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Research Article

The First Molecular Characterization of Picocyanobacteria from the Argentine Sea

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Picocyanobacteria are abundant throughout the world's oceans. Particularly, it has been reported that *Synechococcus* strains have a wide latitudinal distribution, from polar to tropical waters. However, their molecular characterization in the Southwest Atlantic Ocean is still missing. We analyzed *Synechococcus* genetic diversity in a sector of the Argentine Sea, one of the richest biological areas of the world oceans. *16S rRNA* amplicons obtained after PCR amplification of environmental DNA extracted from water samples of this area were used for DGGE and sequenced. Only *Synechococcus* sequences could be retrieved. On the other hand, we isolated two *Synechococcus* strains from the environment. Our analyses revealed that the clade I group was widespread from latitude 38°S to 48°S and that can coexist with clade IV strains in shelf waters. The cooccurrence of these two clades may be related to an adaptation to high-nutrient/low-temperature waters. Our data are the first report on *Synechococcus* ecotypes that would be important contributors to phytoplankton biomass in the Argentine Sea, one of the richest biological areas of the world oceans.

1. Introduction

Oxygenic prokaryotic photoautotrophs (Cyanobacteria) were estimated to constitute about 10% of the total ocean marine picoplankton in the upper 200 m [1]. Strains of *Synechococcus* and *Prochlorococcus* genera dominate the marine picophytoplankton in the world's oceans and contribute significantly to CO_2 fixation and primary production [2, 3]. Although both genera often can coexist in tropical and temperate waters, their distribution patterns differ spatially and seasonally [4, 5]. Whereas *Prochlorococcus* are largely confined to a 40°N–40°S latitudinal band and are mostly abundant in oligotrophic waters, *Synechococcus* are distributed ubiquitously throughout oceanic regions from polar to tropical waters [6–9].

The use of molecular markers based on PCR amplification of different conserved genes, molecular probes, pyrosequencing, and multilocus sequence analysis allowed defining several *Synechococcus* genetic lineages using cultured and natural samples from marine environments [7–14]. Marine *Synechococcus* have been classified into three major subclusters (5.1 to 5.3) [15, 16]. Most strains belong to subcluster 5.1, which contains at least 10 distinct phylogenetic clades according to *16S rRNA* sequences [8]. Additional lineages have been proposed based on other sequences, such as *16S–23S rRNA* ITS, the N-regulatory *ntcA* gene, and the encoding sequence of the cytochrome *b6* subunit of the cytochrome *b6f* complex (*petB*) [9, 11, 14, 17, 18].

Analyses of *Synechococcus* and *Prochlorococcus* lineage distribution at local and ocean basin scale were reported using data from cruises in the Pacific, Atlantic, Indian, and Arctic Oceans and in the Red Sea [4, 5, 13, 18–20]. However, there are unexplored oceanic regions, such as the waters of the Southwest Atlantic Ocean [21], where the Argentine shelf and its shelf-break constitute one of the richest regions for marine life [22]. This was supported by global studies using

Table 1: Localization and environmental parameters of Patagonian stations at the Argentine Sea. Data were registered by the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Gabinete de Oceanografía Física, Base Regional de Datos Oceanográficos (BaRDO).

Station	Date	Location	Depth (m)	Temperature (°C)	Salinity	Abundance cell mL^{-1} 10^{-3}
20	13-03-06	58°53′W 47°19′S	208	10.945	34.061	10 (2.1)*
27	16-03-06	61°48′W 47°22′S	130	13.076	33.564	1,370 (287.7)*
62	29-03-06	58°46′W 43°52′S	201	10.631	33.952	65 (13.7)*
66	29-03-06	61°25′W 43°20′S	88.0	14.730	33.553	26 (5.5)*

*Numbers in brackets indicate biomass expressed in $\mu g\,C\,L^{-1}$.

ocean-color satellite images that revealed bright features denoting high chlorophyll concentrations [23, 24]. However, no molecular characterization of the ultraphytoplankton components has been still reported. Therefore, taking into account the importance of this oceanic region, we started the examination of the population diversity of picocyanobacteria, a crucial plankton component for primary productivity.

