultures) were compared with reference organisms in the same phylogenetic tree (Fig. 2). Second, we reported the number of OTUs (and the number of clones per OTU) from the main groups (Fig. 3(a)). This showed the number of different sequences obtained per group (and thus the extent of intragroup richness) and the redundancy of OTUs inside each group. Third, we investigated the similarity of clones to sequences deposited in GenBank (search on 1 November 2003). This was done by averaging the BLAST best score, and the best score with a known organism, for all sequences of each phylogenetic group (Fig. 3(b)). This revealed which groups were represented by known organisms (high

Table 1
Summary of picoeukaryotic 18S rDNA libraries generated from Blanes Bay

Library	Number of OTUs (number of clones)				Coverage (%)	Estimated number of OTUs	
	Total	Protists	Metazoans	ND		S_{Chao1}	S_{T}
BL000921	44 (78)	39 (71)	1 (1)	4 (6)	54	171	562
BL001221	42 (111)	38 (106)	4 (5)	0 (0)	76	77	177
BL010320	36 (88)	32 (81)	1 (3)	3 (4)	73	92	180
BL010625	54 (106)	41 (81)	11 (23)	2 (2)	70	95	1206

The number of OTUs detected in each library (between parentheses the number of clones) is shown separated among those affiliating to protists, to metazoans and those not sequenced (ND). The distribution of OTUs in each library is used to calculate coverage values and to estimate the total number of OTUs following two estimators (S_{Chao1} and S_{T}).

Table 2
Summary of cultures isolated in this study

Name	Species	Class	Trophic mode	Origin	Date of sampling	Isolation method	Accession Nos.
RCC 500	*Tetraselmis* sp.	Prasinophyceae	Phototrophic	Blanes Bay	25 Jun 2001	<0.6 μm, F/2, W4, SD	AY425299
RCC 434	*Micromonas pusilla*	Prasinophyceae	Phototrophic	Blanes Bay	20 Mar 2001	<1.0 μm, F/2, W1	AY425316
RCC 499	*Nephroselmis pyriformis*	Prasinophyceae	Phototrophic	Barcelona harbor	28 Feb 2001	<3.0 μm, SW, W1, SD	AY425306
RCC 501	*Ostreococcus* sp.	Prasinophyceae	Phototrophic	Barcelona harbor	28 Feb 2001	<0.6 μm, F/2, W2, SD	AY425313
BLA 77	*Bathycoccus prasinos*[*]	Prasinophyceae	Phototrophic	Barcelona harbor	28 Feb 2001	<1.0 μm, SW, W1	AY425315
RCC 498	*Micromonas pusilla*	Prasinophyceae	Phototrophic	Barcelona harbor	28 Feb 2001	<1.0 μm, F/2, W1, SD	
RCC 502	*Emiliania huxleyi*	Haptophyceae	Phototrophic	Blanes Bay	25 Jun 2001	<1.0 μm, SW, W3, SD	
RCC 437	*Pavlova* sp.	Haptophyceae	Phototrophic	Blanes Bay	21 Sep 2000	<3.0 μm, F/2, W2, SD	
RCC 439	*Hemiselmis* sp.	Cryptophyceae	Phototrophic	Blanes Bay	21 Dec 2000	<1.0 μm F/2, W2, SD	
RCC 506	Not determined[*]	Cryptophyceae	Phototrophic	Blanes Bay	25 Jun 2001	<3.0 μm, F/2, W4	
RCC 589	Not determined[*]	Cryptophyceae	Heterotrophic	Blanes Bay	20 Mar 2001	<3.0 μm, SW, W1	
BLA 73	Not determined[*]	Cryptophyceae	Phototrophic	Barcelona harbor	28 Feb 2001	<0.6 μm, F/2, W1	
RCC 435	*Chlorarachnion* sp.	Chlorarachniophyte	Phototrophic	Blanes Bay	21 Sep 2000	<0.6 μm, SW, W3, SD	
RCC 438	*Nannochloropsis* sp.	Eustigmatophyceae	Phototrophic	Blanes Bay	21 Dec 2000	<0.6 μm, F/2, W1	
RCC 504	*Nannochloropsis* sp.	Eustigmatophyceae	Phototrophic	Blanes Bay	20 Mar 2001	<3.0 μm, F/2, W4, SD	
RCC 503	Not determined	Pinguiophyceae	Phototrophic	Blanes Bay	25 Jun 2001	<1.0 μm, F/2, W4, SD	
RCC 505	*Florenciella* -like	Dictyochophyceae	Phototrophic	Blanes Bay	21 Dec 2000	<1.0 μm, SW, W1, SD	
RCC 585	Not determined[*]	Bicosoecida	Heterotrophic	Blanes Bay	21 Dec 2000	<3.0 μm, SW, W2	
RCC 587	Not determined	Bicosoecida	Heterotrophic	Blanes Bay	21 Dec 2000	<1.0 μm, SW, W2	
RCC 586	Not determined[*]	Bicosoecida	Heterotrophic	Blanes Bay	25 Jun 2001	<3.0 μm, HET, W3	
RCC 588	*Paraphysomonas* sp.	Chrysophyceae	Heterotrophic	Barcelona harbor	28 Feb 2001	<3.0 μm, F/2, W1	
RCC 440	Not determined	Bicosoecida	Heterotrophic	Barcelona harbor	28 Feb 2001	<1.0 μm, F/2, W2	

All cultures have been deposited to the Roscoff Culture Collection (RCC) except BLA 77 and BLA 73. Some cultures (marked with [*]) are no longer available. Genus and species names are given only after a formal inspection. Isolation method refers to the initial prefiltration, media used, and inoculation steps (see Section 2.3).

