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Analysis of photosynthetic picoeukaryote diversity at open ocean sites in the Arabian Sea using a PCR biased towards marine algal plastids

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ABSTRACT: Marine photosynthetic picoeukaryotes (PPEs), representing organisms <3 µm in size, are major contributors to global carbon cycling. However, the key members of the PPE community and hence the major routes of carbon fixation, particularly in the open ocean environment, are poorly described. Here, we have accessed PPE community structure using the plastid encoded 16S rRNA gene. Plastid 16S rRNA genes were sequenced from 65 algal cultures, about half being PPEs, representing 14 algal classes. These included sequences from 5 classes where previously no such sequences from cultured representatives had been available (Bolidophyceae, Dictyochophyceae, Eustigmatophyceae, Pelagophyceae and Pinguiophyceae). Sequences were also obtained for 6 of the 7 (according to 18S rRNA gene sequence) prasinophyte clades. Phylogenetic analysis revealed plastids from the same class as clustering together. Using all the obtained sequences, as well as plastid sequences currently in public databases, a non-degenerate marine algal plastid-biased PCR primer, PLA491F, was developed to minimize amplification of picocyanobacteria, which often dominate numerically environmental samples. Clone libraries subsequently constructed from the pico-sized fraction from 2 open ocean sites in the Arabian Sea, revealed an abundance of 16S rRNA gene clones phylogenetically related to chrysophytes, whilst prymnesiophyte, clade II prasinophyte (*Ostreococcus*-like) and pelagophyte clones were also well represented. The finding of a wealth of novel clones related to the Chrysophyceae highlights the utility of a PCR biased towards marine algal plastids as a valuable complement to 18S rDNA based studies of PPE diversity.

KEY WORDS: Photosynthetic picoeukaryotes · Plastid 16S rRNA · Arabian Sea · PCR

INTRODUCTION

Marine phytoplankton are responsible for about half of the global carbon fixation (Field et al. 1998). A significant proportion of this fixed carbon derives from the eukaryotic component <3 µm in size, the photosynthetic picoeukaryotes (PPEs). For example, a study in the central North Atlantic Ocean showed that photosynthetic eukaryotes, despite accounting for only 10% of the ultraphytoplankton by cell number, were responsible for 68% of the primary production (Li 1995). More recently, Worden et al. (2004) showed that PPEs were responsible for, on average, 76% of net C production by the picophytoplankton in Californian coastal waters. That this contribution to C fixation is much greater than that of the far more numerous cyanobacteria *Prochlorococcus* and *Synechococcus*, appears largely due to their greater cell size and rates of cell-specific C uptake (Li 1994).

Compared with the prokaryotic component of the picophytoplankton the PPE community is highly diverse, comprising members from virtually every algal class. Indeed, several new algal classes with picoplanktonic representatives have been described in recent years, such as Pelagophyceae (Andersen et al. 1993), Bolidophyceae (Guillou et al. 1999a) and Pin-

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guiophyceae (Kawachi et al. 2002). Yet despite the evident ecological significance of PPEs, relatively little is known of their diversity in the marine environment, particularly in the open ocean. This has been attributed mainly to difficulties in identification by light microscopy. Only recently, with the advent of molecular techniques, has picoeukaryote diversity begun to be revealed (for a recent review see Moreira & Lopez-Garcia 2002). Thus, phylogenetic studies based on 18S rRNA gene sequence analysis are now beginning to show the extent of taxonomic diversity within this group of organisms (Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001, Stoeck & Epstein 2003, Romari & Vaulot 2004). Such studies, using 'universal' 18S rRNA gene primers (which target both [photo]autotrophs and heterotrophs) and clone library construction from environmental samples have demonstrated the presence of PPEs affiliated with many different algal classes including the Bacillariophyceae, Bolidophyceae, Chrysophyceae, Cryptophyceae, Dictyochophyceae, Dinophyceae, Eustigmatophyceae, Glaucocystophyceae, Pelagophyceae, Prasinophyceae and Prymnesiophyceae (Diez et al. 2001, Romari & Vaulot 2004, Savin et al. 2004). Amongst these classes prasinophyte, prymnesiophyte and cryptophyte sequences were found to be particularly abundant at a French coastal site in the English channel (Romari & Vaulot 2004). Indeed, the development of 18S rRNA gene oligonucleotides targeting specific algal classes, and used in combination with fluorescent *in situ* hybridisation technology has confirmed that prasinophytes, particularly of the order Mamiellales, dominate picoeukaryote communities in both coastal and nutrient rich marine environments e.g. coastal waters of northern France (Not et al. 2002, 2004) and nutrient rich waters of the Norwegian and Barents Seas (Not et al. 2005).

Although these 18S rRNA gene based studies show excellent promise for describing PPE diversity, the fact that these 'universal' primers target both (photo)-autotrophic and heterotrophic components of the <3 µm fraction means that clone libraries constructed using these primers require extensive screening to assess the diversity solely of the autotrophic component. In addition, significant differences in 18S rRNA gene copy number occur in microalgae, ranging from 1 copy in *Nannochloropsis* to over 1000 copies in some nanoplanktonic dinoflagellates (Zhu et al. 2005) whilst PCR bias may be significant with primers used for this gene, as evidenced by little difference in marine eukaryote community structure in light and dark enrichment cultures, possibly due to selective amplification of heterotrophic over photosynthetic organisms irrespective of relative cell abundance (Guillou et al. unpubl.). Furthermore, even specifically targeted 18S rRNA gene oligonucleotides do not necessarily allow analysis purely of 'autotrophs' since several algal classes, e.g. the Dinophyceae and Cryptophyceae, contain representatives whose sole mode of nutrition is heterotrophy (though these do lack plastids). By focusing on the plastid 16S rRNA gene, however, one targets specifically the phototrophic community.

16S rRNA gene sequences have been used extensively to analyse prokaryotic diversity in both cultures and natural environments and hence a large number of these sequences now exist (Cole et al. 2003). However, the generally low numerical abundance of PPEs, and the fact that oligotrophic open ocean environments are dominated numerically by heterotrophic bacteria and the picocyanobacteria *Prochlorococcus* and *Synechococcus*, has meant that PPEs are generally poorly reflected in clone libraries constructed using universal 16S rRNA gene primers (Giovannoni et al. 1990, Britschgi & Giovannoni 1991, Fuhrman et al. 1993). Those libraries that have yielded substantial numbers of plastid clones have tended to be derived from marine coastal waters or from open ocean regions containing elevated nutrients where PPEs are highly abundant (Rappé et al. 1995, Brown & Bowman 2001, Wilmotte et al. 2002). Although more specific PCR primers are currently available, capable of recognising generally 'oxygenic phototrophs', i.e. both plastids and cyanobacteria (Nübel et al. 1997, West et al. 2001), the fact that these primers target picocyanobacteria, organisms dominating most marine water columns, means very few plastid clones appear in libraries using this primer pair (Fuller & Scanlan unpubl. data). Similar problems have arisen using the functional gene *psbA* (encoding the D1 protein of photosystem II) which has also been used for analysing marine picophytoplankton diversity (Zeidner et al. 2003). Thus, although these authors were able to detect prasinophyte, prymnesiophyte, cryptophyte and bacillariophyte sequences from the Red Sea, Mediterranean Sea, and central North Pacific Ocean, this was amongst a plethora of picocyanobacterial sequences. Furthermore, the relative dearth of 16S rRNA gene sequences from cultured isolates (and more so for *psbA* sequences) has made it often impossible to identify the environmental clones which arise, even to the class level. To overcome these problems we report here the sequencing of over 60 algal 16S rRNA genes to considerably 'enrich' the 16S rRNA gene database with cultured isolates, so that virtually every known algal class can now be identified. Using these derived sequences we have designed a new PCR primer, PLA491F, biased towards recognising marine algal plastids over picocyanobacteria. We demonstrate that this primer, in combination with a previously described oxygenic-

phototroph biased primer (OXY1313R) (West et al. 2001), can be used in the PCR to assess the diversity of PPEs at open ocean sites dominated numerically by *Prochlorococcus* or *Synechococcus*. Furthermore, clone libraries constructed from 2 sites along a transect in the Arabian Sea, using the PLA491F-OXY1313R primer pair, reveal that members of the Chrysophyceae appear to be important components of the marine PPE community, in this region at least.