The aim of this study was to contribute to the knowledge of the global biogeography of prokaryotic components of the marine picophytoplankton. Thus we characterized picocyanobacteria in waters from the Argentine Sea, one of the richest biological areas of the world oceans and a still unexplored region. Our data constitute the beginning of more comprehensive studies in this region, and, importantly, it is a contribution to the knowledge of the *Synechococcus* diversity.

2. Materials and Methods

2.1. Sample Collection, Strain Isolation, and Characterization. Water samples were collected at 5 m depth in duplicate using Niskin bottles from (i) a permanent coastal station located off Mar del Plata (EPEA) by the cruises conducted monthly onboard the RV "Capitán Canepa" (INIDEP) and (ii) four stations in the Argentine Sea: two from the continental shelf (stations 66 and 27) and two from adjacent waters (oceanic stations 62 and 20), in March 2006 (RV ARA "Puerto Deseado" cruise GEF II, PNUD ARG 02/018 Project) (Figure 1). Temperature and salinity profiles of water column at each station were recorded by a Seabird SBE1901 CTD (Table 1). Samples of 50 mL were fixed with formaldehyde (0.4% final concentration) and filtered by a polycarbonate membrane of 0.2 μm pore size. *Synechococcus* were characterized by epifluorescence microscopy and carbon biomass was calculated directly from cell number using a conversion factor of 0.21 pg C $cell^{-1}$ [25]. *Synechococcus* isolates were obtained by the serial dilution culture method using f/10 medium without silicate [26].

2.2. DNA Extraction, PCR Amplification, Cloning, and Sequencing. For total DNA extraction from environmental samples, up to 5 L of seawater was collected in surface with a Niskin bottle and transferred through a 200 μm nylon mesh to a black bottle. Sampleswere prefiltered through a 3 μm

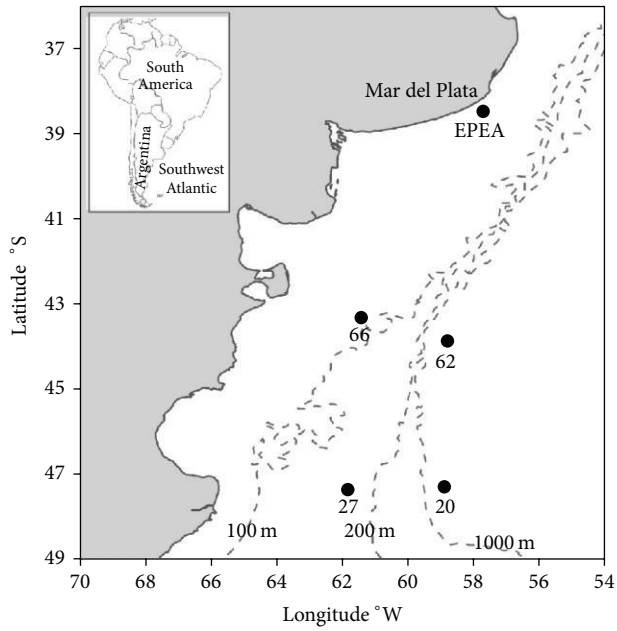

Figure 1: Map showing the location of sampling stations at the Argentine Sea (southwest Atlantic Ocean). Permanent coastal station (EPEA) and four stations (27 and 66 (continental shelf) and 20 and 62 (oceanic waters)) of the RV ARA "Puerto Deseado" cruise (PNUD ARG 02/018 Project).

Nuclepore membrane (Whatman International Ltd, Maidstone, England) to separate picoplankton and subsequently filtered through a Sterivex unit (Milli-pore, Billerica, MA, USA) with a peristaltic pump. The units were kept in liquid nitrogen on board until arrival to the laboratory and stored at −80°C before proceeding. The units were incubated at 37°C for 30 min with lysozyme (1 mg mL^{-1}) and then with proteinase K (0.5 mg mL^{-1}), followed by three freeze-thaw cycles. Finally, DNA was extracted with the DNeasy Plant Mini Kit (Qiagen). DNA from the isolates was extracted as previously described [27].

16S rRNA genes were amplified either from DNA of the isolates or from environmental samples, using primers SYN172F and OXY1313R. This primer pair amplifies all known sequences from *Synechococcus* subclusters 5.1 (clades I–X [8], some subclusters 5.2 strains, no fresh water *Synechococcus* strains, all low-light (LL) *Prochlorococcus* strains,

some high light (HL), HLI *Prochlorococcus* strains, but no HLII *Prochlorococcus* strains [28]). Three environmental libraries from EPEA sampling during summer time were constructed by ligating PCR products to pGEM-T vectors and introduced into *E. coli* cells.