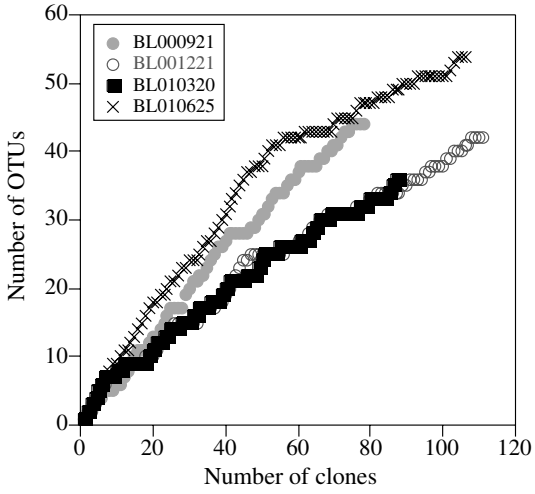

Fig. 1. Accumulation curves for Blanes Bay genetic libraries representing the number of OTUs detected when increasing the number of clones analyzed. Library BL000921 was constructed from a sample collected on 21 September 2000, BL001221 on 21 December 2000, BL010320 on 20 March 2001, and BL010625 on 25 June 2001. OTUs were defined as clones sharing the same RFLP pattern.

scores in the two cases), which groups were represented by environmental sequences only (high score with the best match and low score with the best known match), and which groups are not yet represented in databases (low scores in both cases). Finally, we compared the picoeukaryotic composition of the four libraries by presenting the percentage of clones affiliating to the six main groups (Fig. 4). The particular results of these analyses will be discussed for each group following the main eukaryotic divisions presented in Baldauf [13].

3.1. The marine alveolates

The alveolates form a complex phylogenetic assemblage that includes, among others, dinoflagellates, ciliates and apicomplexans [39]. In addition, novel alveolate groups have been identified from sequences retrieved from the sea. In Blanes libraries, novel alveolates-I [14] accounted for 36% of the total number of clones and dominate in three libraries (Fig. 4). These sequences were often >99% similar to clones from coastal waters [18] and anoxic environments [11,12], and thus presented a high BLAST score (Fig. 3(b)). The score was much lower when considering only known organisms (Fig. 3(b)), a proof of the lack of cultured examples of this ubiquitous and apparently significant marine group. Novel alveolates-I presented a high redundancy in our libraries: on average each OTU represented seven clones instead of the 1–3 of the remaining groups (Fig. 3(a)). A very similar sequence (BL000921.1, BL001221.9, BL010320.12, BL010625.15, >98.6%) accounted for many clones in each library (12, 37, 10 and 9, respectively), suggesting that the corresponding organism was important in these coastal waters. Novel alveolates-II [14] were also present in Blanes libraries but with a modest representation (5% of clones). These sequences were closest to clones retrieved from the marine picoplankton over the world (Fig. 3(b)), but were seldom highly similar (between 92.5 and 98.4%). In Blanes libraries, this group is rather diverse and the same sequence is barely retrieved from different dates (Fig. 2). The presence of the parasite *Amoebophrya* within this group [40] may indicate that novel alveolates-II might be parasites as well [9], and this could explain the apparent high genetic diversity within the group.

The main known alveolate groups were also represented in Blanes libraries (Figs. 2 and 4). Dinoflagellates were the second group in clonal abundance (17% in total), and the retrieved sequences were generally very similar (98–99%) to those of known organisms. Dinoflagellates are known to be larger than 3 μm [3], and their presence in the fraction analyzed could be either due to cell breakage or very small dinoflagellates not yet isolated. Dinoflagellate sequences from Blanes were similar among them, but identical sequences were seldom found on different dates (Fig. 2), suggesting that dinoflagellates were a dynamic component of the plankton. Ciliates were represented by some clones that were moderately related to cultured ciliates (Figs. 2 and 3(b)). Again, ciliates smaller than 3 μm are not known, so cell breakage of these fragile cells [41] might explain their detection in the libraries. Apicomplexans were represented by only one sequence (BL001221.22), whereas BL000921.23 affiliated within alveolates but was not related to any known group.

Contrasting with the exhaustive prevalence of alveolates in the four Blanes libraries (particularly novel alveolates-I and dinoflagellates), it is remarkable that we did not obtain any alveolate isolate. We know that the media and the initial isolation steps (such as filtration) was not adequate for some groups, like dinoflagellates and ciliates. Nevertheless, what is more challenging is our failure to retrieve novel alveolates in culture, which currently constitute one of the uncultivable but seemingly important marine groups.