MATERIALS AND METHODS

Sampling. Seawater for DNA extraction was collected during the NERC funded 'AMBITION' cruise in the Arabian Sea during 1 to 27 September 2001 (for details of the cruise transect see Mazard et al. 2004) aboard the Royal Research Ship 'Charles Darwin'. Samples were taken at Stn 2 (00°00.1'N, 66°59.7'E) on 5 September 2001, from depths of 10 and 50 m (the latter being the deep chlorophyll maximum), and Stn 10 (24°19.8'N, 59°15'E) at depths of 27 m and 50 m on 25 September 2001. PPE cultures were isolated from 10 m depth at 3 stations (Stn 3: 3°47.9'N, 67°00'E, Stn 6: 15°11.6'N, 67°00'E and Stn 9: 23°33.7'N, 59°54.1'E on 7, 15 and 23 September 2001, respectively). Water was collected with a rosette of 24, 20 l Niskin bottles on a hydrographic cable. Conductivity, temperature and hydrostatic pressure were measured simultaneously with a CTD (model Sea-Bird 9/11) with additional fluorescence and oxygen detectors. Seawater was also collected, for clone library construction, from the northwest Mediterranean Sea (DYF, Dyfamed station, Ligurian Sea, 43°24'N, 7°51'E, a 5 to 75 m pooled sample taken on 30 September 1999 during the 'PROSOPE' cruise; van Wambeke et al. 2002), and the northwest coast of Germany (HE, Helgoland Island, 54°11'N, 7°54'E, a surface sample taken on 3 August 2000; (Massana et al. 2004). Seawater (5 l) was pre-filtered through a 47 mm diameter 3 µm pore size filter (MCE MF-Millipore filters, Fisher) and filtered onto a 47 mm diameter 0.45 µm pore size polysulphone filter (Supor®-450, 0.45 µm; Gelman Sciences) under gentle vacuum (10 mm Hg). The filter was placed in a 5 ml cryovial with 3 ml of DNA lysis buffer (0.75 M sucrose, 400 mM NaCl, 20 mM EDTA, 50 mM Tris-HCl [pH 9.0]), and stored at −80°C until extraction.

Environmental DNA isolation. DNA was extracted from the filters in lysis buffer as described by Fuller et al. (2003).

Flow cytometric analysis. Picophytoplankton were analysed from 2.2 ml seawater samples using a FACSort™ flow cytometer (Becton Dickinson). Cells were counted and their chlorophyll fluorescence (>650 nm), phycoerythrin fluorescence (585 nm ±21 nm) and side scatter (light scattered at 90° to the plane of the vertically polarised argon ion laser exciting at 488 nm) measured. Data acquisition was triggered on chlorophyll fluorescence, using laboratory cultures to set rejection gates for background noise. Samples were analysed for 3 min at a flow rate of 95 µl min^{-1} ±2.6 µl and data were stored in listmode format. The listmode data were read using WinMDI v2.8 flow cytometry analysis software to produce scatter-plots of side scatter versus chlorophyll or phycoerythrin fluorescence, from which cell counts were made.

Enrichment cultures. Seawater samples were filtered through 25 mm diameter 3 µm pore-size polycarbonate filters. K medium nutrients (Keller et al. 1987) were added at a 1:10 dilution, and the samples were incubated at 25°C and 10 µE m^{-2} s^{-1} until significant biomass was observed. Cell pellets were then transferred to full-strength K medium for maintenance.

Culture collection isolates. Algal cultures for determining 16S rRNA gene sequences were obtained from the Roscoff Culture Collection (RCC: www.sb-roscoff.fr/Phyto/RCC/) (Vaulot et al. 2004) and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP: ccmp.bigelow.org/; Andersen et al. 1997).

PCR amplification. 16S rRNA gene sequences were amplified from algal cultures using primers OXY359F and OXY1313R (Nübel et al. 1997, West et al. 2001), from DYF and HE environmental DNA using primers OXY107F (West et al. 2001) and OXY1313R, and from Arabian Sea environmental DNA using the newly designed primer PLA491F (5'-GAGGAATAAGCATCGGCTAA-3') in conjunction with OXY1313R. PCR amplification with the OXY107F-OXY1313R and OXY359F-OXY1313R primer pairs was performed as described previously (Fuller et al. 2003). PCR amplification with the PLA491F-OXY1313R primer pair was carried out in a total reaction volume of 50 µl containing 200 µM concentrations of deoxynucleotide triphosphates, 1.2 mM $MgCl_2$, 0.2 µM concentrations of primers and 1.2 U of *Taq* polymerase in 1× enzyme buffer (Fermentas Life Sciences), with 1 mg ml^{-1} of bovine serum albumin for amplification of environmental DNA. Amplification conditions using PLA491F comprised 95°C for 5 min and 80°C for 1 min, at which time *Taq* polymerase was added, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 40 s, with a final extension at 72°C for 6 min. Specificity of the novel plastid PCR was determined by attempting to amplify 10 pg of a PCR product (previously amplified with OXY359F-OXY1313R primer pair) from control strains.

Screening of clone libraries. 16S rRNA gene amplicons derived as described above were purified by using the QIAquick PCR purification kit (Qiagen) and

