

# Diversity of the marine picocyanobacteria *Prochlorococcus* and *Synechococcus* assessed by terminal restriction fragment length polymorphisms of 16S-23S rRNA internal transcribed spacer sequences

Diversidad de las picocianobacterias marinas *Prochlorococcus* y *Synechococcus* por medio de polimorfismos de longitud de fragmentos de restricción terminal en secuencias del espaciador transcrito interno del ARNr 16S - 23S

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## ABSTRACT

In order to assess the appropriateness of the use of internal transcribed spacer (ITS) sequences for the study of population genetics of marine cyanobacteria, we amplified and cloned the 16S rRNA gene plus the 16S–23S ITS regions of six strains of *Prochlorococcus* and *Synechococcus*. We analyzed them by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (T-RFLP). When using the standard application of these techniques, we obtained more than one band or terminal restriction fragment (T-RF) per strain or cloned sequence. Reports in literature have suggested that these anomalies can result from the formation of secondary structures. Secondary structures of the ITS sequences of *Prochlorococcus* and *Synechococcus* strains were computationally modelled at the different temperatures that were used during the polymerase chain reaction (PCR). Modelling results predicted the existence of hairpin loops that would still be present at the extension temperature; it is likely that these loops produced incomplete and single stranded PCR products. We modified the standard T-RFLP procedure by adding the labelled ITS primer in the last two cycles of the PCR reaction; this resulted, in most cases, in only one T-RF per ribotype. Application of this technique to a natural picoplankton community in marine waters off northern Chile, showed that it was possible to identify the presence, and determine the relative abundance, of several phylogenetic lineages within the genera *Prochlorococcus* and *Synechococcus* inhabiting the euphotic zone. Phylogenetic analysis of ITS sequences obtained by cloning and sequencing DNA from the same sample confirmed the presence of the different genotypes. With the proposed modification, T-RFLP profiles should therefore be suitable for studying the diversity of natural populations of cyanobacteria, and should become an important tool to study the factors influencing the genetic structure and distribution of these organisms.

**Key words:** cyanobacteria, DGGE, T-RFLP, ITS, secondary structure.

## RESUMEN

Con el fin de evaluar la utilización de secuencias del espaciador interno transcrito (ITS) en estudios de genética de población de cianobacterias marinas, se amplificó y clonó la secuencia del gen ARNr 16S junto a la región espaciadora 16S-23S ARNr de seis cepas de *Prochlorococcus* y *Synechococcus*. Se analizaron los amplicones del ITS por electroforesis en gel de gradiente de desnaturalización (DGGE) y por polimorfismos de longitud de fragmentos de restricción terminal (T-RFLP). Al aplicar los métodos estándares de estas técnicas, se obtuvo más de una banda o fragmento de restricción terminal (T-RF) por cepa o secuencia clonada. Informes en la literatura han sugerido que estas anomalías podrían ser atribuidas a la formación de estructuras secundarias. Por consiguiente, la estructura secundaria de las secuencias de ITS de las cepas de *Prochlorococcus* y *Synechococcus* fue modelada a las diferentes temperaturas que se utilizaron durante la reacción en cadena de polimerasa (PCR). Dicho modelamiento predijo la existencia de bucles que podrían persistir incluso durante la temperatura de extensión. Es probable que estos bucles generen productos de PCR con fragmentos incompletos y hebras simples. En este trabajo se modificó el procedimiento del método de T-

RFLP añadiendo el partidor marcado en los últimos dos ciclos. Esto resultó, para la mayoría de los casos, la obtención de un solo fragmento de restricción por ribotipo. La aplicación de esta técnica a una muestra del medio ambiente obtenida frente al norte de Chile, demostró que es posible identificar la presencia, y determinar la abundancia relativa, de varios linajes filogenéticos de los géneros *Prochlorococcus* y *Synechococcus* que habitan la zona eufótica. El análisis filogenético de las secuencias de ITS obtenidos por clonación y secuenciación de ADN a partir de la misma muestra confirmó la presencia de los diferentes genotipos. Con la modificación propuesta, el método de T-RFLP debiera ser adecuado para el estudio de la diversidad en poblaciones naturales de cianobacterias, pudiendo transformarse en una importante herramienta para estudiar los factores que influyen en la estructura genética de estos organismos.