3.2. The marine stramenopiles

Like alveolates, stramenopiles form a consistent phylogenetic assemblage of different protist groups [42]. Novel marine stramenopiles (MAST), common in other marine systems [35,43], were very important in Blanes libraries (10% of clones). BLAST scores were high with other marine sequences (generally >98%) and declined drastically when considering only known organisms (Fig. 3(b)). Novel marine stramenopiles include up to 12 different phylogenetic lineages affiliating among the heterotrophic basal stramenopile groups [35], and seven of them were present in Blanes libraries (Fig. 2). MAST-

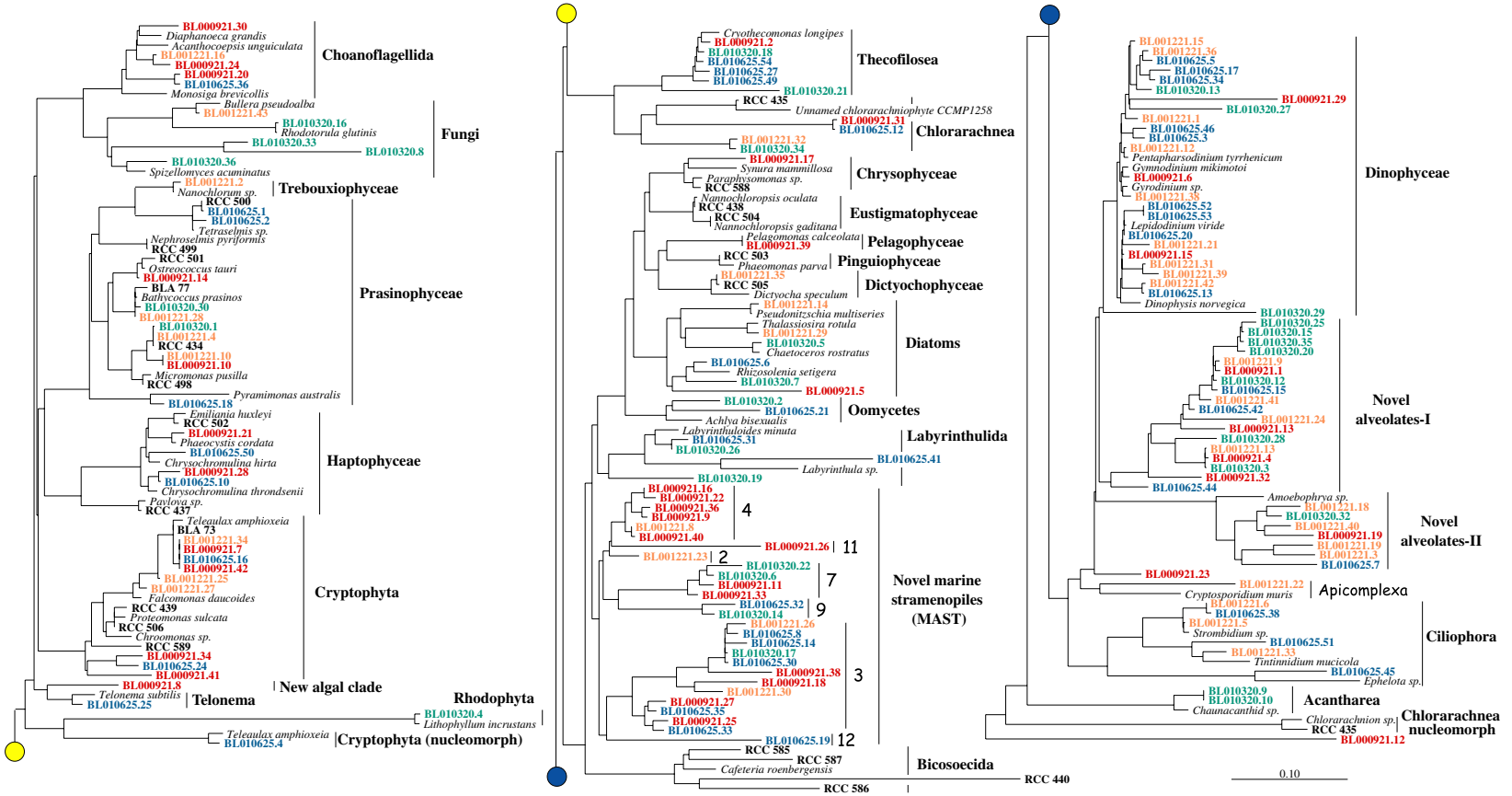

Fig. 2. Phylogenetic tree with partial 18S rDNA sequences retrieved from Blanes Bay (both in environmental libraries and cultures) and their closest cultured organisms. The tree was based on a previous tree in ARB. Environmental clones are named after the code of the library and a number, and appear with a different color for each library. Cultures are shown in black boldface and are named BLA or RCC (those deposited to the Roscoff Culture Collection). The scale bar indicates 0.1 substitutions per position.

