Micro- and macrodiversity in *rbcL* sequences in ambient phytoplankton populations from the southeastern Gulf of Mexico

John H. Paul[1,*], Albin Alfreider[2], Boris Wawrik[1]

[1]Department of Marine Science, University of South Florida, 140 Seventh Avenue South St. Petersburg, Florida 33701, USA
[2]Institute of Zoology and Limnology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria

ABSTRACT: Ribulose-1,5-diphosphate carboxylase/oxygenase (RuBisCO) large subunit genes (*rbcL*) were obtained by amplification and cloning of 554 or 614 bp sequences of indigenous phytoplankton populations at 2 stations in the southeastern Gulf of Mexico. One station (Stn 4) was located in a low salinity, high chlorophyll plume (the 'Green River') which has previously been shown to contain elevated levels of Form IA *rbcL* mRNA while the other (Stn 7) was in oligotrophic, oceanic water. A diversity of *rbcL* sequences was obtained, spanning 3 of the 4 evolutionary clades of Form I RuBisCOs. Six nucleotide sequences obtained from Stn 4 were closely related (92 to 96% similar) to the Form IA-containing *Prochlorococcus* GP2. Flow cytometry and pigment analysis indicated that *Prochlorococcus* was abundant at this site. Other sequences found included a Form IB *rbcL* closely related to prasinophytes, and Form ID sequences related to prymnesiophytes, diatoms, and pelagophytes. One sequence was nearly identical to the pelagophyte, *Pelagomonas calceolata*. At Stn 7, sequences were obtained that were more deeply rooted, and less similar to *rbcL*s in existing databases (77 to 83% similar), and no Form IA *rbcL*s were detected. HPLC pigment signatures and flow cytometry data were consistent with the forms obtained by cloning. The similarity of the 6 *Prochlorococcus* GP2-like sequences (93 to 98%) is consistent with the phenomenon of molecular microdiversity as found at other loci in marine (and other environmental) microorganisms.

KEY WORDS: *rbcL* gene sequences · Phylogeny · *Prochlorococcus* GP-2

INTRODUCTION

Photosynthetic carbon fixation in the world's oceans is catalyzed primarily by the enzyme ribulose-1,5-diphosphate carboxylase/oxygenase (RuBisCO; Raven 1995). A diversity of phytoplankton participate in this process, including the picoplankton (*Synechococcus*, *Prochlorococcus*, and picoeucaryotes) as well as larger micro-, nano- and net plankton. Analysis of diversity in natural phytoplankton populations has usually been done by pigment analysis (Tester et al. 1995, Bidigare & Ondrusek 1996, Peeken 1997), flow cytometry (Campbell et al. 1997, Urbach & Chisholm 1998), or microscopy. Molecular approaches are now being widely used to investigate issues of diversity in heterotrophic and autotrophic picoplankton populations. Molecular phylogeny using 16S rRNA genes has been successfully used to determine the identity of (or nearest relative of) DNA sequences in the marine and other environments (Giovanonni et al. 1990, Moyer et al. 1995, Ferris et al. 1996, Field et al. 1997, Rooney-Varga et al. 1997). The problem with this approach has been that it has been difficult to target groups that perform a physiologic or biogeochemical function in the environment, because 16S genotyping only yields information on the relatedness of one organism to another. A gene which encodes for a critical environmental function in one organism may be absent in a closely related 16S genotype. The diversity of natural populations of *Synechococcus* and *Prochlorococcus* has also been successfully investigated using RNA polymerase genes (Palenik 1994, Ferris & Palenik 1998). By using biogeo-

*E-mail: jpaul@seas.marine.usf.edu

chemically significant gene functions that are somewhat conserved (i.e. *nifH*; Zehr et al. 1998), it is possible to identify organisms with the potential to perform those functions.

The large subunit of RuBisCO has several conserved domains which enable it to be used for phylogenetic purposes (Watson & Tabita 1997). However, there is evidence of many gene transfer events and gene duplications in the evolution of RuBisCO genes, particularly in the large subunit (Delwiche & Palmer 1996). As the *rbcL* database grows, more consistent phylogenies that take into account such lateral transfer events can be constructed.

Little is known concerning the diversity of *rbcL* genotypes in natural phytoplankton populations (Pichard et al. 1997). To this end, we have amplified, cloned and bi-directionally sequenced portions of the *rbcL* genes from 2 distinct environments in the southeastern Gulf of Mexico. Our results indicate the presence of both macro- and microdiversity of the *rbcL* gene in these populations.

MATERIALS AND METHODS

Sampling sites. Samples were taken during a research cruise aboard the RV 'Pelican' in August of 1997 at the sites indicated in Fig. 1. The depths sampled included 3 and 35 m for Stn 4 and 3 m for Stn 7. Stn 4 was located in a low salinity (29 ppt surface water) plume that had a high chlorophyll *a* (chl *a*) content and a shallow (45 m deep) subsurface chlorophyll maximum (SCM; Paul et al. 2000, in this issue). This low salinity feature was believed to have originated from the estuaries and rivers in the northern Gulf of Mexico, and has been termed the 'Green River'. This station also had elevated levels of Form IA *rbcL* mRNA and *Synechococcus* cell counts in the surface waters (8.6 × 10^4 cells ml^{-1}), maximal *Prochlorococcus* cell counts (1.4 × 10^5 cells ml^{-1}) at 40 m depth, and maximal picoeucaryotes (2.3 × 10^3 cells ml^{-1}) at the SCM. Maximal carbon fixation occurred in the surface (3 m depth) sample (Paul et al. 2000, this issue). Stn 7 was more typical of the stratified, oligotrophic waters found in the Gulf of Mexico (Pichard et al. 1997), with a SCM at 83 m, and near constant salinity (~36 ppt) with depth. *Synechococcus* was in the surface waters (2.22 × 10^4 cells ml^{-1}), *Prochlorococcus* maximal abundance (1.83 × 10^5 cells ml^{-1}) at 50 m, and picoeucaryotes at the SCM (2.76 × 10^3 cells ml^{-1}). Stn 10 was sampled by SCUBA divers over a reef in the Dry Tortugas.