Table 1. Origin and characteristics of the cultures used in this study

Class	Genus	Species	Strain	RCC No.	CCMP No.	Ocean origin
Bicosoecid[a]	*Symbiomonas*	*scintillans*	OLI120SDA	24	1950	South Pacific
Bicosoecid[a]	*Symbiomonas*	*scintillans*	MIN33-5m-13	25	1951	Mediterranean Sea
Bolidophyceae	*Bolidomonas*	*mediterranea*	Min129-20m Aa	238	1867	Mediterranean Sea
Chlorarachniophyceae	*Bigelowiella*	*natans*	CCMP 621	623	621	North Atlantic
Chlorarachniophyceae	*Gymnochlora*	*stellata*	CCMP 2057	626	2057	North Pacific
Chlorarachniophyceae	Unknown		PROSOPE_99	365		Mediterranean Sea
Chlorarachniophyceae	*Chlorarachnion*		BL_33-3	435		Mediterranean Sea
Chrysophyceae	*Chrysosphaera*		CCMP296	616	296	Freshwater
Chrysophyceae	*Ochromonas*	*distigma*	Caen	21		North Atlantic
Chrysophyceae[a]	*Picophagus*	*flagellatus*	OLI11SC2D	22	1953	Equatorial Pacific
Chrysophyceae[a]	*Paraphysomonas*		CCMP1604	620	1604	North Pacific
Cryptophyceae	*Campylomonas*	*reflexa*	CCMP1177		1177	Freshwater
Cryptophyceae	*Chroomonas*	*pauciplastida*	CCMP268		268	North Atlantic
Cryptophyceae	*Proteomonas*	*sulcata*	CCMP1175		1175	South Pacific
Cryptophyceae	*Storeatula*	*major*	CCMP320		320	
Cryptophyceae	*Storeatula*		CCMP1868		1868	Indian Ocean
Dictyochophyceae	Unknown		PROSOPE_32-1	332		Mediterranean Sea
Dictyochophyceae	*Mesopedinella*	*arctica*	PROSOPE_2	382		North Atlantic
Dictyochophyceae	*Rhizochromulina*		CCMP1253	94	1253	North Atlantic
Euglenophyceae	*Eutreptiella*	*marina*	CCMP 390	615	390	North Pacific
Eustigmatophyceae	*Nannochloropsis*	*salina*	CCMP527	92	527	North Atlantic
Eustigmatophyceae	*Nannochloropsis*		BL_39	438		Mediterranean Sea
Pavlophyceae	*Pavlova*		BL_20/5	437		Mediterranean Sea
Pavlophyceae	*Pavlova*		BL_146	441		Mediterranean Sea
Pavlophyceae	*Pavlova*	*pinguis*	CCMP609		609	North Atlantic
Pavlophyceae	*Pavlova*		CCMP1234		1234	North Atlantic
Pelagophyceae	*Ankylochrysis*	*lutea*	Caen	286		
Pelagophyceae	*Aureococcus*	*anophagefferens*	CCMP1784	96	1784	North Atlantic
Pelagophyceae	*Aureoumbra*	*lagunensis*	CCMP1681	97	1681	North Atlantic
Pelagophyceae	*Pelagomonas*	*calceolata*	CCMP1214	100	1214	North Pacific
Pelagophyceae	*Pelagomonas*	*calceolata*	MAXEuk 71	101	1954	North Atlantic
Pelagophyceae	*Pelagomonas*	*calceolata*	REDSEA2C	103	1865	Red Sea
Pelagophyceae	*Pelagomonas*		PROSOPE_63	341		Mediterranean Sea
Phaeophyceae	*Bodanella*	*lauterbornii*	CCMP148		148	Freshwater
Pinguiophyceae	*Glossomastix*	*chrysoplasta*	CCMP 1537	624	1537	Indian Ocean
Pinguiophyceae	*Glossomastix*	*chrysoplasta*	CCMP 1623	625	1623	Indian Ocean
Pinguiophyceae	*Pinguiococcus*	*pyrenoidosus*	CCMP 1144	621	1144	North Atlantic
Pinguiophyceae	*Pinguiococcus*	*pyrenoidosus*	CCMP 2078	622	2078	North Atlantic
Pinguiophyceae	Unknown		BL_149-10	503		Mediterranean Sea
Prasinophyceae	*Bathycoccus*	*prasinos*	CCMP1898	113	1898	Mediterranean Sea
Prasinophyceae	genus nov.	species nov.	NOUM15	287		Equatorial Pacific
Prasinophyceae	*Mamiella*	species nov.	PROSOPE_1-10	391		North Atlantic
Prasinophyceae	*Micromonas*		NOUM17	299		Equatorial Pacific
Prasinophyceae	*Micromonas*		BL_122	434		Mediterranean Sea
Prasinophyceae	*Micromonas*		CCMP489	450	489	North Atlantic
Prasinophyceae	*Nephroselmis*	*pyriformis*	CCMP717	618	717	North Atlantic
Prasinophyceae	*Ostreococcus*	*tauri*	OTTH 0595	116		Mediterranean Sea
Prasinophyceae	*Ostreococcus*		PROSOPE_3	344		North Atlantic
Prasinophyceae	*Ostreococcus*		RA000412-9	356		English Channel
Prasinophyceae	*Ostreococcus*		PROSOPE_122-4	393		Mediterranean Sea
Prasinophyceae	*Pseudoscourfieldia*	cf. *marina*	TAK9801	261		Pacific Ocean
Prasinophyceae	*Prasinoderma*		CCMP1220	137	1220	North Atlantic
Prasinophyceae	*Pycnococcus*	*provasolii*	CCMP1199	135	1199	Atlantic Ocean
Prasinophyceae	*Pycnococcus*		VillF I 5m-4	445		Mediterranean Sea
Prasinophyceae	*Tetraselmis*		BL_148-9	500		Mediterranean Sea
Prasinophyceae	Unknown		CCMP1205	369	1205	
Prasinophyceae	Unknown		AS3	672		Indian Ocean
Prasinophyceae	Unknown		AS9	673		Indian Ocean
Prymnesiophyceae	*Chrysochromulina*	*acantha*	PROSOPE_1-5	339		Atlantic Ocean
Prymnesiophyceae	*Emiliania*	*huxleyi*	CCMP625	192	625	
Prymnesiophyceae	*Imantonia*	*rotunda*	VillFII 50m	305		Mediterranean Sea
Prymnesiophyceae	*Imantonia*	*rotunda*	RA000609-18-5	406		English Channel
Prymnesiophyceae	*Phaeocystis*	*cordata*	MED Disco203 NS2	186		Mediterranean Sea

Table 1 (continued)

Class	Genus	Species	Strain	RCC No.	CCMP No.	Ocean origin
Prymnesiophyceae	*Phaeocystis*	*globosa*	PLY147	187		English Channel
Trebouxiophyceae	*Chlorella*		CCMP253	368	253	North Atlantic
Trebouxiophyceae	*Chlorella*	*autotrophica*	CCMP243		243	North Atlantic
Trebouxiophyceae	*Nannochloris*		Embiez	9		Mediterranean Sea
Trebouxiophyceae	*Nannochloris*		OLI 26 FC	13		Equatorial Pacific
Trebouxiophyceae	*Nannochloris*		OLI 26 FH	99		Equatorial Pacific
Trebouxiophyceae	*Nannochloris*	*atomus*	CCMP508		508	English Channel
Trebouxiophyceae	*Picochlorum*		OLI 26 SA	289		Equatorial Pacific

"Organisms solely heterotrophic: No PCR product with either the 359F-OXY1313R or PLA491F-OXY1313R primer pairs

cloned into the TA vector pCR2.1-TOPO (Invitrogen). Transformants were then screened by restriction fragment length polymorphism (RFLP) analysis as follows: cells from the colonies were transferred to 50 µl of sterile water and heated to 95°C for 5 min to lyse cells. Samples of 1 µl of these lysates were then subject to further PCR amplification with the primers OXY359F and OXY1313R (for libraries from mixed cultures and DYF or HE environmental DNA), or PLA491F and OXY1313R (for the libraries from Arabian Sea environmental DNA). PCR amplicons (5 µl) were subject to restriction digestion in 20 µl reaction mixtures with 2 U (each) of *Eco*RI and *Hae*III in 1× NEBuffer 2 (New England BioLabs), with incubation at 37°C for 2 h. Fragments were then resolved by gel electrophoresis on a 2% (w/v) agarose gel at 60 mA for 40 min. For the Arabian Sea environmental DNA, 4 libraries were constructed and between 30 and 130 clones were screened from each.

DNA sequencing. 16S rRNA gene sequences were determined bidirectionally from PCR products by using Big Dye Terminator v3.1 Chemistry (Applied Biosystems) and run on the 3100 Genetic Analyser.

Phylogenetic analysis. Sequences were checked for the presence of chimeric artefacts as described previously (Fuller et al. 2003). Sequence alignment was performed by using the ARB program (Ludwig et al. 2004). Phylogenetic analysis was carried out using the neighbour joining algorithm with Jukes-Cantor correction and a maximum frequency filter for plastids. Bootstrap analysis was done with the ARB parsimony bootstrap algorithm with independent Nearest Neighbour Interchange (NNI).