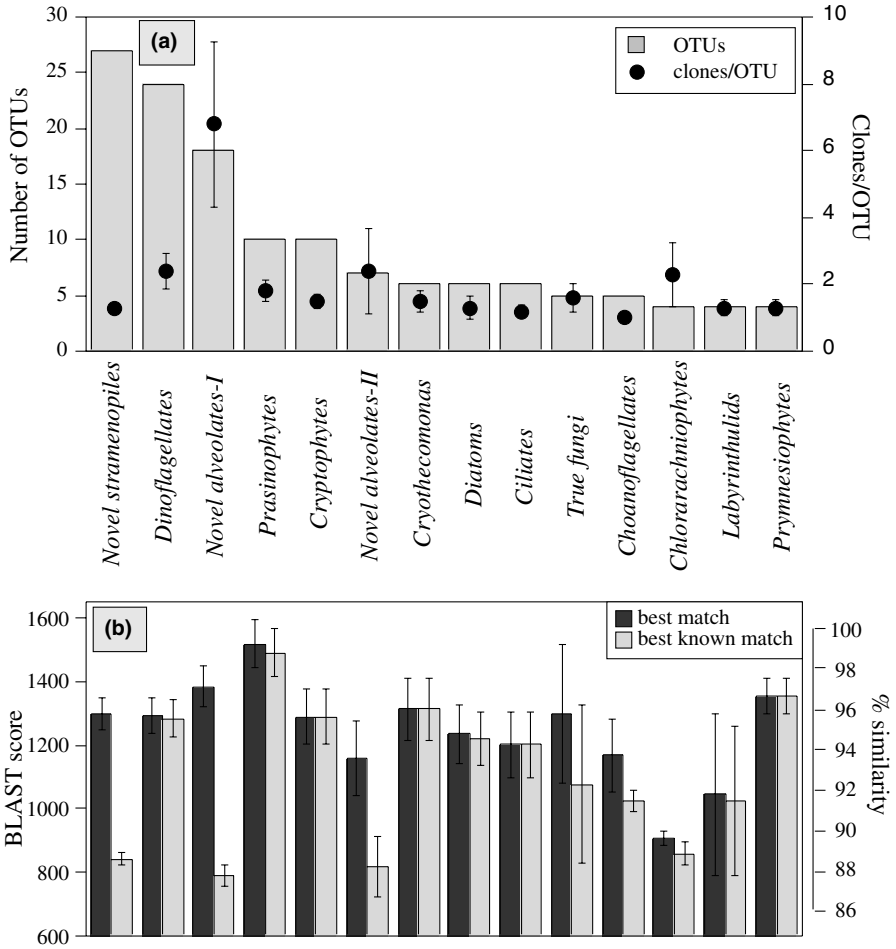

Fig. 3. (a) Number of OTUs identified in each major phylogenetic group (14 OTUs from 11 additional groups are excluded from the graph). The average redundancy of each OTU within each group (number of clones per OTU) is shown overlaid. Vertical bars are standard errors. (b) Averaged BLAST score for all sequences within the major phylogenetic groups. Dark bars show the score with the best match, whereas light bars show the score considering only known organisms (thus excluding environmental sequences). The separated right axis gives an idea of the sequence similarity corresponding to BLAST scores (calculated with the averaged sequence length of 767 bp).

3 was the dominant lineage (12 OTUs and 15 clones), MAST-4 (seven clones) and MAST-7 (six clones) were moderately important, whereas the remaining lineages were represented by one or two clones. The high number of MAST clusters detected, plus the fact that the same sequence was seldom found on different dates, showed MAST organisms as a diverse and dynamic component of this coastal system. A few sequences affiliated within known heterotrophic basal stramenopile groups (Fig. 2), such as labyrinthulids (five clones) and oomycetes (four clones), groups that could be more represented in coastal than in open sea waters [35]. Concerning stramenopiles within the photosynthetic radiation [44], we retrieved six OTUs (eight clones) related to diatoms, each one to a different genus, one sequence identical to a picoeukaryotic pelagophyte, one moderately related to a chrysophyte and another one related to a dictyochophyte (Fig. 2).

Some of the Blanes isolates affiliated with stramenopiles. Within the photosynthetic radiation, four algal isolates were very similar to established cultures within Dictyochophyceae (RCC 505, similar to the new genus *Florenciella ultra* (Eikrem et al., submitted), not shown in the tree), Eustigmatophyceae (RCC 438 and RCC 504) and Pinguiophyceae (RCC 503), and one heterotrophic isolate (RCC 588) to Chrysophyceae. Heterotrophic organisms within chrysophytes have been explained by an evolutionary loss of the chloroplast [44]. The other four heterotrophic isolates (RCC 440, RCC 585, RCC 586 and RCC 587) belonged to bicosoecids, a basal stramenopile group of heterotrophic flagellates commonly found in cultures. No MAST organism was retrieved in culture. Therefore, except for the dictyochophyte example (the culture was identical to an environmental sequence), molecular and culturing approaches gave different pictures of the

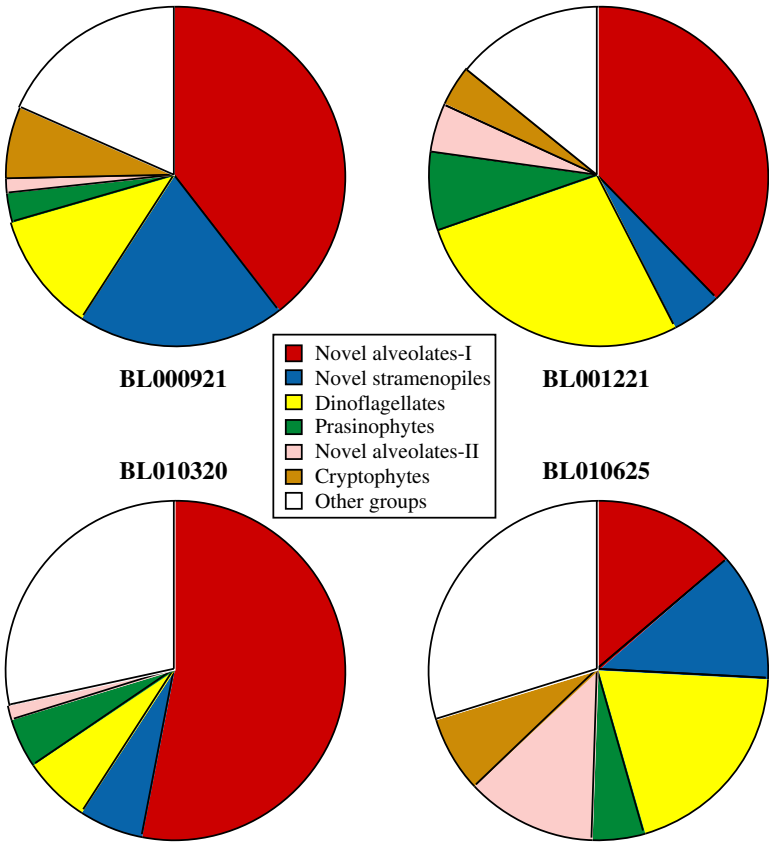

Fig. 4. Relative abundance of clones within the six most represented phylogenetic groups in the four genetic libraries from Blanes Bay.

diversity of picoeukaryotic stramenopiles, highlighting the biases that can occur when studying diversity by culture dependent methods [24,25].