DNA sampling. Seawater samples (500 to 1000 ml) were filtered onto 25 mm, 0.45 µm polyvinylidene difluoride filters (Millipore Durapore), the filters placed into 2.2 ml microfuge tubes, and 1.0 ml of STE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) added and the filters frozen on liquid nitrogen. Samples were stored until extraction at –20°C. The extraction of environmental *rbcL* DNA were performed by boiling lysis in STE containing 1% sodium dodecyl sulfate as previously described in Pichard et al. (1993).

PCR. Two sets of degenerate oligonucleotide primers were designed for PCR amplification of *rbcL* gene fragments. The nucleotide sequences of the Form ID primers, which amplified a 554 bp fragment of the *rbcL* gene, were: forward primer: GATGATGA(AG)AA-(CT)ATTAACCT-3'.

Reverse primer: ATTTG(A/G/T)CCACAGTG(A/G/T)-ATACCA-3'. Nucleotide sequences of primers which amplified a 615 bp fragment of the Form IA and Form IB *rbcL* gene consisted of: forward primer: TCIGCITG-(A/G)AACTA(C/T)GGTCG and reverse primer: GGC-AT(AG)TGCCAIAC(AG)TG(AG)AT. Extracted DNA from different sampling stations was resuspended in sterile water and 1 to 5 µl of DNA was used for amplification in a reaction mixture containing 50 pmol each of the appropriate primers, 2 µl of each deoxyribonucleoside triphosphate (final conc. 0.2 mM each), 10 µl of 10X reaction buffer (Promega Inc, Madison, WI), 6 µl 25 mM MgCl$_2$ and 3 U of *Taq* DNA polymerase (Promega Inc, Madison, WI) in a total volume of 100 µl. To prevent nonspecific amplification and primer-dimer formation, *Taq* Start antibodies were used according to the instructions supplied by the manufacturer (Clontech Laboratories Inc., Palo Alto, CA).

Cycle parameters were as follows: 3 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 52°C, and 1 min 30 s at 72°C. These cycles were followed by a 20 min incu-
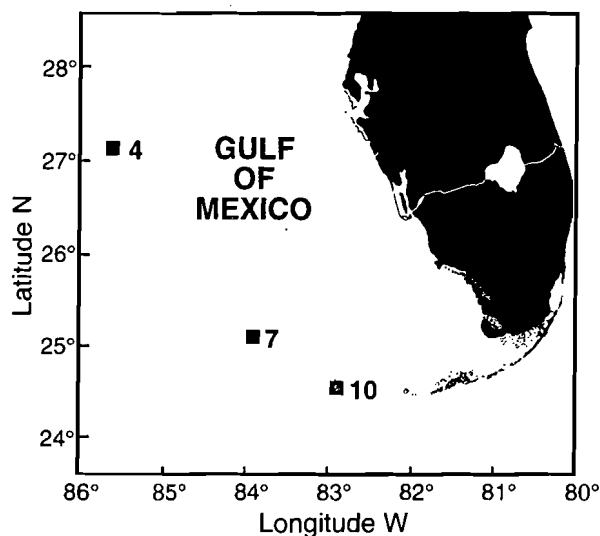

Fig. 1. Location of stations sampled in this study. Stns 4 and 7 were open ocean profiles, whereas Stn 10 was from a shallow reef environment

bation at 72°C. The retrieved amplicons were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels stained with fluorescent stain Hoechst 33258.

Cloning and variant screening of PCR amplification products. Fresh PCR products were purified (QIAquick PCR purification kit, Qiagen Inc., Valencia, CA) and TA cloned into pCR®2.1 vector (Invitrogen Corp., Carlsbad, CA) according to the protocols provided by the manufacturers. A total of 110 white colonies were selected for study, and 76 of these were shown to contain inserts of approximately the expected size. Plasmid DNA was isolated using a plasmid isolation kit (Qiagen Inc., Valencia, CA), digested with restriction enzyme *Eco*RI and screened for variation using denaturing gradient gel electrophoresis (DGGE). DGGE was performed with a vertical gel electrophoresis apparatus (Hoefer SE 600) as described previously (Muyzer et al. 1993). In total 76 *Eco*RI restricted clones were applied onto 6% (wt/vol) polyacrylamide gels containing 0.5× TAE (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2-EDTA) with different denaturing gradients ranging from 0 to 80% denaturant (100% denaturant corresponds to 7 M Urea and 40% [vol/vol] deionized formamide). The gels were electrophoresed for 12 h at 60°C and 65 V. After electrophoresis, the gels were stained with SYBR Green I nucleic acid stain (Molecular Probes Inc., Eugene, OR).

DNA sequencing and phylogenetic analysis. Plasmids containing PCR-amplified inserts were sequenced with an Applied Biosystems model 373 sequencer at the Sequencing Core Laboratory (University of Florida, Gainesville, FL).

Deduced amino acid sequences were aligned with Clustal W as provided by OMIGA 1.1 software (Oxford Molecular group, Oxford, UK) using a pairwise, weighted alignment method. The gap penalty was 50, while gap elongation penalty was set to 10. The alignment was visually inspected for obvious misalignments and was then exported to the MEGA 1.02 software (Kumar et al. 1993). MEGA was used to compute neighbor-joining tress applying gamma distribution as a distance method to correct for multiple hits. While different gamma parameters were explored the default value of 2 was applied in order to generate the trees (using TreeView [Page 1966]) in Figs. 2 & 3. Closest relatives to *rbcL* clones were obtained by using NCBI's sequence similarity search tool BLASTN 2.0.8 (Basic Local Alignment Search Tool; Altschul et al. 1997). GenBank accession numbers for *rbcL* sequences for the organisms used to build the trees in Figs. 2 & 3 are as follows:

Synechococcus PCC7002, D13971; *Anabaena* sp., L02520; *Bathycoccus prasinos*, U30275; *Calyptroshpaera sphaeroidea*, D45842; *Chlorella ellipsoidea*, M20655;