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database under the following accession numbers: for algal cultures, AY702104 to AY702136 and AY702138 to AY702170; for environmental clones, AY702171 to AY702197 and DQ013162 to DQ013172.

RESULTS

Sequencing of plastid 16S rRNA genes

Plastid 16S rRNA genes were sequenced from 65 cultures representing 14 classes (Table 1). Of these, about half were picoeukaryotes. In addition, enrichment cultures of PPEs were isolated from 3 locations along a transect in the Arabian Sea, and clone libraries were constructed from 16S rRNA gene amplicons from each of the 3 enrichment cultures. 16S rRNA gene clone libraries were also generated, as part of the EU project 'PICODIV', from the northwest Mediterranean Sea (DYF site) and the northwest coast of Germany (HE site off Helgoland island). Phylogenetic analysis of all these sequences was performed (Fig. 1).

The 16S rRNA gene was sequenced for organisms from 3 classes for which no such sequences had previously been available (Bolidophyceae, Dictyochophyceae and Pinguiophyceae), and from 2 further classes for which no such sequences from cultured representatives had been available (Eustigmatophyceae and Pelagophyceae). Of the 7 prasinophyte clades, according to 18S rRNA (Fawley et al. 2000, Guillou et al. 2004), sequences were obtained for 6 clades, of which 1 (clade VI) previously had no 16S rRNA gene sequences available, and another (clade V) previously had no such sequences from cultured representatives (Fig. 1). Amongst all the 65 cultures, sequences from the same algal class generally clustered together. An exception was the class Prasinophyceae, whose 7 clades clustered separately. Yet sequences from each prasinophyte clade clustered together. Bolidophytes may form a distinct cluster, but they fall at the base of, and slightly within, the bacillariophytes, partially dividing them. The only other exception are the plastid sequences from dinophytes: *Dinophysis* sequences from the database fall within the cryptophyte branch, as previously described (Hackett et al. 2003).

Clone sequences from the Arabian Sea PPE enrichment cultures revealed that each of these cultures appears to be dominated by clade VI prasinophytes. The coastal Mediterranean clone library (DYF) yielded sequences of prymnesiophytes, together with a dinophyte (*Dinophysis*) and what is probably a bolidophyte. The German coastal clone library (HE) yielded only a prymnesiophyte and a chrysophyte. These latter 2 clone libraries were dominated by picocyanobacterial clones, which were the more numerous organisms (data not shown).

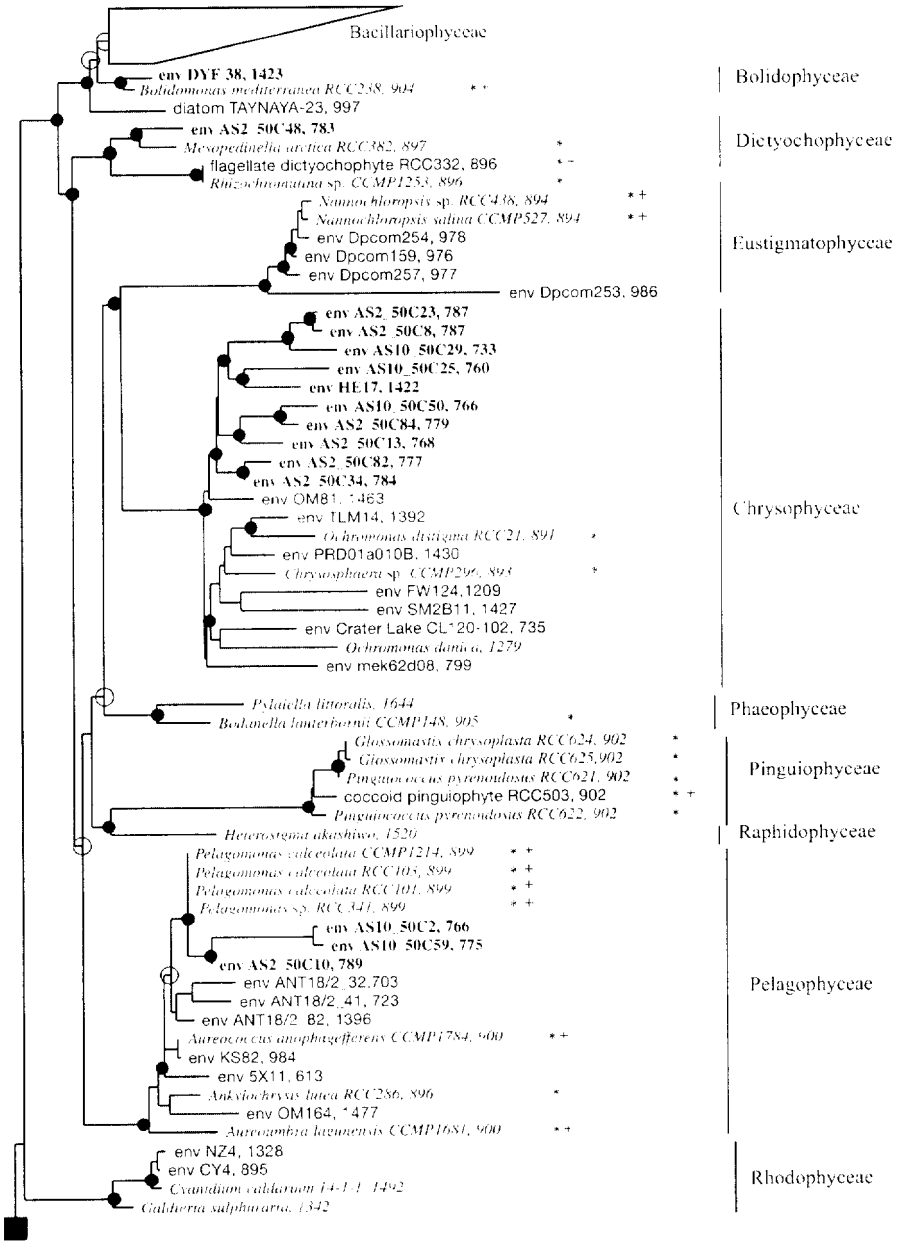
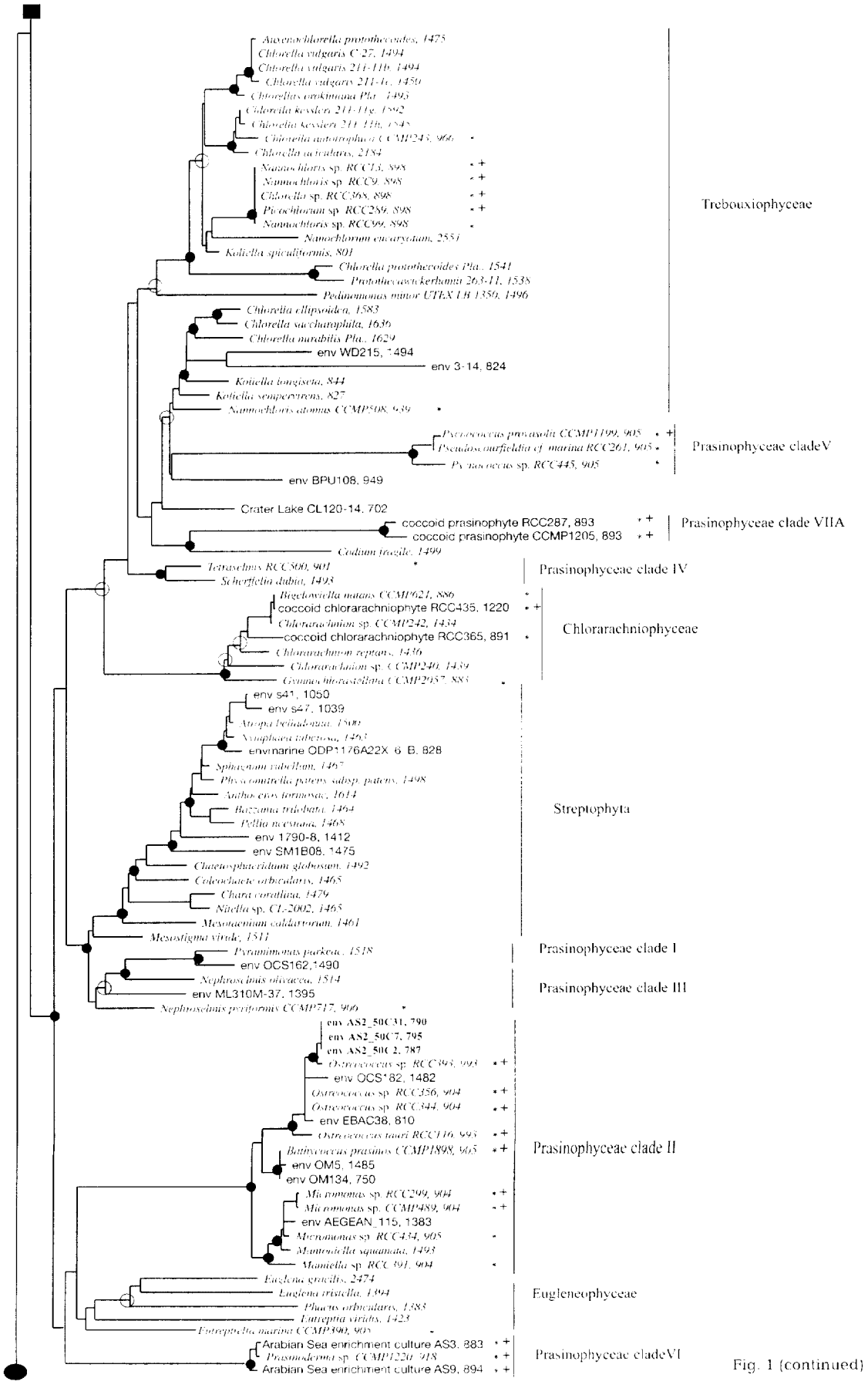