3.3. The marine green algae and relatives

This group was represented in Blanes libraries mostly by prasinophytes together with a few sequences within Trebouxiophyceae, Rhodophyta, and a novel algal clade (Fig. 2), the latter being related to Glaucocystophyceae (Valentin et al., submitted). Prasinophyceae, a class known to include many picoeukaryotic strains [34], were the fourth group in abundance in our libraries (5% of clones, after novel alveolates-I, dinoflagellates and novel stramenopiles) and accounted always for a constant fraction of clones (Fig. 4). Prasinophyte sequences from Blanes libraries were always very similar to described cultures and thus presented the highest BLAST score (Fig. 3(b)). The composition was different in the June library, when *Tetraselmis* and *Pyramimonas* were found, and the rest of the year, dominated by *Micromonas* (10 of the 18 prasinophyte clones), *Bathycoccus* and *Ostreococcus*. It was interesting to note that the *Micromonas* found in consecutive dates in Blanes was the same that dominated in other marine systems [34]. Many of the cultures obtained in our study were prasinophytes (Table 2), and the rDNA gene of some of them (RCC 500, RCC 501, BLA 77 and RCC 434) was nearly identical to environmental clones (Fig. 2). Therefore, prasinophytes offer the best example of a group of relevant picoeukaryotes in nature that can be readily studied by culturing approaches.

3.4. The marine cercozoans

The Cercozoa form another phylogenetically coherent assemblage of different protist groups [45]. In Blanes libraries we retrieved two cercozoan groups in most samples at low abundances (nine clones total each). Some clones affiliated with Thecofilosea (Fig. 2) and were similar to the marine amoeboflagellate with pseudopodial feeding *Cryothecomonas* (96–99%, excluding the divergent BL010329.21). Similar sequences have been retrieved from the Mediterranean Sea (ME1-26) and the Antarctic Ocean (ANT37-28), indicating a wide distribution of this genus [7]. The second cercozoan group was composed by four sequences (one per sample) that formed two clusters of almost identical sequences within Chlorarachnea (Fig. 2). These sequences were quite distant with its closest culture (<93%, with strain CCMP1258) and were remarkably absent from other marine open sea and coastal systems (clone NA37-5

from the North Atlantic, very similar to BL001221.32, was the exception, [7]). Thus, BLAST scores are low for both searches in Fig. 3(b). These novel chlorarachniophytes could be significant members of coastal picoplankton in Blanes Bay.

We obtained only one cercozoan isolate (RCC 435). It was relatively similar to the chlorarachniophyte strain CCMP1258, and this was confirmed by a second sequence from the same culture related to the nucleomorph of this strain (Fig. 2). It is interesting that, within chlorarachniophytes, environmental sequences represent novel and undescribed organisms (which are likely the most abundant in the environment), whereas isolates coincide with organisms already described. This highlights how the cultivable fraction of the community represents in some cases organisms that are not necessarily the most representative.

3.5. The marine opisthokonts

This phylogenetic assemblage contains, among other groups, metazoans, choanoflagellates and true fungi. As mentioned before, metazoans are not considered here since their presence in our libraries was an undesired artifact. Choanoflagellates, generally considered significant members of marine heterotrophic flagellate assemblages [5], were detected year-round (three of the four libraries) although in low numbers (five clones). Their sequences were generally distant from cultured organisms (Fig. 2), indicating that there is still a large diversity that needs to be retrieved in culture. Fungi were detected mostly from one library (seven of eight clones from BL010320) and often their sequences did not have a cultured counterpart (Fig. 2). These fungal sequences could reflect terrestrial or sediment influence in this particular sampling date. Nevertheless, the existence and distribution of truly planktonic fungi has not been well explored and deserves further research. No choanoflagellate or fungi culture have been retrieved from Blanes samples.

3.6. The remaining groups

Cryptophytes and haptophytes are two important marine algal groups and are discussed here since their phylogenetic position among the eight major eukaryotic divisions is unclear [13]. Cryptophytes were found in all libraries except one (nine OTUs and 14 clones) and accounted for a significant fraction of clones (Fig. 4). Half of these clones were very close to *Teleaulax amphioxeia* and another sequence affiliated with its nucleomorph (Fig. 2). In addition, one of the cultures obtained (BLA 73) was nearly identical to these sequences. This points towards the presence of *Teleaulax amphioxeia* (or some highly related cryptophyte) in Blanes Bay throughout the year. The other cryptophyte sequences retrieved from the libraries and the isolated cultures were not similar among them or to known organisms, reflecting a large diversity in this group not yet characterized (Fig. 2). One of these cultures (RCC 589) was heterotrophic.