Chrysochromulina hirta, D45846; *Cryptomonas* sp., X62349; *Cylindrotheca* sp., M59080; *Ectocarpus siliculosus*, X52503; *Emiliania huxleyi*, D45845; *Heterosigma akashiwo*, X61918; *Hibberdia magna*, AF015572; *Hydrogenovibrio marinus*, D43622; Mn-oxidizing bacterium, L32182; *Nitrobacter vulgaris*, L22885; *Odontella sinensis*, Z67753; *Pavlova salina*, D45847; *Synechococcus* PCC6301, J01536; *Pelagococcus subviridis*, AF15580; *Pelagomonas calceolata*, U89898; *Pleurochrysis carterae*, D11140; *Prochlorococcus* GP2, D21833; *Prochlorothrix hollandica*, X57359; *Rhodobacter sphaeroides*, M64624; *Prochloron* sp., D21834; *Skeletonema costatum*, AF015569; *Spinacia oleracea*, V00168, J01443; *Thiobacillus* sp., D13539; *Synechococcus* WH7803, U46156; *Xanthobacter flavus*, X17252; *Gonyaulax polyedra*, L41063. *rbcL* sequences appearing in Fig. 3 from the Gulf of Mexico or cultures and published in our previous paper (Pichard et al. 1997) include: GOMst8 mRNA 5m, U93860; GOMst4 mRNA 80m, U93861; GOMst4 mRNA 50m, U93856; GOMst4 DNA 5m, U93290; GOMst4 DNA2 5m, U93855; *Prochlorococcus* PAC1, U93858; *Prochlorococcus* MED, U93857; and *Synechococcus* sp. WH8007, U93859. *rbcL* clone sequences obtained in this study can be accessed from GenBank using the following accession numbers: 10GH4, AF179005; 10GH8, AF179006; 10GY1, AF179007; 4AH1, AF179008; 4AH10, AF179009; 4AH3, AF179010; 4AH6, AF179011; 4AH8, AF179012; 4AH9, AF179013; 4AY1, AF179014; 4AY2, AF179015; 4DCH1, AF179016; 4DCH11, AF179017; 4DCH12, AF179018; 4DCH2, AF179019; 4DCH6, AF179020; 4DCH7, AF179021; 4DCH9, AF179022; 7HCY2, AF179023; 7ACH3, AF179024; 7ACH5, AF179025; 7ACH9, AF179026; 7ACY7, AF179027.

RESULTS

Fig. 2 is a neighbor joining tree of deduced *rbcL* amino acid sequences from Genbank and those obtained from Stns 4, 7, and 10. The prefix number of the clone designation indicates the station from which it was obtained. For example, clone 4AY2 was obtained from Stn 4, depth 'A' or surface (3 m). Table 1 presents the most closely related sequence and the % similarity based upon deduced amino acid sequence and nucleotide sequence, rather than displaying a very large similarity table for all sequences. In total 6 Form IA *rbcL* sequences were cloned from Stn 4 that were most closely related to *Prochlorococcus* GP2. The degree of similarity of these clones to this organism based on inferred amino acid sequences ranged from 88% (clone 4DCH9) to 99% (clones 4DCH6, 4DCH11, and 4DCH12). Nucleotide similarities ranged from 93% (4DCH9) to 96% (4DCH12). Clones were isolated from
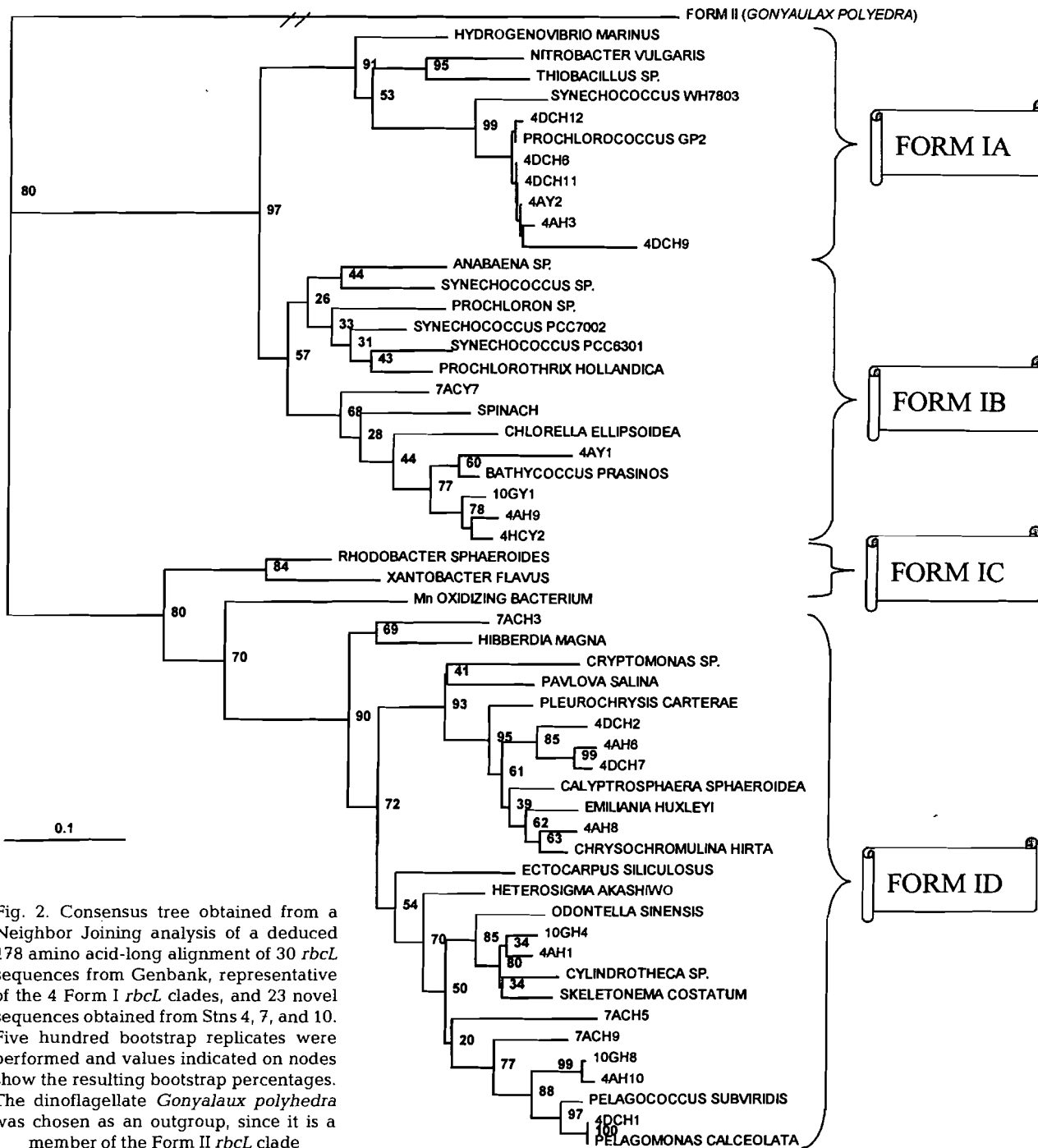
Fig. 2. Consensus tree obtained from a Neighbor Joining analysis of a deduced 178 amino acid-long alignment of 30 *rbcL* sequences from Genbank, representative of the 4 Form I *rbcL* clades, and 23 novel sequences obtained from Stns 4, 7, and 10. Five hundred bootstrap replicates were performed and values indicated on nodes show the resulting bootstrap percentages. The dinoflagellate *Gonyalaux polyhedra* was chosen as an outgroup, since it is a member of the Form II *rbcL* clade