The amplification of the nitrogen regulatory gene *ntcA* (approach that specifically targets cyanobacteria) was used to identify novel *Synechococcus* clades with considerably higher resolution than the *16S rRNA* sequences [11, 13]. The primer pair 1F/4R [29] was used to amplify *ntcA* genes from the isolates. In Table S1 we summarize the oligonucleotide sequences of primer pairs used in this study (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/237628). PCR products were cloned using a pGEM-T Easy kit according to the manufacturer's instructions (Promega Corporation). To screen for *Synechococcus 16S rRNA* and *ntcA* clones, inserts were digested with the restriction endonucleases *Eco*RI and *Pst*I (Promega Corporation), respectively. Plasmid DNA was isolated from three representatives of each restriction pattern, using a QIAprep Spin Miniprep Kit (QIAGEN), and inserts were sequenced (Macrogen, Korea).

2.3. Denaturing Gradient Gel Electrophoresis (DGGE). As a diversity study approach, the environmental samples were analyzed by DGGE. The amplification products of the *16S rRNA* genes (either from the isolates or the environmental samples from Patagonian shelf and oceanic waters) were used as templates in a nested PCR with the primer pair 358fGC/907r [30] (Table S1). The amplification products of the *16S rRNA* genes from the isolates Syn-EPEA.1 and Syn-GEF.1 were used as references. The products were subjected to DGGE (2001 system, CBS Scientific Company) [30]. A 6% polyacrylamide gel with a gradient of DNA-denaturant agent was casted by mixing solutions of 40 and 80% denaturant agent (100% denaturant agent was 7 M urea and 40% deionized formamide). Electrophoresis was performed at 100 V and 60°C for 18 h in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). DGGE gels were stained with 100 μL L^{-1} SybrGold (Molecular Probes Europe, Leiden, The Netherlands) in TAE buffer for 45 min, rinsed with the same buffer for 20 min, removed from the glass plate to a UV transparent gel scoop, visualized, and registered by using a Photodyne system apparatus (Photodyne Technologies, LA, USA) [31]. Prominent DNA bands were excised from the DGGE gels and eluted from the gel by sterile water at 4°C overnight. These eluted products were reamplified with primers 358fGC, without the GC-clamp, and 907r and the PCR products were sequenced.

2.4. Sequence and Phylogenetic Analyses. All *16S rRNA* and *ntcA* gene sequences were first compared with those present in public databases using the basic local alignment search tool BLAST network service (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed with the neighbour-joining and maximum parsimony methods, and bootstrap analyses (1,000 replicates) were done with *MEGA* version 5 [32].

3. Results and Discussion

3.1. Isolation and Molecular Identification of Two Synechococcus Strains from Coastal and Shelf Waters. For further diversity studies, we initially isolated *Synechococcus* strains from different regions of the Argentine Sea. In EPEA station, the maximum phytoplankton biomass value was observed during summer (December–March), with being *Synechococcus* the major contributor [33]. Firstly, from surface waters at this station, we PCR amplified environmental DNA and obtained only three differentpartial *16S rRNA* sequences (EMBL accession numbers HE600704, HE600705, and HE600706), which were 98.9 to 99.7% identical to the sequence of *Synechococcus* sp. Almo3. Then we isolated under microscope a strain (named Syn-EPEA.1), whose complete *16S rRNA* sequence was amplified for molecular characterization (EMBL accession number HE600707), and being around 98.9% identical to sequences of members of the genus *Synechococcus*.

On the other hand, from shelf waters corresponding to the GEF-III cruise (at a location equivalent to station 27, 61°48′W 47°22′S), we isolated a picophytoprokaryote strain (named Syn-GEF.1), whose complete *16S rRNA* sequence (EMBL accession number HE601900) was 99.8% identical to marine *Synechococcus* sequences.