Fig. 1. (Above and next 2 pages.) Neighbour joining tree of 16S rRNA gene sequences from algal plastids. The tree was constructed from sequences >1200 nucleotides using the Jukes-Cantor correction and a maximum frequency filter for plastids. Shorter sequences were added by parsimony using the same filter. The length of each sequence is shown in the tree. *Escherichia coli* was used as the root. Bootstrap analysis was done with ARB parsimony bootstrap (Ludwig et al. 2004). ●: values >95%; ○: 70–95%; values <70% are not shown. *: sequenced in this study; +: picoeukaryotes; env: environmental sequences. Environmental sequences in bold were obtained from environmental clone libraries constructed within this study. AS: clones from the Arabian Sea; DYF: clones from the northwest Mediterranean Sea Dyfamed station; HE: clones from the northwest coast of Germany, Helgoland island



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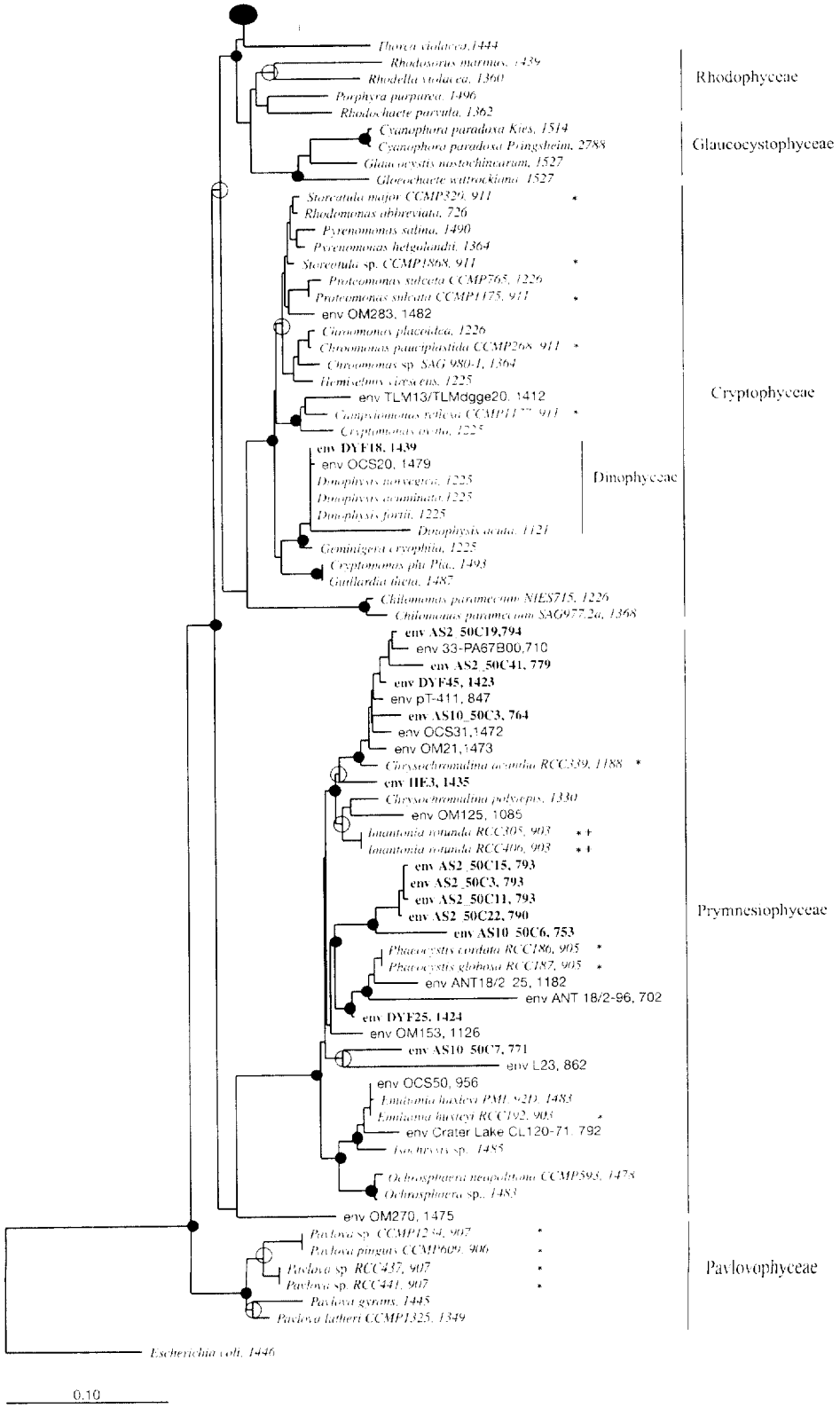

Fig. 1 (continued)

Development of an algal plastid-biased PCR

Current PCR primers used to amplify plastid 16S rRNA genes are not capable of selectively amplifying plastids over picocyanobacteria. This was observed, for instance, in the paucity of plastid sequences from the DYF and HE clone libraries above. Furthermore, comparison of current 'oxygenic phototroph-specific' primers against algal 16S rRNA gene sequences revealed that the primers CYA106F (Nübel et al. 1997), OXY107F (West et al. 2001), and CYA781R (Nübel et al. 1997) have significant mismatches to many algal sequences, especially prasinophytes and chrysophytes, which would hinder their amplification (data not shown). Based on all the new sequences from this study, together with those available in the database, a forward primer, PLA491F, was designed, to be used in conjunction with OXY1313R (West et al. 2001). The specificity of PLA491F to individual algal classes, higher plants and cyanobacterial genera is shown both in silico and empirically, by PCR with the OXY1313R primer (Table 2). Notably, under the correct PCR conditions, this primer pair can selectively amplify all known algal plastids, though with a slightly decreased efficiency for members of the Eustigmatophyceae, over the marine picocyanobacteria *Synechococcus* and *Prochlorococcus*. While this primer pair will amplify several cyanobacteria with no mismatches, these are either not known in the marine environment (e.g. *Nostoc*) or are >3 µm (e.g. the unicellular nitrogen fixing '*Crocosphaera*' [see Montoya et al. 2004]), and so would not be included in the picoplanktonic component of size-fractionated marine DNA samples.