Haptophyte sequences were retrieved from the September and June libraries (four OTUs and five clones) and were related to *Phaeocystis* and *Chrysochromulina* species (Fig. 2). The low representation of this group in the libraries probably does not reflect its importance in marine systems, where pigment signature analyses suggest it to be one of the most important algal groups in Blanes Bay (Mikel Latasa, personal communication) as well as in open waters [46]. This still remains an open question that should be soon addressed by FISH probes. The two isolates obtained from Blanes samples were related to *Emiliania* and *Pavlova* (Fig. 2), giving another example of disparate diversity from molecular and culturing approaches.

Finally, some sequences affiliated with uncertain phylogenetic groups. Clone BL010625.25 was similar to the heterotrophic flagellate *Telonema subtilis*, and its particular phylogenetic placement will be investigated in detail in a separate study (Schalchian-Tabrizi et al., submitted). Two sequences from the BL010320 library (four clones) were similar (>97%) to a recently sequenced acantharid [47]. These are typical open-sea marine protists but generally of larger size. Finally, clone BL000921.12 appeared at the base of the tree and was different to any known eukaryotic sequence.

3.7. General conclusions

Our data show some clear trends in the diversity of picoeukaryotes in this coastal oligotrophic environment. First, the diversity was very large in the four samples analyzed, each one corresponding to a different season of the year. This was shown by the high number of OTUs and different phylogenetic groups retrieved (representing at least five of the eight major eukaryotic divisions, [13]) even though the libraries were clearly undersampled, as indicated by the non-saturating accumulation curves (Fig. 1), low coverage values, and estimated number of OTUs several times larger than the actual number (Table 1). It is obvious that additional OTUs would have been retrieved if we had analyzed more clones, but also that a more exhaustive analysis of the libraries would not change significantly the overall picture. Essentially all the sequences retrieved corresponded to marine organisms or to sequences found in marine studies, indicating the usefulness of this approach to investigate marine assemblages. Our data confirms and expands the large diversity of marine picoeukaryotes found in previous studies [7,9,18].

Second, the groups better represented in Blanes libraries were the novel alveolates-I (36% of clones), dinoflagellates (17%), novel stramenopiles (10%), pra-

sinophytes (5%), novel alveolates-II (5% of clones), and cryptophytes (4%). Except the latter, these groups were found in the four libraries (Fig. 4). The eukaryotic picoplankton in Blanes Bay appeared dominated by known phytoplankton groups (dinoflagellates, prasinophytes and cryptophytes) and novel groups (alveolates and stramenopiles), although a proper quantification of the abundance of each one would require the application of other molecular tools, such as FISH with specific probes. For instance, the dominance of novel alveolates-I could derive from a higher copy number of the rDNA operon (and so its higher OTU redundancy, Fig. 3(a)) and not to higher cell abundance. In any case, our data show again the contrast between the potential importance of novel marine groups [14,43] and our lack of knowledge of their morphology, functional capabilities and ecological role. Obviously culturing efforts on these novel groups are encouraged, and recent success with previously though ''uncultivable'' bacteria [48], gives hope that these groups will be cultured in the future when finding the appropriate conditions.

Third, even though the composition of picoeukaryotes remained relatively stable in terms of phylogenetic groups detected (Fig. 4), a closer look revealed that particular sequences were often found only in one or two consecutive dates. This could be partially explained by the low coverage of our genetic libraries. There were exceptions to this general trend pointing to some organisms found year-round, as the most common sequence within novel alveolates-I, some MAST-3 examples, and the sequences related to *Micromonas*, *Teleaulax* and *Cryothecomonas*. Whole picoeukaryotic assemblages could be better compared by a fingerprinting technique such as DGGE, which revealed a highly variable band pattern in consecutive monthly samples (unpublished results). Therefore, the results obtained in our genetic libraries are just four snapshots of the picoeukaryotic composition and do not represent an exhaustive compendium of all organisms that live in this coastal environment. The large temporal variability of picoeukaryotic assemblages contrasts with the relatively small seasonal changes of the bacterioplankton in the same site [19]. Mechanisms driving these different dynamics should be better identified.

Fourth, the picoeukaryotes from this coastal oligotrophic system were similar to those found in open sea and other coastal habitats (Fig. 3(b) [7–9,18]). Only a few sequences, such as those of chlorarachniophytes and perhaps labyrinthulids, appear rather restricted to Blanes Bay, showing a low averaged BLAST score (Fig. 3(b)). The detection of similar sequences in distant places (many clones show >99% similarity to clones retrieved in marine systems thousands km apart) seems to indicate ubiquitous marine picoeukaryotic populations, as has been suggested for marine bacteria [49] and archaea [50]. However, some particularities of coastal habitats are evident, mostly in the percentages of specific groups. Prasinophytes and prymnesiophytes are more common in open sea libraries whereas novel alveolates-I and dinoflagellates dominated in Blanes libraries. Other groups more represented in Blanes libraries were cryptophytes, oomycetes, labyrinthulids, chlorarachniophytes, fungi and choanoflagellates. The only coastal study so far, from a colder and more eutrophic system, gives again a different picture in the proportions of clones, with a lower representation of dinoflagellates and novel alveolates-I and a higher representation of novel alveolates-II and ciliates [18].