To better differentiate between genotypes, we amplified and sequenced *ntcA* gene fragments from the Syn-EPEA.1 and Syn-GEF.1 isolates (EMBL accession numbers HE601901 and HE601902, resp.). Syn-EPEA.1 and Syn-GEF.1 sequences were about 91.6, 90.6, and 70.2% identical to partial *ntcA* sequences of *Synechococcus* sp. WH 8020, CC9311, and RS9905, respectively and 97.9% identical among them.

3.2. Molecular Identification of Synechococcus Strains from Shelf and Adjacent Waters of the Argentine Sea. DGGE technique was used to analyze *Synechococcus* diversity in shelf and adjacent waters at the Patagonian region of the Argentine Sea. The amplification products of the *16S rRNA* genes from the isolates Syn-EPEA.1 and Syn-GEF.1 (used as references) and amplicons from the environmental samples corresponding to stations 66 and 27 (shelf waters) and 62 and 20 (adjacent waters) were subjected to DGGE (Table S1 and Figure 1). Although some bands appeared dispersed across the gel gradient, obvious and representative bands (Figure 2) could be selected for DNA reamplification and further sequencing. All environmental sequences were about 93 to 100% identical to sequences of marine *Synechococcus* (Table 2). Although bands of samples corresponding to a shelf and adjacent waters migrate at similar positions on the gel (Table 2 and Figure 2), their sequences shown high degree of identity to sequences of different *Synechococcus* strains: 27.2 and 62.2 bands were 100% and 99.3 identical to CC 9902 and CC 9311, respectively.

3.3. Phylogenetic Analyses. Partial *16S rRNA* sequences were aligned with representative sequences of *Synechococcus* belonging to clades I to IX [8] and to clades XV and XVI [9]. Neighbour-joining phylograms were constructed after

Table 2: Analysis of *16S rRNA* sequences from the DGGE excised bands (Figure 2) of environmental samples from the Argentine Sea. The isolated strains Syn-EPEA.1 and Syn-GEF.1 were used as reference. *E* values of all sequences were 0.0.

Site	Sample	Bands selected	Closest relatives	Identity (%)	Clade	Accession number
GEF	Syn GEF.1	GEF.1	*Synechococcus* sp. Almo3 *16S rRNA* gene, partial sequence.	99.8	I	HE792894
		GEF.2	*Synechococcus* sp. Almo3 *16S rRNA* gene, partial sequence.	100	I	HE687328
EPEA	Syn EPEA.1	EPEA.1	*Synechococcus* sp. Almo3 *16S rRNA* gene, partial sequence.	99.6	I	HE687320
		EPEA.2	*Synechococcus* sp. Almo3 *16S rRNA* gene, partial sequence.	99.8	I	HE687321
27	Environmental	27.1	*Synechococcus* sp. CC9311 *16S rRNA* gene, partial sequence.	100	I	HE687322
		27.2	*Synechococcus* sp. CC 9902, complete genome.	100	IV	HE687323
20	Environmental	20.1	*Synechococcus* sp. Almo3 *16S rRNA* gene, partial sequence.	99.6	I	HE687324
66	Environmental	66.1	*Synechococcus* sp. CC9902, complete genome.	99.6	IV	HE792895
		66.2	*Synechococcus* sp. CC 9902, complete genome.	100	IV	HE687325
62	Environmental	62.1	*Synechococcus* sp. RCC307 genomic DNA sequence.	93.5	subcluster 5.3	HE687326
		62.2	*Synechococcus* sp. CC9311 *16S rRNA* gene, partial sequence.	99.3	I	HE687327

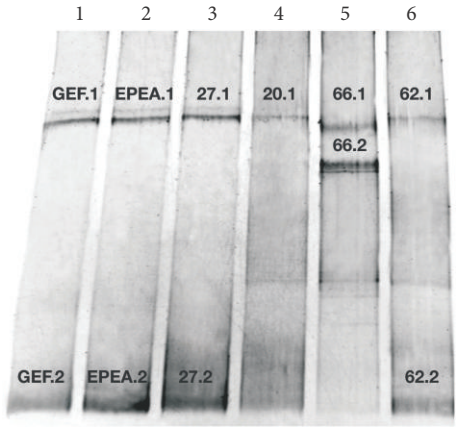

Figure 2: Denaturing gradient gel electrophoresis analysis of PCR amplified *16S rRNA* gene segments obtained from environmental samples of the Argentine Sea. Syn-GEF.1 (lane 1) and Syn-EPEA.1 (lane 2) isolates were used as references. Lanes 3 and 5: shelf stations 27 and 66, respectively; lanes 4 and 6: oceanic stations 20 and 62, respectively. Labeled bands were excised for further sequencing.