Table 2. Specificity of the primer PLA491F, illustrated by an alignment of the primer against algal and higher plant plastid, and cyanobacterial 16S rRNA gene sequences. Dots represent bases identical to the primer sequence. PCR amplification is defined as very good (+++), moderate (++), poor (+), no product (–) and not determined (ND)

Target organism	5'-GAG	GAA	TAA	GCA	TCG	GCT	AA-3'	PCR amplification
Plastids								
Bacillariophyceae (*Thalassiosira weissflogii*)	..A	...	...	...	...	...	...	+++
Bolidophyceae (RCC238)	..A	...	...	...	...	...	...	+++
Chlorarachniophyceae (CCMP621)	A..A	...	...	...	...	...	...	+++
Chrysophyceae (CCMP296)	...	...	...	...	...	...	...	+++
Cryptophyceae (CCMP1868)	...	...	...	...	...	...	...	+++
Dictyochophyceae (RCC382)	..A	...	...	...	...	...	...	+++
Euglenophyceae (CCMP390)	...	...	...	...	C..	...	...	+++
Eustigmatophyceae (RCC92)	.G.	TGG	A..	...	...	...	...	++
Pavlovophyceae (CCMP609)	.G.	...	...	...	...	...	...	+++
Pelagophyceae (RCC100)	..A	...	...	...	...	...	...	+++
Pinguiophyceae (RCC503)	..T	...	...	...	...	...	...	+++
Prasinophyceae clade I (*Pyramimonas parkeae*)	...	...	...	...	...	...	...	ND
Prasinophyceae clade II (RCC344)	...	...	...	...	...	...	...	+++
Prasinophyceae clade III (RCC618)	A..	...	C..	...	...	...	...	+++
Prasinophyceae clade IV (RCC500)	A..	...	...	...	...	...	...	+++
Prasinophyceae clade V (RCC261)	.G.	...	...	...	...	...	...	+++
Prasinophyceae clade VI (RCC137)	A..	...	...	...	...	...	...	+++
Prasinophyceae clade VII (RCC287)	A..	...	...	...	...	...	...	+++
Prymnesiophyceae (RCC186)	...	...	...	...	...	...	...	+++
Streptophyta (*Arabidopsis thaliana*)	.G.	...	...	...	...	...	...	+++
Streptophyta (*Nicotiana tabacum*)	.G.	...	...	...	...	...	...	+++
Trebouxiophyceae (CCMP243)	...	...	...	...	...	...	...	+++
Cyanobacteria (marine)								
Synechococcus sp. (WH 7803)	...	...	...	C..	A..	...	...	+
Prochlorococcus sp. (GP2)	...	...	...	C..	A..	...	...	+
Cyanobium sp. (WH 5701)	...	...	...	C..	A..	...	...	+
Trichodesmium sp. (RS9602)	A..	...	C..	C..	...	...	...	–
Crocosphaera sp. (WH 8501)	...	...	...	...	...	...	...	+++
Cyanobacteria (freshwater)								
Synechococcus sp. (PCC 7942)	..A	...	...	C..	...	...	...	++
Synechocystis sp. (PCC 6803)	..T	...	...	...	...	...	...	+++
Nostoc sp. (PCC 7120)	...	...	...	...	...	...	...	+++
Plectonema boryanum	..T	...	C..	...	...	...	...	++
Planktothrix rubescens	...	...	A..	...	...	...	...	+++
Gloeotrichia sp.	...	...	...	...	...	...	...	+++
Microcystis aeruginosa (PCC 7806)	...	...	C..	C..	...	...	...	–

Hydrological and community structure characteristics of Stns 2 and 10, Arabian Sea

Profiles of temperature and chlorophyll *a* fluorescence (Fig. 2) revealed a stratified water column with a surface mixed layer down to about 40 m, and a deep chlorophyll maximum (DCM) at 50 m (Stn 2) or a large sub-surface chlorophyll maximum around 30 m (Stn 10). Picophytoplankton cell counts showed the Stn 2 community to be dominated numerically by the cyanobacterium *Prochlorococcus* throughout the entire photic zone. PPEs were at their maximum concentration at the DCM, yet were still vastly outnumbered by cyanobacteria. Cell counts at 50 m were 900 PPEs ml^{-1}; 8000 *Synechococcus* ml^{-1}; and 282 000 *Prochlorococcus* ml^{-1}, i.e. over 300 times more cyanobacteria than PPEs. In contrast, Stn 10 was dominated by *Synechococcus*, with over 67 000 cells ml^{-1} at 27 m, coincident with numbers of *Prochlorococcus* and picoeukaryotes of 21 000 and 2100 cells ml^{-1}, respectively. These stations hence represented a rigorous test for the use of the PLA491F-OXY1313R primer pair given the contrasting ratios of picocyanobacteria (*Prochlorococcus*/*Synechococcus*): picoeukaryote cell number at these sites.

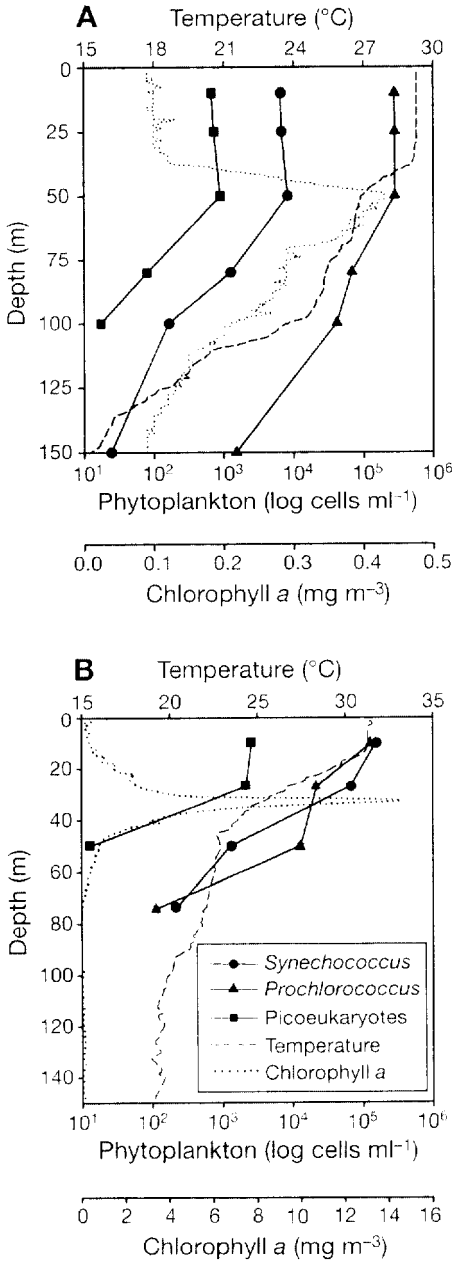

Fig. 2. Ancillary CTD and flow cytometry data from depth profiles at (A) Stn 2 and (B) Stn 10, both open ocean sites in the Arabian Sea