**Palabras clave:** cianobacteria, DGGE, T-RFLP, ITS, estructura secundaria.

## INTRODUCTION

The unicellular cyanobacteria *Prochlorococcus* and *Synechococcus* are the most abundant photosynthetic microorganisms on Earth, playing an important role in the ocean's carbon cycle and contributing significantly to the global marine primary production (Li 1995, Liu et al. 1997, Partensky et al. 1999, Maranon et al. 2003). They form part of the picoplankton (cells with a size of  $< 2 \mu\text{m}$ ). These cyanobacteria are distributed especially in tropical and subtropical open oceans, playing an important role in the ecology of the planktonic community, especially in oligo- and mesotrophic regions (Campbell et al. 1994, Partensky et al. 1999).

In marine ecosystems, environmental clines exist in temperature, light intensity, spectral composition, and nutrient availability that can affect the distribution, composition and diversity of organisms. *Prochlorococcus* and *Synechococcus* have ecotypes which have identifiable geographic ranges that correspond to particular temperature, nutrient concentration, as well as light regimes (Ferris & Palenik 1998, Partensky et al. 1999, Fuller et al. 2003).

In oligotrophic waters, *Prochlorococcus* usually dominate the microbial plankton, both in terms of numbers and biomass (Zubkov et al. 2000). Along trophic gradients, *Prochlorococcus* abundance shows opposite patterns to *Synechococcus* abundance, becoming a less important component of the carbon standing stock from oligo- to eutrophic conditions, even though *Prochlorococcus* can also grow at these higher nutrient levels (Partensky et al. 1996, Partensky et al. 1999, Zubkov et al. 2000, Grob et al. 2007). *Prochlorococcus* is geographically limited to latitude ranges of  $40^\circ \text{S}$ - $40^\circ \text{N}$ , inhabits the

entire photic zone. It can be found as deep as 200 m below the surface, being abundant in warmer, oligotrophic ecosystems where it is the major primary producer (Partensky et al. 1999, Grob et al. 2007, Zwirgmaier et al. 2007).

*Prochlorococcus* and *Synechococcus* co-occur in many oceanographic regions, but *Synechococcus* tolerates a broader temperature range, not being limited by temperatures as low as  $2^\circ \text{C}$  (Shapiro & Haugen 1988, Moore et al. 1995, Alvain et al. 2005) *Synechococcus* is more ubiquitous and has a broader latitudinal distribution than *Prochlorococcus*, but is often less abundant in warmer, oligotrophic ecosystems and is usually limited to shallower depths ( $< 100 \text{m}$ ) (Chisholm et al. 1988, Partensky et al. 1999, Grob et al. 2007, Zwirgmaier et al. 2007).

Even when both populations can coexist in the ocean, molecular and physiological studies of cultured strains of *Prochlorococcus* and analyses of the photosynthetic pigment in natural *Prochlorococcus* populations, have demonstrated the existence of genetically distinct ecotypes that can be found at different depths, adapted to different average irradiance (Partensky et al. 1993, Moore et al. 1995, Moore et al. 1998, Urbach & Chisholm 1998, Partensky et al. 1999, Hess et al. 2001). These ecotypes are known as "low light" (LL) ecotype, which is adapted to the low light and high nutrient conditions of the deep euphotic zone, and "high light" (HL) ecotype, adapted to the high light and low nutrient conditions of the surface (Chisholm et al. 1988, Campbell & Vault 1993, Goericke & Repeta 1993, Partensky et al. 1996). Moreover, the relative abundance of *Prochlorococcus* ecotypes (HL:LL) in surface waters has shown to be related to changes in vertical mixing (Bouman et al. 2006).

Based on phylogenetic analyses of the 16S rRNA gene and the 16S-23S internal

transcribed spacer (ITS) from *Prochlorococcus*, six lineages have been recognized: two well defined “high-light-adapted” (HL-I and HL-II) ecotype subgroups, clustered in the HL clade, and a loosely defined clade of “low-light-adapted” (LL-I, LL-II, LL-III and IV) ecotypes (Partensky et al. 1999, Rocap et al. 2002).

In the case of *Synechococcus*, molecular phylogeny based on 16S rRNA genes and the 16S-23S rRNA ITS region has also proved that this genus contains genetically diverse lineages (Honda et al. 1999, Fuller et al. 2003, Chen et al. 2006, Jones et al. 2006) giving more than 10 distinct *Synechococcus* lineages described to date (Ferris & Palenik 1998, Rocap et al. 2002, Fuller et al. 2003). *Synechococcus* ecotypes differ in their pigment content, capacity for chromatic adaptation, nutrient utilization and motility (Brahamsha 1996, Toledo et al. 1999, Moore et al. 2002, Fuller et al. 2003, Moore et al. 2005) allowing these organisms to exploit specific spectral niches (Palenik 2001, Stomp et al. 2004, Everroad et al. 2006). However, *Synechococcus* phylotypes are often restricted to the upper half of the euphotic zone and do not show a clear distinctive partitioning with depth, as *Prochlorococcus* does, but do show a clear geographical partitioning of specific lineages (Ferris & Palenik 1998, Fuller et al. 2003, Zwirgmaier et al. 2007).