both the surface waters and the 35 m depth. The clones were obtained by using both the cyano (Form IA/B) primers and the chromo (Form ID) primers. These clones were all relatively similar to each other (98 to 99% at the amino acid level, 94 to 99% at the nucleotide level) with the exception of 4DCH9, which had a lower similarity (88 to 89% at the amino acid level, and 93 to 99% at the nucleotide level).

Also present at Stn 4 were green algal sequences (Form IB *rbcL*; Table 2) most closely related to prasinophytes. These ranged in similarity between 87 to 90% at the amino acid level and 80 to 91% at the nucleotide level to *Bathycoccus prasinos*.

Form ID (chromophytic algal) *rbcL* sequences were amplified from Stn 4 samples using both the cyano and chromophytic primers. Four such clones were in
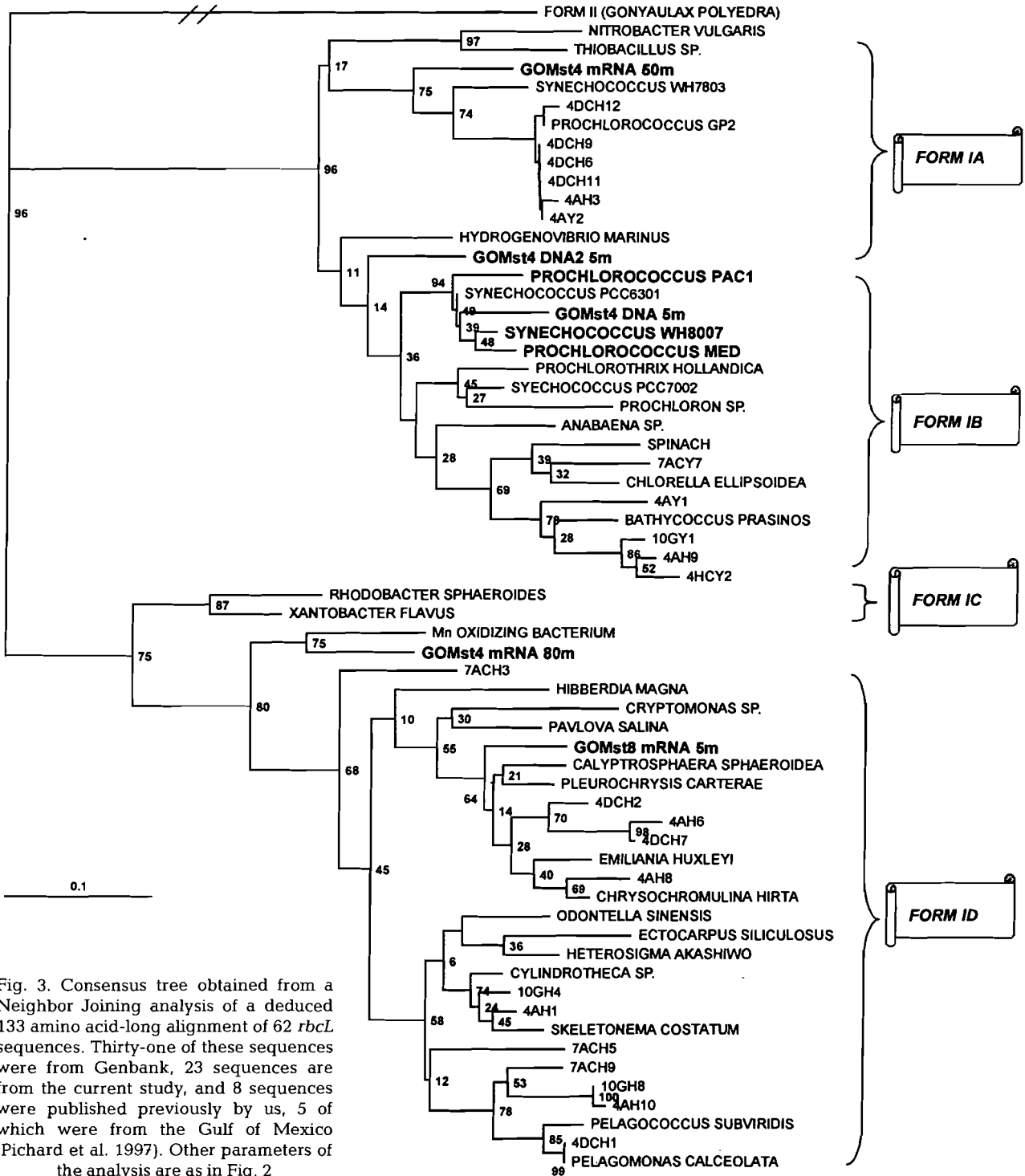
Fig. 3. Consensus tree obtained from a Neighbor Joining analysis of a deduced 133 amino acid-long alignment of 62 *rbcL* sequences. Thirty-one of these sequences were from Genbank, 23 sequences are from the current study, and 8 sequences were published previously by us, 5 of which were from the Gulf of Mexico (Pichard et al. 1997). Other parameters of the analysis are as in Fig. 2

the prymnesiophyte group (Fig. 2 and Table 1). The closest to a cultivated alga was that of 4AH8 which was 94 and 89% similar at the amino acid and nucleotide levels, respectively, to *Chrysochromulina hirta*. Other prymnesiophyte-like clones included 4AH6, 4DCH2, and 4DCH7, which were similar to

Calyptosphaera sphaeroideae and *Pleurochrysis cartereae*. Also present in the surface waters was 1 diatom-like clone, 4AH1, which was 94% similar to *Skeletonema costatum*. This sequence showed a lesser similarity to the diatoms *Cylindrotheca* and *Odontella*.