Plastid-biased PCR in the Arabian Sea

Picoplanktonic DNA from Stn 2 (10 m and 50 m) and Stn 10 (27 m and 50 m) was amplified by PCR using the PLA491F-OXY1313R primer pair with subsequent clone library construction, to test the efficacy of the newly developed marine algal plastid-biased primer PLA491F. Clones with the same RFLP pattern (Fig. 3) were grouped, and representatives from each were sequenced, to identify the group. Most algal classes were represented by a single RFLP type (Fig. 3), the exception being Prymnesiophyceae where 3 RFLP types were discernible. Although *Prochlorococcus* sequences, mainly of the high-light adapted HLII type (West & Scanlan 1999), dominated the Stn 2 libraries

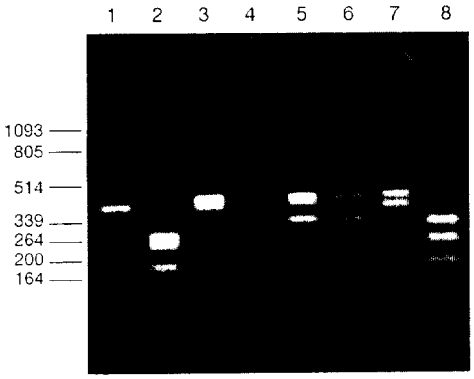

Fig. 3. RFLP analysis of PPE clones from the Arabian Sea. 16S rRNA gene PCR amplicons were digested with *Hae*III and *Eco*RI and separated by electrophoresis on a 2% agarose gel. Representative patterns for the different PPE classes are indicated as well as for the picocyanobacterial genus *Prochlorococcus*. Track 1: *Prochlorococcus*; Track 2: Chrysophyceae; Track 3: Pelagophyceae; Track 4: Prasinophyceae; Tracks 5–7: Prymnesiophyceae; Track 8: Dictyochophyceae. Bars indicate the size of the λ *Pst*I markers in base pairs

Table 3. Results of RFLP analysis of the 4 Arabian Sea clone libraries

Library	No. of clones	No. of RFLP types	No. (%) of clones representative of each group					
			Prochlorococcus/ *Synechococcus*	Chryso-phyte	Pelago-phyte	Prasino-phyte	Prymnesio-phyte	Dictyocho-phyte
Stn 2, 10 m	27	3	17 (63)	9 (33)	1 (4)	–	–	–
Stn 2, 50 m	135	7	89 (65)	12 (9)	8 (6)	8 (6)	17 (13)	1 (0.75)
Stn 10, 27 m	44	3	9 (20)	24 (55)	–	11 (25)	–	–
Stn 10, 50 m	61	6	2 (3)	27 (45)	6 (10)	16 (26)	10 (16)	–

(though 1 clone of the HLI type and 2 low-light adapted *Prochlorococcus* clones were also found in the Stn 2, 50 m library), PPE sequences were highly enriched (35 to 40% of the clones, Table 3), certainly compared to our previous experiences of clone library construction with the OXY107F-OXY1313R primer pair (<1% of the clones, data not shown). This enrichment of PPE sequences was also well evident in the Stn 10 clone libraries, with relatively few picocyanobacterial clones observed (Table 3). Within all these PPE sequences chrysophyte sequences were relatively dominant in all 4 libraries, with lower proportions of prymnesiophyte, pelagophyte and prasinophyte clones. A single dictyochophyte sequence was also identified.

Phylogenetic analysis of representative clones from the different algal classes that were sequenced, revealed their identity more fully (env AS sequences in Fig. 1). The prasinophyte sequences all appear to represent clade II clones, and can be identified as being *Ostreococcus* sequences whilst the pelagophyte clones appear to be *Pelagomonas* sequences. All the other clones form distinct lineages within their class, and may represent novel species or genera (prymnesiophyte clones), or even higher order taxa (chrysophyte clones).

DISCUSSION

Plastid sequences

By sequencing the plastid 16S rRNA gene from many algal strains we initially set out to considerably augment the 16S rRNA gene database for these sequences, particularly for those PPE classes that were poorly represented or not represented at all. For the 5 algal classes for which there were previously no cultured representatives, we can now compare plastid 16S rRNA (Fig. 1) with nuclear 18S (small sub-unit, SSU) rRNA and plastid *rbc*L gene phylogenies. All 5 classes are members of the stramenopiles, and plastid 16S rRNA gene phylogeny places them clearly within this group (Fig. 1), in accordance with nuclear SSU (Andersen et al. 1993, Saunders et al. 1995, Guillou et al. 1999a, Kawachi et al. 2002), large sub-unit (LSU) rRNA (Van der Auwera & De Wachter 1997, Ben Ali et al. 2001) and plastid *rbc*L (Daugbjerg & Andersen 1997, Kawachi et al. 2002) phylogenies. Furthermore, plastid 16S rRNA gene phylogeny relates bolidophytes to the bacillariophytes, a feature in agreement with the 18S rRNA (Guillou et al. 1999a) and plastid *rbc*L (Daugbjerg & Guillou 2001) phylogenies. In addition, according to the plastid 16S rRNA, albeit with relatively low bootstrap support, pinguiophytes fall near to the raphidophyte *Heterosigma akashiwo*, a feature recovered with plastid *rbc*L, but not 18S rRNA, gene phylogeny (Kawachi et al. 2002). The multi-clade nature of prasinophytes is clearly seen according to 16S rRNA gene phylogeny (Fig. 1), yet this class does not appear to be monophyletic, in agreement with 18S rRNA phylogeny (Fawley et al. 2000, Guillou et al. 2004).

As shown in Fig. 1, there are now 16S rRNA gene sequences for picoplanktonic isolates from 9 algal classes. Furthermore, a clone, DYF18, from the <3 µm size fraction Mediterranean Sea derived picoplankton clone library, obtained in this study, clustered phylogenetically with members of the Dinophyceae. Known *Dinophysis* species are much larger (Larsen & Moestrup 1992), so although this sequence may have resulted from inefficient prefiltration or cell breakage during sample collection, a pico-sized stage in the life-cycle, or an as yet undescribed picoplanktonic member of this genus is also possible. Certainly, since *Dinophysis* is known to harbour plastids of cryptophyte origin (Hackett et al. 2003) an endosymbiotic origin of this sequence is likely.

The plastid 16S rRNA gene sequences reported here also aid in taxonomic assignment of several environmental clones from previous studies that had been left unidentified even to the class level, such as the OM and OCS clones from early coastal libraries (Rappé et al. 1998). For example, clone OCS182 relates to a sequence of *Ostreococcus*, and OM5 and OM134 are likely *Bathycoccus* sequences.

Environmental clone libraries

The small number of plastid 16S rRNA gene clones obtained from the DYF and HE libraries we constructed prevents any general conclusions of PPE composition at those sites. Even so, the presence of 2 prymnesiophyte sequences, DYF45 and HE3, closely related to *Chrysochromulina* sp. from these sites, is consistent with their presence in coastal waters of northwest France. Similarly, a bolidophyte sequence, found in the DYF library from the northwest Mediterranean Sea, correlates with the known presence of this group, albeit in very low abundance, in coastal waters of northwest France (Not et al. 2002) and the Mediterranean Sea (Guillou et al. 1999a,b).