In the last years, culture-independent molecular methods of microbial identification and characterization have been used and applied in studies of the structure and the dynamics of microbial communities. In several ecosystems, these techniques have proven to be valuable for the comparison of the structures of complex microbial communities and for monitoring their dynamics in relation to environmental factors (Bernard et al. 2000, Scala & Kerkhof 2000, Hollibaugh et al. 2002, Sakano et al. 2002, Freitag & Prosser 2003, Jasti et al. 2005). Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (T-RFLP), have been applied in the study of microbial ecology community structure and dynamics, detecting differences in the nucleotide sequence of 16S rRNA genes that belong to different genotypes (Heuer et al. 1997, Bernard et al. 2000, Scala & Kerkhof 2000). DGGE is an electrophoretic separation method based on differences in the melting

response of double stranded DNA fragments; it has been extensively used for the identification of point mutations (Fodde & Losekoot 1994). T-RFLP analysis is a highly reproducible and robust technique that yields high-quality fingerprints that consist of fragments of precise sizes, which, in principle, could be phylogenetically assigned if an appropriate database is available (Blackwood et al. 2003, Engebretson & Moyer 2003). In spite of the usefulness of the 16S rRNA gene sequence analysis in revealing genetic diversity in bacterial communities, these sequences often do not allow for the discrimination among phylogenetically tightly related strains (Fox et al. 1992). For example, *Prochlorococcus* and *Synechococcus* show more than 96 % identity in their 16S rRNA sequences (Rocap et al. 2002). Therefore, several studies have focused on the ITS sequences (Boyer et al. 2001, Laloui et al. 2002, Rocap et al. 2002, Janse et al. 2003, Brown & Fuhrman 2005) to discriminate between highly related strains. The greater degree of ITS sequence heterogeneity, as well as the considerable number of published ITS sequences, make the ITS region more appropriate for high-resolution analyses of *Prochlorococcus* and *Synechococcus* genotypes. However, initial discriminatory and quantitative investigations between related lineages of *Prochlorococcus* and *Synechococcus* have been done by DNA-DNA dot blot hybridization to 16S rRNA gene using genotype-specific oligonucleotide probes to each lineage (West & Scanlan 1999, Fuller et al. 2003). Recently, some diversity studies have been done using quantitative PCR (qPCR) for the ITS region. This method, while powerful, is still costly, and is not used widely. For each environmental sample, separate qPCR analyses are necessary with each of the ecotype primer set, and with the appropriate different standards (Johnson et al. 2006, Zinser et al. 2006).

Previous studies using epifluorescence microscopy and flow cytometry (Cuevas & Morales 2006, Grob et al. 2007) and high performance liquid chromatography (HPLC) (Stuart et al. 2004), have suggested the presence of cyanobacteria in waters off northern Chile, a coastal upwelling system characterized by the presence of large phytoplankton cells, such as diatoms and dinoflagellates (Stuart et al. 2004). However,

the genetic diversity of such cyanobacteria has not been determined.

In this study, we tested on cultures the ability of the DGGE and T-RFLP approaches to reveal information about the genetic diversity of ribosomal ITS sequences in *Prochlorococcus* and *Synechococcus* populations. We also describe a modified T-RFLP procedure that should allow the use of the T-RFLP technique in accessing the diversity and distribution of these globally important cyanobacteria. Finally, we assessed the molecular diversity of picocyanobacteria in coastal waters off northern Chile using T-RFLP, and showed that is consistent with that obtained by cloning and sequencing.