Table 1. Closest relatives to *rbcL* clones obtained from Stn 4

Depth	Clone design	Primer pair used	Relative	%NT similarity	%AA similarity
Form IA clones					
Surface	4AY2	IA/IB	*Prochlorococcus* GP2	92	98
	4AH3	ID	*Prochlorococcus* GP2	92	98
35 m	4DCH12	ID	*Prochlorococcus* GP2	96	99
	4DCH6	IA/IB	*Prochlorococcus* GP2	93	99
	4DCH11	IA/IB	*Prochlorococcus* GP2	94	99
	4DCH9	IA/IB	*Prochlorococcus* GP2	93	88
Form IB clones					
Surface	4AY1	IA/IB	*Bathycoccus prasinos*[a]	80	87
	4AH9	ID	*Bathycoccus prasinos*	91	87
100 m	4HCY2	IA/IB	*Bathycoccus prasinos*	91	90
Form ID clones					
Surface	4AH8	ID	*Chrysochromulina hirta*[b]	89	94
			Emiliani huxleyi[b]	88	91
	4AH6	ID	*Pleurochysis carterae*[b]	85	89
			Calyptosphaera sphaeroidea[b]	88	89
	4AH10	ID	*Pelagococcus subviridis*[c]	89	91
	4AH1	ID	*Skeletonema costatum*[d]	90	94
			Cylindrotheca[d]	88	92
			Odontella[d]	87	90
35 m	4DCH7	ID	*Pleurochysis carterae*	84	88
			Calyptosphaera sphaeroidea	88	89
	4DCH2	ID	*Pleurochysis carterae*	84	89
	4DCH1	ID	*Pelagomonas calceolata*[c]	99	100
			Pelagococcus subviridis	94	96

[a]Prasinophyte; [b]prymnesiophytes; [c]pelagophyte; [d]diatoms

Pelagophyte-like clones were also present at Stn 4. Clone 4DCH1 was identical to *Pelagomonas calceolata* showing that this organism is present in the Gulf of Mexico. A surface clone (4AH10) was 91% similar to *P. subviridis*.

Phylogenetic similarities of clones isolated from Stn 7 appear also in Fig. 2 and Table 2. No Form IA *rbcL* clones were found, even though hybridization occurred with the Form IA probe (Paul et al. 2000). One chlorophyte-like (Form IB) sequence, clone 7ACY7, was found at this station in the surface waters. This clone was not closely related to any *rbcL* in the database, being most similar to *Chlorella ellipsoidea* (88 and 77% similar at the nucleotide and amino acid levels, respectively). Several chromophytic (Form ID) clones were obtained from Stn 7 as well, although these were all fairly deeply rooted amongst the existing *rbcL* sequences. Clone 7ACH3 was between the Form ID and Form IC *rbcLs*. The closest relative was *Hibberida magna*, which is a heterokont alga, a chromophytic autotrophic stramenopile, also known as a true chrysophyte (Daugs-

berg & Anderson 1997a). Clones 7ACH9 and 7ACH5 were isolated from the surface waters of Stn 7 and were phylogenetically grouped between the pelagophytes and diatoms. Clone 7ACH9 was most likely a pelagophyte, 87 and 81% similar at the amino acid and nucleotide levels, respectively, to *Pelagococcus subviridis*. Clone 7ACH5 was more distantly related to the pelagophytes *P. subviridis* and *P. calceolata* (82 and 81% similar, respectively, at the amino acid level) than clone 7ACH9. Clone 7ACH5 showed a similar degree of relatedness to the diatoms *Skeletonema costatum* and *Odontella* as to the raphidophyte *Heterosigma akashiwo*.

Three clones were also obtained from the surface of corals in the Dry Tortugas. These included a green algal-like clone (10GY1) most closely related to the prasinophyte *Bathococcus prasinos*, a diatom-like clone related to *Cylindrotheca* (10GH4; 93% similar), and a pelagophyte-like clone related to *Pelagococcus subviridis* (10GH8, 92% similar).

DISCUSSION

Twenty unique *rbcL* sequences were obtained from the water column at Stns 4 and 7 in the Gulf of Mexico and 3 additional sequences were obtained from the coral surface in the Dry Tortugas. Of the clones obtained at Stn 4, 6 were in the Form IA clade and most closely related to *Prochlorococcus* GP2 (Shimada et al.

Table 2. Closest relatives to *rbcL* clones obtained from Stns 7 and 10

Depth	Clone design	Primer pair used	Closest relative	%NT similarity	%AA similarity
Stn 7—Form IB *rbcLs*					
Surface	7ACY7	IA/IB	*Chlorella*[a]	77	88
Stn 7—Form ID *rbcLs*					
Surface	7ACH3	ID	*Hibberdia magna*[b]	83	83
	7ACH9	ID	*Pelagococcus subviridis*[c]	81	87
	7ACH5	ID	*Pelagococcus subviridis*	81	82
Stn 10—Form IB *rbcL*					
Surface	10GY1	IA/IB	*Bathycoccus prasinos*[d]	90	87
Stn 10—Form ID *rbcL*					
Surface	10GH4	ID	*Cylindrotheca* sp.[e]	87	93
	10GH8	ID	*Pelagococcus subviridis*	89	92

[a]Green alga; [b]heterokont chrysophyte; [c]pelagophyte; [d]prasinophyte; [e]diatom