The 150 PPE clones obtained from the 4 Arabian Sea libraries comprised 5 algal classes: chrysophytes, prymnesiophytes, pelagophytes, prasinophytes and dictyochophytes. Surprisingly, chrysophyte sequences dominated each of these libraries particularly in surface waters (Table 3). This algal class is well recognised as being an important component of freshwater phytoplankton assemblages (Sandgren et al. 1995) and, although the presence of chrysophytes in the open ocean has been suggested by pigment studies (Mackey et al. 1998), generally information from the marine environment regarding the occurrence and distribution of photosynthetic members of this class is limited. An exception are Parmales, an order that has been classified inside the chrysophytes based on field sample observations since no strain is available in culture. Parmales encompass a group of tiny, solitary cells 2 to 5 µm in diameter each with a chloroplast and a silicified cell wall composed of 5 to 8 plates (Booth & Marchant 1987, Bravo-Sierra & Hernández-Becceril 2003). Parmales appear to be widespread in both cold and tropical waters. However, the lack of cultured representatives of this order prevents the identification of environmental plastid 16S rRNA gene sequences reported here with members of this order. It is possible though that some of these chrysophyte plastid sequences are derived from pico-sized dinoflagellates, or a life cycle stage of that group, which would harbour a plastid of chrysophyte origin. Certainly dinoflagellates appear to have an unparalleled ability to capture plastids via endosymbiosis e.g. dinoflagellate plastids of cryptophyte, haptophyte and prasinophyte origin have been described (Tengs et al. 2000, Hackett et al. 2003). In order to resolve this point, it will be necessary in the future to obtain 16S sequences from more dinoflagellate species. Alternatively, since chrysophytes encompass organisms with both photosynthetic and heterotrophic lifestyles, a subset of these sequences may be derived from solely heterotrophic chrysophyte genera that contain a non-photosynthetic plastid remnant (or leucoplast, see Sekiguchi et al. 2002), e.g. members of the genus *Paraphysomonas* (environmental clones of which have been identified in 18S rRNA gene clone libraries from the North Atlantic and Mediterranean Sea). However, this genus appears to be a fairly uncommon component of the flagellate community *in situ*, though common in enrichments (Lim et al. 1999). Moreover all our attempts to amplify 16S rDNA plastid genes from the heterotrophic strains listed in Table 1 have been unsuccessful.

The relative abundance of prymnesiophyte sequences within these libraries (Table 3) is generally consistent with other studies, though there is some disagreement between pigment and molecular data in this previous work. Thus, in the equatorial Pacific, pigment studies (Higgins & Mackey 2000, Mackey et al. 2002) have estimated a contribution of 30 to 40% of the total chlorophyll *a* by this class, whereas molecular data suggests that prymnesiophytes represent between 1 to 12% of the total eukaryotic picoplankton community (Moon-van der Staay et al. 2000). Problems of 19'-hexanoylfucoxanthin being a diagnostic pigment for prymnesiophytes, or of the 18S rRNA gene probes not targeting all prymnesiophytes, but more likely the fact that the general eukaryotic 18S probe targets all eukaryotes while chlorophyll *a* only takes into account photosynthetic ones, may explain these discrepancies. Certainly, the relative abundance of these plastid prymnesiophyte sequences in these pico-size-fractionated Arabian Sea libraries suggests that pico-sized members of this class are important in this oceanic region at least, whilst the lack of phylogenetic affiliation of some of these prymnesiophyte plastid sequences with cultured counterparts, e.g. clones including AS2_50C15 (see Fig. 1), suggests they likely represent novel genera or lineages. Other clones, such as AS10_50C3, may derive from members of the *Chrysochromulina*, known to be a diverse genus, and for which picoplanktonic members are known to exist, e.g. *Chrysochromulina leadbeateri* (Eikrem & Throndsen 1998).

Sequences of *Ostreococcus*, a clade II prasinophyte (order Mamiellales), the smallest known PPE, were also well represented in these Arabian Sea clone libraries. Although initially isolated from a coastal Mediterranean Sea lagoon, *Ostreococcus* strains have subsequently been obtained from the tropical Atlantic (Guillou et al. 2004), Red Sea (Vaulot et al. 2004) and at a Pacific Ocean coastal site in the Southern California Bight (Worden et al. 2004). In addition, *Ostreococcus* 18S rRNA gene sequences have been recorded from an open ocean Mediterranean Sea location (Díez et al. 2001) as well as the English Channel (Romari & Vaulot 2004). Hence, with the Arabian Sea sequences described here, there is added weight to the idea that this

is a widely distributed prasinophyte genus. Interestingly, no clones related to prasinophyte clade VI (order Prasinococcales) were obtained in these libraries, suggesting that, although they were the dominant PPE in our enrichment cultures, they represented only a minor component of the *in situ* PPE community at the 2 stations. Clade VI prasinophytes have however, also been isolated from open ocean locations in the North Atlantic Ocean and eastern Mediterranean Sea (Andersen et al. 1997, Guillou et al. 2004) indicating that members of this prasinophyte clade are widely distributed at least.

Pelagophyte sequences comprised up to 10% of the sequences in any 1 library and appear to derive from the genus *Pelagomonas*. *Pelagomonas calceolata*, the type strain of this class, was isolated from the open ocean in the North Pacific (Andersen et al. 1993), whilst pelagophyte 18S rRNA gene sequences have also been obtained from the oligotrophic South Pacific (Moon-van der Staay et al. 2001) and Mediterranean Sea (Díez et al. 2001). The fact that several clone libraries from marine coastal sites do not include this class (e.g. see Romari & Vaulot 2004) may suggest that this genus is restricted to more open ocean regions. Interestingly, environmental 16S rRNA gene sequences (e.g. 5X11) which we are now able to identify as pelagophytes (Fig. 1) were also common in 2 clone libraries from the Southern Ocean (Wilmotte et al. 2002).

In addition to the Arabian Sea dictyochophyte sequence reported here, sequences of this class have also been detected in the oligotrophic South Pacific (Moon-van der Staay et al. 2001) and from the North Atlantic and Mediterranean Sea (Díez et al. 2001). Since only a single dictyochophyte sequence was amplified from these libraries however it is likely that this class is present only at low abundance in these Arabian Sea waters.

CONCLUSIONS

The development of a PCR biased towards marine algal plastids represents significant progress in the assessment of PPE diversity in the many marine habitats dominated numerically by the picocyanobacteria *Synechococcus* and *Prochlorococcus*, particularly oligotrophic open ocean systems. Whilst our clone library data showed that this novel PCR will still amplify picocyanobacteria, PPE plastid sequences were considerably enriched, even from samples where flow cytometry data indicated a vast excess of picocyanobacterial cells. Interestingly, recent clone libraries constructed with the PLA491F-OXY1313R primer pair from the Gulf of Naples contain only PPE sequences (S. McDonald, A. Zingone & D. J. Scanlan unpubl. data).

We should add that some caveats exist with this approach. Thus, rRNA gene copy number and plastid copy number per cell, as well as any inherent biases associated with the PCR with this primer pair, need to be borne in mind when, for example, interpreting the number of clones of a specific class obtained in clone libraries, as does the fact that some solely heterotrophic picoeukaryotes might possess a non-photosynthetic plastid (e.g. the dictyochophyte *Pteridomonas danica* [Sekiguchi et al. 2002] or members of the chrysophyte genus *Paraphysomonas*) which may be amplified by these plastid-biased primers. Furthermore, a dinoflagellate origin of some of these amplified sequences cannot be ruled out at present. Even so, since we now have a significantly increased database of plastid 16S rRNA gene sequences from algal cultures, with correct application of this marine algal plastid-biased PCR to the picoplanktonic fraction of marine samples, we anticipate further improvements in our understanding of PPE diversity and community dynamics.

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