## MATERIAL AND METHODS

### Strains

The strains of *Prochlorococcus* and *Synechococcus* used in this study were obtained from the Roscoff Culture Collection ([www.sb-roscoff.fr/Phyto/RCC/](http://www.sb-roscoff.fr/Phyto/RCC/)): *Prochlorococcus* MED4 (RCC151) - HL Clade I (Moore et al. 1995), *Prochlorococcus* SB (RCC147) - HL Clade II (Shimada et al. 1995), (*Prochlorococcus* SS120 (RCC156) - LL Clade II, (Chisholm et al. 1992), *Synechococcus* WH7803 (RCC752) - Marine *Synechococcus* clade V, *Synechococcus* WH8103 (RCC29) - Marine *Synechococcus* Clade I, *Synechococcus* WH8020 (RCC751) - Marine *Synechococcus* Clade III (Waterbury et al. 1986). All strains were grown in the PRO99 media developed at the Chisholm's Lab (MIT, USA) and slightly modified by the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) ([http://ccmp.bigelow.org/CI/Pro99\\_family.html](http://ccmp.bigelow.org/CI/Pro99_family.html)) and maintained at 25 °C with a photosynthetic photon flux density of approximately 8  $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ , and a light regime of 12:12 h (Light: Dark).

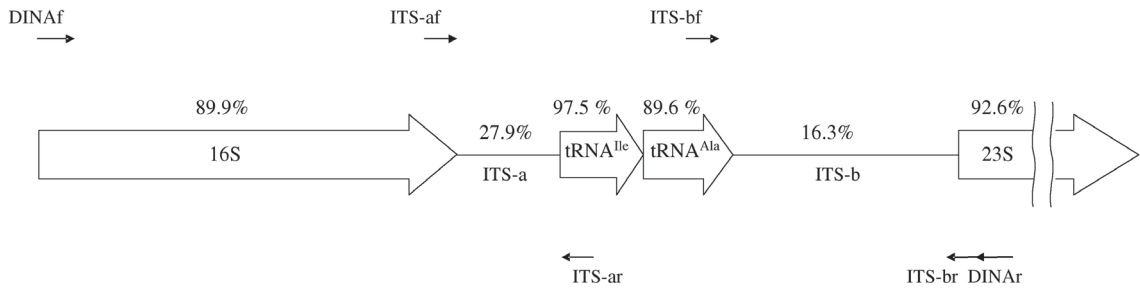
### Sample collection

Seawater (~ 10 L) was collected for environmental DNA isolation from site off northern Chile during the DINAMO cruise onboard R/V Carlos Porter (Station DBGQ, 20°9.9' S, 70°21.09' W, March 2004). The

sample was collected with a rosette Niskin bottle from 20 m depth. The sample was drawn through 47 mm diameter 3 mm pore size polysulfone filter and filtered using a 0.45 mm pore size polysulfone filter, under gentle vacuum. This filter was stored separately at -80 °C in 1 mL of DNA buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5 M NaCl) until DNA extraction. Samples for flow cytometric analyses were taken from the same site and fixed with paraformaldehyde (1 % final concentration) and quick frozen in liquid nitrogen. Cytometric analyses for picophytoplankton were performed with a FACSCalibur (Becton Dickinson) flow cytometer according to Grob et al. (2007).

### Primer design

We designed new specific primers to amplify the 16S rRNA gene sequence plus the ITS region (DINaf 5'-GAA TCT GCC CTC AGG AGG GGG-3' and DINAr 5'-GGG TTG CCC CAT TCG GAA AT-3'), and the two variable zones of the ITS region (ITS-af 5'-GGA TCA CCT CCT AAC AGG GAG-3', ITS-ar 5'-GGA CCT CAC CCT TAT CAG GG-3' for the 16S-tRNA<sup>Ile</sup> spacer, and ITS-bf 5'-GTT GGT AGA GCG CCT GCT TTG-3' and ITS-br 5'-CCG TGA GCC CTT TGT AGC TTG-3' for the tRNA<sup>Ala</sup>-23S). The positions where these six primers anneal are shown in Fig. 1. PCR primers were designed based on the 16S rRNA gene and the ITS sequences of 11 *Prochlorococcus* and 7 *Synechococcus* strains, respectively, which are available in the GenBank with the following access numbers: AF115269, AF397683, AF115270, AF397702, AF115271, AF397684, AF053396, AF397686, AF053398, AF397688, AF053399, AF397704, AF001467, AF397695, AF001471, AF397696, AF001472, AF397678, AF397678, AF397679, AF001473, AF397693, AY172832, AF397729, AY172833, AF397707, AY172834, AF397718, AY172835, AF397719, AF001480, AF397728, NC\_005070, AY172836, AF397710. For DGGE analysis, we used a primer with a 40 nucleotide GC-rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCG CGG CCG C-3') added to the 5' end of the ITS-bf primer sequence. The ITS-af and ITS-bf primers used for T-RFLP analysis were labelled in their 5' ends with the NED fluorochrome.