1995, 1996), being 93 to 96% similar at the nucleotide level. *Prochlorococcus* has been shown to be a diverse taxonomic assemblage (Moore et al. 1998, Urbach & Chisholm 1998, Urbach et al. 1998, Partensky et al. 1999, West & Scanlan 1999). Analysis of the 16S rRNA (Ferris & Palenik 1998), *petB/D* loci (Urbach & Chisholm 1998), and location in the water column (Moore et al. 1998, West & Scanlan 1999) indicates that there are at least 2 major genotypes in this taxa, the HL (high light) and LL (low light) clades. Additionally, subgroups of the high light clade (HLI and HLII) have been identified (West & Scanlan 1999). It is not known how *rbcL* phylogeny fits into this taxonomy, as to date only 3 *Prochlorococcus rbcL* sequences have been published. *Prochlorococcus* GP2, a member of the HLII clade by 16S rRNA taxonomy, also possess a Form IA *rbcL* (Shimada et al. 1995). The *Prochlorococcus* PAC1 strain, a low light strain, contains a Form IB *rbcL*, consistent with other cyanobacteria, *Synechococcus*, and the prochlorphytes *Prochlorthrix* and *Prochloron*. The *Prochlorococcus* MED strain, identified as a HLI clone (West & Scanlan 1999), also contains a Form IB *rbcL* (Pichard et al. 1997). Of the 6 *Prochlorococcus* GP2-like clones, 2 were from the surface waters of Stn 4 and 4 were from the 35 m depth sample. In most oceans this depth corresponds to that which is dominated by high light clones (Moore et al. 1998, Urbach & Chisholm 1998, West & Scanlan 1999). It may be that the HLII group of *Prochlorococcus* contains the Form IA *rbcL*, while the LL clade contains the Form IB *rbcL*. However, further *Prochlorococcus* clone sequences are needed before such a generalization can be made.

A second possibility for the identity of these 6 Form IA sequences is that they belong to a *Synechococcus* strain that has yet to be sequenced. This is unlikely in part because of the G+C content of these Form IA sequences. An observation of many *Prochlorococcus* gene sequences is that they have an overall low G+C content (average 36.8%; Partensky et al. 1999). The average G+C content of the 6 Form IA sequences found in this study is 40.6%, which compares favorably to the *Prochlorococcus* GP2 *rbcL* sequence of 40.2% (Shimada et al. 1995) but unfavorably with *Synechococcus* WH7803 Form IA *rbcL* (60.7%; Watson & Tabita 1996).

At Stn 7, no Form IA clones were obtained, even though some Form IA *rbcL* transcripts were detected in the surface and subsurface waters by direct hybridization. Eleven-fold greater levels of Form IA *rbcL* mRNA were encountered at Stn 4 than at Stn 7 (Paul et al. 2000). We are not certain as to the efficiency of our amplification strategy in obtaining representative genotypes or our capability to screen unique clones by DGGE. For example, many of the clones obtained with the FormIA/IB primer set had similar mobilities in the DGGE gel yet unique sequences. The lower levels of

Form IA transcriptional activity may indicate a smaller biomass of these organisms. Because DNA was amplified, perhaps other genotypes were preferentially amplified. Additionally, there were 2 peaks in the Form IA *rbcL* mRNA at Stn 7, a surface 1 (which may have corresponded with *Synechococcus*) and a subsurface 1 (perhaps corresponding to *Prochlorococcus*; Paul et al. 2000). Such studies will await flow cytometric sorting in conjunction with cloning and sequencing to answer such questions.

No *Synechococcus*-like Form IB sequences were found at either station, whereas Form IB green algal sequences were found at both. These were most closely related to *Bathycoccus prasinos*, a prasinophyte. Prasinophytes are found in the surface waters of many oceanic environments, including the Antarctic (Stoecker et al. 1998), the Pacific near Japan (Miyashita, et al. 1993), and the Gulf of Mexico. *B. prasinos* was first described in the Gulf of Naples, Italy (Eikrem & Throndsen 1990).

A diversity of chromophytic (Saunders et al. 1995) *rbcL* sequences were found at Stn 4, including diatoms, prymnesiophytes, and pelagophytes. Of the prymnesiophytes, 1 was most closely related to *Chrysochromulina hirta* (4AH8), while the other clones fell clearly in the prymnesiophyte 'clade'. Prymnesiophytes are known to form a separate clade within the Form ID *rbcL* sequences distinct from diatoms, pelagophytes, red algae, and brown algae (Daugbjerg & Andersen 1997b). These algae, also known as haptophytes of the 'heterokont' chromophytic algae, are known to contain chl *a* and *c*, as well as the carotenoid 19'-hexanoyloxyfucoxanthin (Mejanelle et al. 1995). These pigments were found both in the surface and subsurface waters at Stns 4 and 7, with the highest concentrations at the SCM. These clones were found in waters with abundant picoeucaryotic cell counts as determined by flow cytometry (Paul et al. 2000). Prymnesiophytes have been described as important contributors to phytoplankton communities in the Southern Ocean (Peeken 1997) equatorial Pacific (Bidigare & Ondrusek 1996, Verity et al. 1996) and in the NW Mediterranean (Bustillos-Guzman et al. 1995).

The 4AH1 sequence fell clearly in the diatom clade. Only a few diatom *rbcL* sequences exist at this time (Daugbjerg & Anderson 1997a), and the sequence obtained was not identical to any in the database (87 to 90% similar at the nucleotide level). There were diatoms present in the surface water plume of Stn 4 as indicated by the presence of Fucoxanthin and chl *c*1 and *c*2 (Paul et al. 2000).

Pelagophyte sequences obtained at Stn 4 were related to *Pelagococcus subviridis* (clone 4AH10) and 1 (4DCH1) was identical to *Pelagomonas calceolata* (Andersen et al. 1993). This is compelling evidence that *P.*

calceolata was a member of the phytoplankton in these waters. The presence of pelagophytes as components of the picoplankton at this station was also verified by detection of 19'-butanoyloxyfucoxanthin (Paul et al. 2000), a pigment found in pelagophytes (Bidigare & Ondrusek 1996, Peeken 1997).

Unlike the clones obtained from Stn 4, the *rbcL* sequences obtained from Stn 7 were not as closely related to existing DNA sequences. For example, the green alga Form IB clone 7ACY7 was related to both *Chlorella ellipsoidea* (77%) and spinach (72%). The 7ACH3 clone was related to *Hibberida magna* (83%), a chrysophyte related to synurophytes, and somewhat between the Form IC and ID lineages (Daugbjerg & Andersen 1997a). Sequence 7ACH5 was between the pelagophytes (81% similar to *Pelagococcus subviridis*) and the raphidophytes (*Heterosigma akashiwo*; 82% similar). It may be that waters at Stn 7 were more oligotrophic than Stn 4, and contained species similar to true oceanic taxa, and perhaps not as easily cultivated or readily studied as those from Stn 4. Thus, there may be less oligotrophic oceanic taxa in the *rbcL* database than those from coastal oceanic waters, which may contribute to the dissimilarity of the clones obtained at Stn 7 to those in the database.