Finally, the simultaneous use of molecular and culturing approaches to study picoeukaryotic diversity clarifies and confirms some trends that have been often suspected but seldom directly assessed. It is evident that culturing biases the estimation of microbial diversity in a more dramatic way than molecular approaches, despite the inherent biases of PCR-based methods [23]. This has been shown both for marine picoeukaryotes [24,25] and for planktonic bacteria and archaea [49]. The extent of culturing bias, however, depends on the group considered. Thus, some groups like the prasinophytes can be properly studied by culturing [51], since their isolates match perfectly the sequences from genetic libraries [34]. Other groups such as haptophytes, cryptophytes, chlorarachniophytes and phototrophic stramenopiles are represented in both approaches, but isolates generally do not coincide with the sequences in libraries. Since this data has been gathered from the same samples, this suggests some isolation bias. Nevertheless, there are many isolates whose 18S rDNA gene has not been obtained, and could account for part of the discrepancies. Bicosoecids offer an example of a group not found in libraries but dominating the isolates of heterotrophic flagellates. Finally, novel stramenopiles and novel alveolates have never been obtained in culture and they are only known by environmental sequences in genetic libraries. Given the recurrence of these groups they must play significant roles in marine ecosystems. For instance, novel stramenopiles have been found to be members of heterotrophic flagellates [43], a key functional group in marine microbial food webs as grazers of bacteria, food for larger zooplankton, and remineralizers of nutrients. The large diversity of picoeukaryotes in most marine systems must be linked to a wide array of functional roles that deserve further research.

Acknowledgments

This work was funded by EU Contracts PICODIV (EVK3-CT1999-00021) and BASICS (EVK3-CT-2002-00078). We also thanked the EU Contract INPRIMAT (QLRI-CT-2002-01325) for logistical support. We appreciate the help from Ramon Terrado in sequence

management and Tom Curtis for the routine to calculate species richness.

References

[1] Sherr, E.B. and Sherr, B.F. (2000) Marine Microbes. An Overview In: Microbial Ecology of the Oceans (Kirchman, D.L., Ed.), pp. 13–46. Wiley-Liss, New York.

[2] Li, W.K.W. (1994) Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: measurements from flow cytometric sorting. Limnol. Oceanogr. 39, 169–175.

[3] Thomsen, H.A. (1986) A survey of the smallest eukaryotic organisms of the marine phytoplankton. In: Photosynthetic Picoplankton (Platt, T. and Li, W.K.W., Eds.), pp. 121–158. Can. Bull. Fish. Aquat. Sci. vol. 214, pp. 121–158.

[4] Caron, D.A., Peele, E.R., Lim, E.L. and Dennett, M.R. (1999) Picoplankton and nanoplankton and their trophic coupling in the surface waters of the Sargasso Sea south of Bermuda. Limnol. Oceanogr. 44, 259–272.

[5] Fenchel, T. (1986) The ecology of heterotrophic microflagellates. Adv. Microb. Ecol. 9, 57–97.

[6] Giovannoni, S.J., Britschgi, T.B., Moyer, C.L. and Field, K.G. (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345, 60–63.

[7] Díez, B., Pedrós-Alió, C. and Massana, R. (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl. Environ. Microbiol. 67, 2932–2941.

[8] López-García, P., Rodríguez-Valera, F., Pedrós-Alió, C. and Moreira, D. (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409, 603–607.

[9] Moon-van der Staay, S.Y., De Wachter, R. and Vaulot, D. (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409, 607–610.

[10] Dawson, S.C. and Pace, N.R. (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc. Natl. Acad. Sci. USA 99, 8324–8329.

[11] Edgcomb, V.P., Kysela, D.T., Teske, A., de Vera Gomez, A. and Sogin, M.L. (2002) Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. Proc. Natl. Acad. Sci. USA 99, 7658–7662.

[12] Stoeck, T., Taylor, G.T. and Epstein, S.S. (2003) Novel eukaryotes from the permanently anoxic Cariaco Basin (Caribbean Sea). Appl. Environ. Microbiol. 69, 5656–5663.

[13] Baldauf, S.L. (2003) The deep roots of eukaryotes. Science 300, 1703–1706.

[14] Moreira, D. and López-García, P. (2002) The molecular ecology of microbial eukaryotes unveils a hidden world. Trends in Microbiology 10, 31–38.

[15] Cebrián, J. and Valiela, I. (1999) Seasonal patterns in phytoplankton biomass in coastal ecosystems. J. Plankton Res. 21, 429–444.

[16] Worden, A.Z., Nolan, J.K. and Palenik, B. (2004) Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. Limnol. Oceanogr. 49, 168–179.

[17] Bricelj, V.M. and Lonsdale, D.J. (1997) *Aureococcus anophagefferens* : Causes and ecological consequences of brown tides in U.S. mid-Atlantic coastal waters. Limnol. Oceanogr. 42, 1023–1038.

[18] Romari, K. and Vaulot, D. (2004) Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnol. Oceanogr. 49, 784–798.

[19] Schauer, M., Balagué, V., Pedrós-Alió, C. and Massana, R. (2003) Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. Aquat. Microb. Ecol. 31, 163–174.

[20] Duarte, C.M., Agustí, S., Kennedy, H. and Vaqué, D. (1999) The Mediterranean climate as a template for Mediterranean marine ecosystems: the example of the northeast Spanish littoral. Progr. Oceanogr. 44, 245–270.

[21] Calbet, A., Garrido, S., Saiz, E., Alcaraz, M. and Duarte, C.M. (2001) Annual zooplankton succession in coastal NW Mediterranean waters: the importance of the smaller size fractions. J. Plankton Res. 23, 319–331.