sequence alignments (Figure 3). Similar tree topologies were observed by maximum parsimony analyses (not shown). The *16S rRNA* sequences from the EPEA environmental samples and the two *Synechococcus* isolates grouped in clade I. For the analysis of *ntcA* sequences, we also included *Synechococcus* sequences of clades XI to XIV, which were identified in *ntcA* phylogenies [11]. The phylogenetic analysis using *ntcA* sequences showed similar results for the isolates to those obtained with the *16S rRNA* sequences (Figure 4), which suggests a good congruence with the two genetic markers used. On the other hand, whereas most of the *16S rRNA* sequences obtained from the DGGE bands produced significant alignments with *Synechococcus* sequences belonging to clade I, three sequences from shelf waters (66 and 27) grouped with those of clade IV, and another one from station 62 clustered with sequences of subcluster 5.3 (Table 2 and Figure 3).

According to our results, clade I *Synechococcus* seems to be the most common group in the Southwest Atlantic from latitude 38°S to 48°S. Also, sequences from clade IV were found at 43°S and 47°S at the Patagonian shelf (stations 66 and 27) but not in oceanic waters. The coexistence of clades I and IV has been well-documented in other mesotrophic temperate regions (latitudes above 30°N/S), and the ratio of abundances of the two clades is likely to vary both spatially and temporally suggesting that both clades are adapted to high-nutrient/low-temperature waters [4, 5, 19, 34, 35]. Interestingly, comparative genome studies show that strains of those clades lack regulatory genes that are important for phosphate scavenging when this nutrient is under limiting conditions [16]. Yet, there is a glaring lack of understanding about the genetic and physiological features that allow clades I and IV to compete with other *Synechococcus* clades in mesotrophic regions. Strikingly, our data show an extraordinarily high level of biomass (287.7 μg C L^{-1}) in station 27 (Table 1), which could be ascribed exclusively to picocyanobacteria (Silva, personal communication). A recent report, studying six *Synechococcus* strains isolated along a gradient of latitude in the North Atlantic Ocean, revealed