We have looked for similarities between the current Gulf of Mexico clones and those published by us previously (Pichard et al. 1997), which appear in bold in Fig. 3. None of the previously obtained clones were closely related to those obtained in the current study. The lack of similarity may not be surprising. First, the primer sets used between the 2 studies were different, even though they targeted the same general area of the *rbcL* gene (a ~350 bp overlap exists between the 2 amplicons). However, the primer sets designed in the current study are based upon much more sequence data than those designed in 1994 for the 1997 publication (Pichard et al. 1997). Secondly, the relative biases of the primers are unknown. Finally, the differences in sequences could be the result of variation of the phytoplankton populations in space and time in the Gulf of Mexico as would be expected.

Xu & Tabita (1996) took a similar approach to the isolation of *rbcL* sequences from Lake Erie, using a primer set that we had previously designed. Diatom-like *rbcL* sequences were obtained from subsurface samples while green algal-like sequences were found in the surface and subsurface waters (Xu & Tabita 1996).

The occurrence of very similar yet non-identical sequences, as observed for the *Prochlorococcus* GP2-like sequences of Stn 4, has been termed 'molecular microdiversity' (Fuhrman & Campbell 1998, Moore et al. 1998). In all these studies (including the present one) the term 'diversity' is misleading, because inherent in diversity is an indication of the abundance of

each of the 'species' or genotypes present (Shannon & Weaver 1949). A better term for what has been measured in these types of studies may be 'genotype richness', analogous to species richness, which is the total number of species present. However, the types of amplicons obtained are limited by primer biases, relative efficiency of amplification, and other factors.

We do not think that the 'molecular microdiversity' found in this study is simply PCR error caused by misincorporation of nucleotides. Using a similar calculation as that of Scala & Kerkhoff (1999), 1 PCR error in 10^4 bases (a high misincorporation rate) would result in 99.99% similarity after 1 PCR cycle. If 35 PCR cycles occur, then the % similarity between a true and 'corrupted' sequence would be 99.99^{35} or 99.7%. Thus, clones that are >99.7% similar are considered identical. Because our clones ranged from 92 to 96% similar to 1 another, we feel that the microdiversity measured was real and not an artifact of PCR.

The microdiversity observed with the Form IA sequences seems to be a common occurrence in many loci examined in natural populations of marine microorganisms, and may be a fundamental precept in the ecology of genes in marine microbial populations. For example, microdiversity has been observed in the Sar 11 cluster, an as yet unidentified but prominent group of the proteobacteria in many oceans (Field et al. 1997). Scala & Kerkhof (1999) measured diversity of the nitrous oxide synthetase gene (*nosZ*) which encodes the final step in denitrification. These investigators found similarities ranging from between 33 to 99% for clones from the sediments of the Atlantic and Pacific continental shelves. Ferris et al. (1996) found microdiversity in thermophilic *Synechococcus* populations in a thermal spring microbial mat. Zehr & coworkers (1998) have observed both microdiversity and macrodiversity in the *nifH* genes obtained from environmental samples, again with differences only being a few % between clones, yet greater than that caused by PCR. Both macro- and microdiversity has been observed in the *rpo* locus in *Prochlorococcus* and *Synechococcus* (Palenik 1994, Palenik & Swift 1996, Ferris & Palenik 1998).

The issue of molecular microdiversity raises some interesting evolutionary and ecological questions. For example, to what advantage would it be to have a population genetically similar yet not identical (presumably not clonally derived) competing for the same resources? Hutchinson (1961) raised this issue to question phytoplankton diversity in his paper *Paradox of the Plankton*. That paper was dealing with what we would term 'macrodiversity', or differing phyla, classes, orders, and genera in the phytoplankton in a somewhat homogeneous environment, the water column. However, the *Paradox of the Plankton* is even more appropriate when considering molecular microdiver-

sity. Fuhrman & Campbell (1998) considered this issue in *Prochlorococcus*, and wondered if the small changes in sequence are simply genetic drift or microevolution in physiologically similar, clonal populations, or if it is the result of macroevolution of distinct populations, each with unique niches? Genetic drift in a culture is either neutral or results in replacement of the original genotype with one more fit for the culture conditions, not a widespread diversification of the loci in question. Perhaps constantly changing conditions, as per the water column, promote growth and perpetuation of distinct clones, transient conditions favoring one genovar over the other. Molecular microevolution occurs in every loci thus far examined, and may be a method for adaptation to environmental conditions and evolution of successful genotypes.

LITERATURE CITED

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Andersen RA, Saunders, GW, Paskind MP, Sexton JP (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

Bidigare RR, Ondrusek ME (1996) Spatial and temporal variability of phytoplankton pigment distributions in the Central Equatorial Pacific Ocean. Deep-Sea Res 43:809–833

Bustillos-Guzman J, Claustre H, Marty JC (1995) Specific phytoplankton signatures and their relationship to hydrographic conditions in the coastal northwestern Mediterranean Sea. Mar Ecol Prog Ser 124:247–258

Campbell L, Liu H, Nolla HA, Vaulot D (1997) Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991–1994 ENSO event. Deep-Sea Res 44:167–192

Daugbjerg N, Andersen RA (1997a) A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded *rbcL* sequence data. J Phycol 33:1031–1041

Daugbjerg N, Andersen RA (1997b) Phylogenetic analyses of the *rbcL* sequences from haptophytes and heterokont algae suggest their chloroplasts are unrelated. Mol Biol Evol 14:1242–1251

Delwiche CF, Palmer JD (1996) Rampant horizontal transfer and duplication of Rubisco genes in eubacteria and plastids. Mol Biol Evol 13:873–882

Eikrem W, Throndsen J (1990) The ultrastructure of *Bathycoccus* gen. nov. and *B. prasinos* sp. nov., a non-motile picoplanktonic alga (Chlorophyta, prasinophyceae) from the Mediterranean and Atlantic. Phycologia 29:344–350