*Fig. 1:* Schematic view of the rRNA operon from *Synechococcus* and *Prochlorococcus* strains depicting the annealing positions of the PCR primers used in this study. Sequence similarity values based on the information from 18 sequences of *Synechococcus* and *Prochlorococcus* strains are shown as percentages.

Esquema del operón ARNr de cepas de *Synechococcus* y *Prochlorococcus* que representa las posiciones de los partidores de PCR utilizados en este estudio. Se muestran los porcentajes de los valores de similitud de secuencia basados en la información de 18 secuencias de cepas de *Synechococcus* y *Prochlorococcus*.

### PCR amplification

PCR amplifications were performed in a GeneAmp 2400 PCR System (PerkinElmer®, Applied Biosystems, Foster, California, USA) in 25  $\mu$ L reaction mixtures containing approximately 50 ng of DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200  $\mu$ M (each) deoxynucleotides, 3 mM MgCl<sub>2</sub>, 2.5 U of *Taq* DNA polymerase (Invitrogen, São Paulo, Brazil), and 0.2  $\mu$ M (each) primer. Amplification conditions for the DINAf/DINAr primer pair were: 95 °C for 5 min, followed by 33 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products (~ 2.4 kb) were visualized under UV light after electrophoresis at 100 mV for 35 min on 1 % agarose gels prepared in TAE 1X buffer containing 1X SYBR Safe™ DNA gel stain (Invitrogen). Amplification conditions for T-RFLP with the ITS-af/ITS-ar and ITS-bf/ITS-br primer pairs were: 95 °C for 5 min, followed by 28 cycles of 95 °C for 1 min, 55 °C (61°C for ITS-bf/ITS-br) for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplification conditions for DGGE with the ITS-bf/ITS-br primer pair were: 95 °C for 5 min, followed by 28 cycles of 95 °C for 1 min, 64 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Cloned sequences were amplified using the universal primers that are present in the TOPO TA cloning® Kit (Invitrogen): M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13

Reverse (5'-CAGGAAACAGCTATGAC-3'), as specified by the supplier.

### DNA extraction and cloning

DNA was obtained from a 50  $\mu$ L sample of late-exponential-phase cultures of cyanobacterial strains, centrifuged at 20,817 g for 10 min, then washed with 50  $\mu$ L of ultra pure deionised water, and finally lysed by denaturation at 95 °C for 10 min and posterior centrifugation at 20,817 g for 1 min. The supernatant was used as a template to amplify the 16S rRNA gene + ITS region with the DINa primer pair. The PCR products were cloned with the TOPO TA cloning® kit (Invitrogen), as specified by the supplier, and these clones were used as templates in PCR amplification for DGGE and T-RFLP analysis.

For DNA extraction a modified version of the West and Scanlan protocol was used (West & Scanlan 1999). Briefly, the filter was cut into small pieces and placed in 3 ml of lysis solution (45 mM glucose, 23 mM Tris [pH 8.0], 59 mM EDTA) containing lysozyme at a concentration of 1 mg mL<sup>-1</sup>. This mixture was incubated 1 h at 37 °C, frozen at -20 °C (15 min) and then thawed at 50 °C. DNA was extracted by adding 350  $\mu$ L of 10 % sodium dodecyl sulfate and 33.5  $\mu$ L of a proteinase K solution (10 mg mL<sup>-1</sup>), followed by incubation at 37 °C (30 min) and at 55 °C (10 min). This solution was mixed gently with the pipette, frozen at -20 °C (15 min) and then thawed at 50 °C. The thawed mixture was extracted with water saturated phenol

(neutralized with 0.5 M Tris-HCl buffer, pH 7.8 to 8.5) - chloroform-isoamyl alcohol (25:24:1) and centrifuged at 4,000 rpm (5 min). After centrifugation, the aqueous phase was carefully removed, and again the DNA was extracted with chloroform-isoamyl alcohol (24:1) and centrifuged at 4,000 rpm (5 min). DNA was precipitated by adding 0.4 volumes of sodium acetate (7.5 M) and 2 volumes of cold ethanol (95 %). The mixture was kept at -80 °C for 20 min and then centrifuged at 13,000 rpm (10 min). The DNA pellet was washed with 70 % ethanol, left to dry for 10 to 15 min, resuspended in TE buffer (100 µL) and stored at -80 °C. DNA was examined in an agarose gel. Environmental DNA was amplified with DINA primer pair in order to be used in T-RFLP analysis and cloning, as described above.