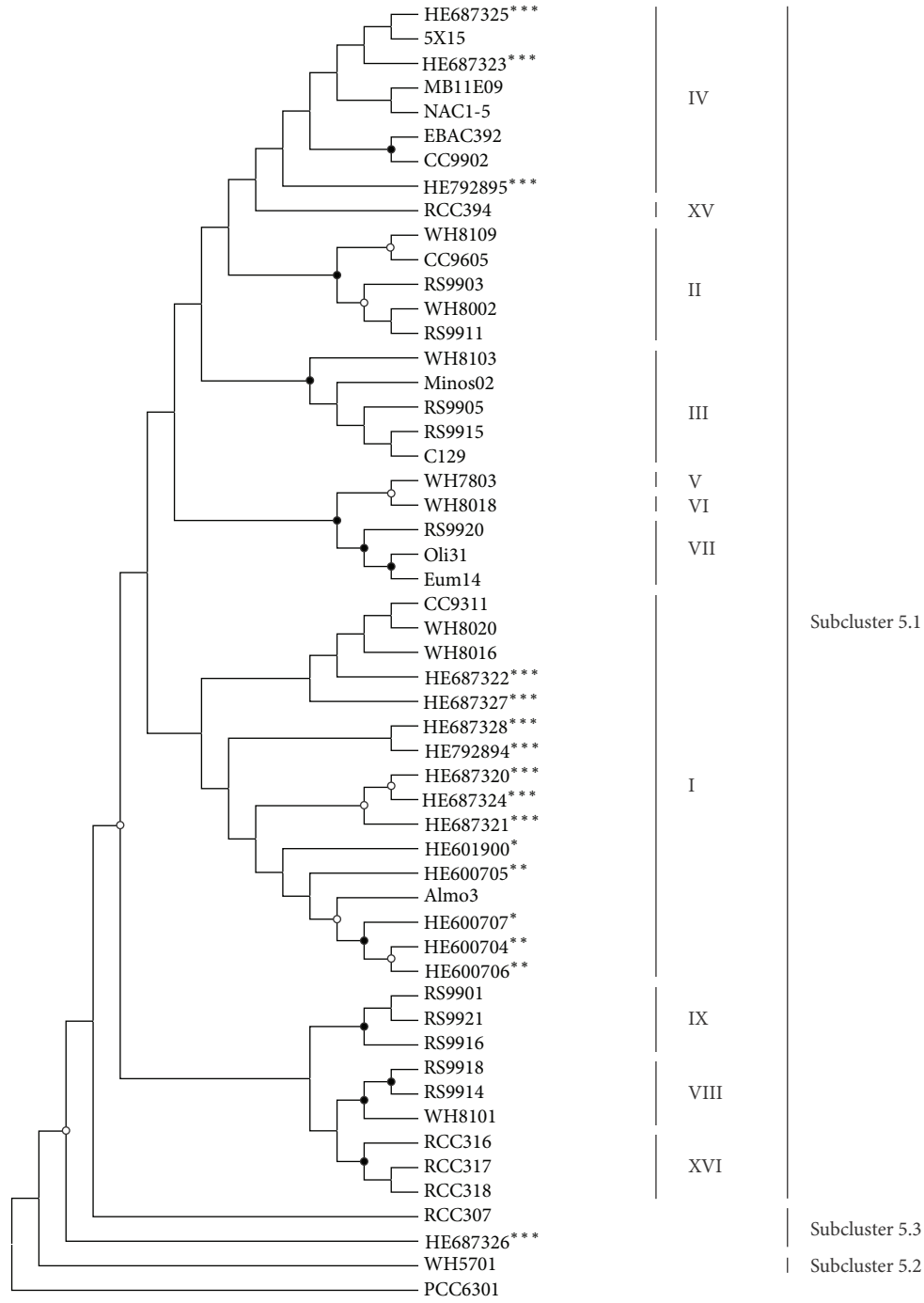

Figure 3: Neighbor-joining phylogenetic trees based on *16S rRNA* gene sequences. Bootstrap values were obtained through 1,000 repetitions. Similar tree topologies were observed by maximum parsimony analyses (not shown). (•) indicates a value >90% and (◦) indicates a value >60%. The freshwater strain *Synechococcus* sp. PCC 6301 was used as outgroup. *Synechococcus* clades are marked with roman numbers. Sequences from *Synechococcus* isolates from environmental samples and from DGGE bands are indicated with one, two, or three asterisks, respectively.

the existence of lineages of marine *Synechococcus* physiologically specialized in different thermal niches, suggesting the existence of temperature ecotypes within the marine *Synechococcus* radiation [36]. Moreover, the authors suggest that clade I and probably clade IV *Synechococcus* have a physiology preferentially adapted to cold thermal niches. Our results are in line with those findings since the range of water temperatures at the sites of sampling was between 10.6 and 14.7°C (Table 1).

Finally, a sequence belonging to subcluster 5.3 (formerly included in clade X [8]) was detected in the oceanic station 62. *Synechococcus* subcluster 5.3 has been recently reestablished