[22] Andersen, R.A., Saunders, G.W., Paskind, M.P. and Sexton, J.P. (1993) Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the *Pelagophyceae classis* nov. J. Phycol. 29, 701–715.

[23] Von Witzingerode, F., Göbel, U.B. and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229.

[24] Guillou, L., Moon-van der Staay, S.Y., Claustre, H., Partensky, F. and Vaulot, D. (1999) Diversity and abundance of Bolidophyceae (Heterokonta) in two oceanic regions. Appl. Environ. Microbiol. 65, 4528–4536.

[25] Lim, E.L., Dennet, M.R. and Caron, D.A. (1999) The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures. Limnol. Oceanogr. 44, 37–51.

[26] Paul, J.H. and Myers, B. (1982) Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. Appl. Environ. Microbiol. 43, 1393–1399.

[27] Medlin, L., Elwood, H.J., Stickel, S. and Sogin, M.L. (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71, 491–499.

[28] Good, I.J. (1953) The population frequencies of species and the estimation of the population parameters. Biometrika 40, 237–264.

[29] Chao, A. (1984) Non-parametric estimation of the number of classes in a population. Scand. J. Statist. 11, 265–270.

[30] Curtis, T.P., Sloan, W.T. and Scannell, J.W. (2002) Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. USA 99, 10494–10499.

[31] Elwood, H.J., Olsen, G.J. and Sogin, M.L. (1985) The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. Mol. Biol. Evol. 2, 399–410.

[32] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

[33] Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker Jr., C.T., Saxman, P.R., Stredwick, J.M., Garrity, G.M., Li, B., Olsen, G.J., Pramanik, S., Schmidt, T.M. and Tiedje, J.M. (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res. 28, 173–174.

[34] Guillou, L., Eikrem, W., Chrétiennot-Dinet, M.-J., Le Gall, F., Massana, R., Romari, K., Pedros-Alió, C. and Vaulot, D. (2004) Diversity of picoplanktonic Prasinophyceae assessed by direct SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist 155, 193–214.

[35] Massana, R., Castresana, J., Balagué, V., Guillou, L., Romari, K., Groisillier, A., Valentin, K. and Pedrós-Alió, C. (2004) Phylogenetic and ecological analysis of novel marine stramenopiles. Appl. Environ. Microbiol. 70, 3528–3534.

[36] Guillard, R.R.L. and Ryther, J.H. (1962) Studies on marine planktonic diatoms. 1 *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gram. Can. J. Microbiol. 8, 229–239.

[37] Doyle, J.J. and Doyle, J.L. (1987) A rapid DNA isolation for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

[38] Coma, R., Ribes, M., Gili, J.M. and Zabala, M. (2000) Seasonality in coastal benthic ecosystems. TREE 15, 448–453.
[39] Kuvardina, O.N., Leander, B.S., Aleshin, V.V., Myl'nikov, A.P., Keeling, P.J. and Simdyanov, T.G. (2002) The phylogeny of Colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. J. Eukaryot. Microbiol. 49, 498–504.
[40] Gunderson, J.H., Goss, S.H. and Coats, D.W. (1999) The phylogenetic position of *Amoebophrya* sp. infecting *Gymnodinium sanguineum*. J. Euk. Microbiol. 46, 194–197.
[41] Sime-Ngando, T., Hartmann, H.J. and Groliere, C.A. (1990) Rapid quantification of planktonic ciliates: Comparison of improved live counting with other methods. Appl. Environ. Microbiol. 56, 2234–2242.
[42] Patterson, D.J. (1989) Stramenopiles: chromophytes from a protistan perspective In: Chromophyte Algae: Problems and Perspectives (Green, J.C., Leadbeater, B.S.C. and Diver, W.L., Eds.), pp. 357–379. Clarendon Press, Oxford.
[43] Massana, R., Guillou, L., Díez, B. and Pedrós-Alió, C. (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl. Environ. Microbiol. 68, 4554–4558.
[44] Leipe, D.D., Tong, S.M., Goggin, C.L., Slemenda, S.B., Pieniazek, N.J. and Sogin, M.L. (1996) 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. Eur. J. Protistol. 32, 449–458.
[45] Cavalier-Smith, T. and Chao, E.-Y. (2003) Phylogeny and classification of Phylum Cercozoa (Protozoa). Protist 154, 341–358.
[46] Moon-van der Staay, S.Y., van der Staay, G.W.M., Guillou, L., Vaulot, D., Claustre, H. and Medlin, L.K. (2000) Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. 45, 98–109.
[47] Amaral Zettler, L.A. and Caron, D.A. (2000) New insights into the phylogeny of the Acantharea based on SSU rRNA gene sequences. Eur. J. Protistol. 36, 34–39.
[48] Rappé, M.S., Connon, S.A., Vergin, K.L. and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418, 630–633.
[49] Giovannoni, S. and Rappé, M. (2000) Evolution, Diversity, and Molecular Ecology of Marine Prokaryotes In: Microbial Ecology of the Oceans (Kirchman, D.L., Ed.), pp. 47–84. Wiley-Liss, New York.
[50] Massana, R., DeLong, E.F. and Pedrós-Alió, C. (2000) A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. Appl. Environ. Microbiol. 66, 1777–1787.
[51] Zingone, A., Sarno, D. and Forlani, G. (1999) Seasonal dynamics in the abundance of *Micromonas pusilla* (Prasinophyceae) and its viruses in the Gulf of Naples (Mediterranean Sea). J. Plankton Res. 21, 2143–2159.