Ferris MJ, Palenik B (1998) Niche adaptation in ocean cyanobacteria. Nature 396:226–228

Ferris MJ, Ruff-Roberts ED, Kopczynski ED, Bateson MM, Ward DM (1996) Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a

single hot spring microbial mat habitat. Appl Environ Microbiol 62:1045–1050

Field KG, Gordon D, Whight T, Rappe M, Urgach E, Vergin K, Giovannoni SJ (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Appl Environ Microbiol 63:63–70

Fuhrman JA, Campbell L (1998) Microbial microdiversity. Nature 393:410–411

Giovanonni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 344:60–63

Hutchinson GE (1961) The paradox of the plankton. Am Nat 45:137–145

Kumar S, Tamura K, Nei M (1993) MEGA: Molecular evolutionary genetic analysis, version 1.0. The Pennsylvania State University, University Park, PA

Mejanelle L, Laureillard J, Fillaux J, Saliot A, Lambert C (1995) Winter distribution of algal pigments in small and large-size particles in the northeastern Atlantic. Deep-Sea Res 42:117–133

Miyashita H, Ikemoto H, Kurano N, Miyachi S, Chihara M (1993) *Prasinococcus capsulatus* gen. et sp. nov., a new marine coccoid prasinophyte. J Gen Appl Microbiol 39: 571–582

Moore LR, Rocap G, Chisholm SE (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature 393:464–467

Moyer CL, Dobbs FC, Karl DM (1995) Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl Environ Microbiol 61:1555–1562

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Palenik B (1994) Cyanobacterial community structure as seen from RNA polymerase gene sequence analysis. Appl Environ Microbiol 60:3212–3219

Palenik B, Swift H (1996) Cyanobacterial evolution and prochlorphyte diversity as seen in DNA-dependent RNA polymerase gene sequences. J Phycol 32:638–646

Partensky F, Hess WR, Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol Mol Biol Rev 63:106–127

Paul JH, Alfrieder A, Kang JB, Stokes RA, Griffin D, Campbell L, Ornolfsdottir E (2000) Form IA *rbcL* transcripts associated with a low salinity/high chlorophyll plume ('Green River') in the eastern Gulf of Mexico. Mar Ecol Prog Ser 198:1–8

Peeken I (1997) Photosynthetic pigment fingerprints as indicators of phytoplankton biomass and development in different water masses of the Southern Ocean during austral spring. Deep-Sea Res 44:261–282

Pichard SL, Frischer ME, Paul JH (1993) Ribulose bisphosphate carboxylase gene expression in subtropical marine phytoplankton populations. Mar Ecol Prog Ser 101:55–65

Pichard SL, Campbell L, Paul JH (1997) Diversity of ribulose bisphosphate carboxylase oxygenase Form I gene (*rbcL*) in natural phytoplankton communities. Appl Environ Microbiol 63:3600–3606

Raven JA (1995) Inorganic carbon assimilation by marine biota. J Exp Mar Biol Ecol 203:39–47

Rooney-Varga JN, Devereux R, Evans RS, Hines ME (1997)

Seasonal changes in the relative abundance of unculti-vated sulfate-reducing bacteria in a salt marsh sediment and in the rhizosphere of *Spartina alterniflora*. Appl Environ Microbiol 63:3895–3901

Saunders GW, Potter D, Paskind MP, Andersen RA (1995) Cladistic analyses of combined traditional and molecular data sets reveal an algal lineage. Proc Natl Acad Sci USA 92:244–248

Scala DJ, Kerkhof LJ (1999) Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl Environ Microbiol 65:1681–1687

Shannon CE, Weaver W (1949) The mathematical theory of communication. Univ Illinois, Champagne

Shimada A, Kanai S, Maruyama T (1995) Partial sequence of ribulose-1,5-bisphosphate carboxylase/oxygenase and phylogeny of *Prochloron* and *Prochlorococcus* (Prochlorales). J Mol Evol 40:671–677

Shimada A, Maruyama T, Miyachi S (1996) Vertical distributions and photosynthetic action spectra of two oceanic picophytoplankters, *Prochlorococcus marinus* and *Synechococcus*. Mar Biol 127:15–23

Stoecker DK, Gustafson DE, Black MMD, Baier CT (1998) Population dynamics of microalgae in the upper land-fast sea ice at a snow-free location. J Phycol 34:60–69

Tester PA, Geesey ME, Guo C, Paerl HW, Millie DF (1995) Evaluating phytoplankton dynamics in the Newport River estuary (North Carolina, USA) by HPLC-derived pigment profiles. Mar Ecol Prog Ser 124:237–245

Urbach E, Chisholm SW (1998) Genetic diversity in *Prochlorococcus* populations flow cytometrically sorted from the Sargasso Sea and Gulf Stream. Limnol Oceanogr 43: 1615–1630

Urbach E, Scanlan DJ, Distel DL, Waterbury JB, Chisholm SW (1998) Rapid diversification of marine picophytoplankton with dissimilar light-harvesting structures inferred from sequences of *Prochlorococcus* and *Synechococcus* (Cyanobacteria) J Mol Evol 46:188–201

Verity PG, Stoecker DK, Sieracki ME, Nelson JR (1996) Microzooplankton grazing of primary production at 140° W in the Equatorial Pacific. Deep-Sea Res 43:1227–1256

Watson GMF, Tabita FR (1996) Regulation, unique gene organization and unusual primary structure of carbon fixation genes from a marine phycoerythrin-containing cyanobacterium. Plant Mol Biol 32:1103–1106

Watson GMF, Tabita FR (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. FEMS Microbiol Lett 146:13–22

West NJ, Scanlan DJ (1999) Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the Eastern North Atlantic Ocean. Appl Environ Microbiol 65: 2585–2591

Xu EH, Tabita FR (1996) Ribulose-1,5-bisphosphate carboxylase/oxygenase gene expression and diversity of Lake Erie planktonic microorganisms. Appl Environ Microbiol 62:1913–1921

Zehr JP, Mellon MT, Zani S (1998) New nitrogen-fixing microorganisms detected in olgiotrophic oceans by amplification of nitrogenase (*nifH*) genes. Appl Environ Microbiol 64:3444–3050