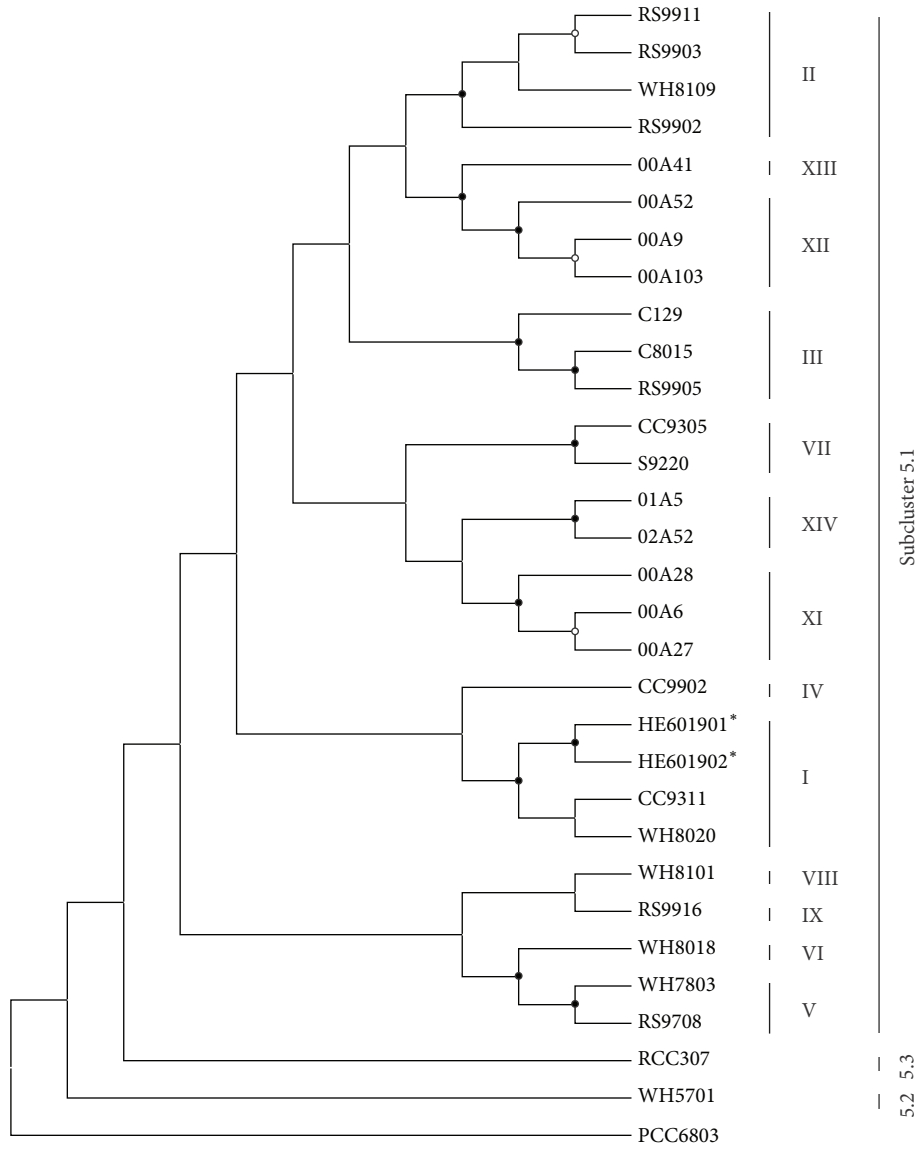

Figure 4: Neighbor-joining phylogenetic trees based on *ntcA* gene sequences. Bootstrap values were obtained through 1,000 repetitions. Similar tree topologies were observed by maximum parsimony analyses (not shown). (•) indicates a value >90% and (◦) indicates a value >60%. The *ntcA* sequence from *Synechocystis* sp. PCC 6803 was used as outgroup. *Synechococcus* clades are marked with roman numbers. Sequences from the two *Synechococcus* isolates are indicated with an asterisk.

[15, 16] and includes *Synechococcus* RCC307 [9], KORDI-15, and KORDI-30 [17]. Much less is known about the biogeography of those *Synechococcus* strains compared with subcluster 5.1 [18]. Our results showed that members of the subcluster 5.3 are also present in oceanic waters.

4. Conclusions

This study is the first report on the molecular characterization of *Synechococcus* in the Southwest Atlantic Ocean, contributing to the knowledge of their global distribution. *Synechococcus* were identified not only in coastal waters, as reported in EPEA, a permanent coastal station located off Mar del Plata [37], but also in the shelf and oceanic waters.

While *Synechococcus* clade IV sequences could only be retrieved from Patagonian shelf waters (stations 66 and 27), *Synechococcus* clade I seems to be widespread in the Argentine Sea, from latitude 38°S to 48°S. Moreover, strains of clades I and IV, suggested as low temperature ecotypes [36], can coexist at the Patagonian shelf (stations 27). The cooccurrence of both ecotypes could be associated with the biomass increment in station 27; however, further studies are needed to support this speculation. Data presented are a starting point to evaluate the role of *Synechococcus* ecotypes, as powerful bioindicators to monitor the impact

of possible climatic changes in the South Atlantic Ocean. However, a large-scale and a long-term effort will be needed to understand seasonality and population dynamic not only of picocyanobacteria but also of other ultraphytoplankton components.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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