#### *DGGE analysis*

The 16S rRNA-ITS fragment was amplified in each cloned sequence using the DINA primers as a selective pre-amplification step. Then, 1 µL of this PCR product was used as a template for nested PCR with primers ITS-bf and ITS-br. The Dcode Universal Mutation Detection system (Bio-Rad, Hercules, California, USA) was used for DGGE analysis. Electrophoreses were performed in a 0.8 mm thick polyacrylamide gel (8 % [wt/vol] acrylamide-bisacrylamide at 37.5:1), using a denaturant gradient from 20 to 80 % (100 % corresponding to 7 M urea and 40 % [wt/vol] formamide). Electrophoretic runs were carried out at a constant temperature of 60 °C in 1.25x TAE for 5 h at 200 V. After electrophoresis, gels were stained for 20 min in water containing 1X SYBR Safe™ DNA gel stain and visualized under UV light. Images were digitally captured, and analyzed with the Kodak Electrophoreses Documentation and Analysis System (EDAS 120).

#### *Standard T-RFLP analysis*

PCR products of selective pre-amplification with the DINA primer pair were obtained, and 1 µL of each PCR product was used as a template for nested PCR with ITS-af/ITS-ar and ITS-bf/ITS-br labelled primers. PCR products (200-500 bp) were visualized under UV light after electrophoresis at 100 mV for 15 min on 1 % agarose gels prepared in TAE 1x

buffer containing 1x SYBR Safe™ DNA gel stain. Eight microliters of PCR product were digested with 10 U of the restriction enzyme *AluI* (Fermentas, Lithuania; sequence: AG<sup>^</sup>CT) and the associated Tango restriction buffer (Fermentas) at 37 °C for 4 h, as specified by the supplier. Digested PCR products were precipitated with 0.1 volume of 5 M sodium acetate (pH 5.2) and 2.5 volume of ethanol, incubated for 1 h at -80 °C, centrifuged at 20,817 g for 15 min at 4 °C, washed with 70 % (v/v) ethanol, and vacuum-dried. The DNA fragments were separated by capillary electrophoresis using a ABI Prism 310 sequencer. Fragment sizes were determined using the internal standard ROX 1000 as a reference.

#### *Modified T-RFLP analysis*

The modification of the T-RFLP procedure consisted of performing a nested PCR with only two cycles. Ten µL of the PCR products of selective pre-amplification with the DINA primer pair were mixed with 0.68 µL of the new reaction mix containing 1 µL of ITS labelled forward primer (10 µM), 1 µL of ITS reverse primer (10 µM), 1 µL of dNTP mix (10 µM) and 0.4 µL of *Taq* polymerase. Amplification conditions used for ITS-af/ITS-ar and ITS-bf/ITS-br primer pairs were 95 °C for 5 min followed by two cycles of 95 °C for 1 min, 55 °C (61 °C for ITS-bf/ITS-br) for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR product digestion and T-RFLP analysis were performed as described above.

#### *T-RFLP data handling*

Raw data sets consisted of peaks reflecting the size of terminal restriction fragments (T-RFs), measured in base pairs. The area of each peak was measured in fluorescence units. T-RFs from 50 to 700 bp were included in the analysis, whereas those representing less than 0.5 % of the total area were excluded (Dunbar et al. 2001). Data were standardized by calculating the area of each peak as a percentage of the total area.

#### *Secondary-structure simulations*

DNA folding prediction was carried out with the online “mfold” software (<http://>

frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi) for prediction of secondary structure (Mathews et al. 1999, Zuker 2003). DNA folding parameters were kept as default, except for the folding temperatures, which were adjusted to 61 °C (PCR annealing temperature) and 72 °C (PCR extension temperature). Ionic conditions were changed to  $[Na^+] = 50 \text{ mM}$  and  $[Mg^{++}] = 3 \text{ mM}$ .

### Phylogenetic analysis

All phylogenetic analysis was carried out using the Bosque software developed at PROFCA, Universidad de Concepción, Chile (Ramírez-Flandez & Ulloa 2008). Only ITS fragment located between 600f and 340r primer sequences (Laloui et al. 2002) were used and aligned to sequences from the NCBI using MUSCLE (Edgar 2004) in order to perform phylogenetic analysis. The Maximum Likelihood analyses were performed with tree-puzzle 5.2 (Schmidt et al. 2002) using 697 positions of alignment. It uses the heuristic quartet-puzzling algorithm to compute likelihood trees. The HKY85 model was selected (Hasegawa et al. 1985). Parameters of the models of sequence evolution and rate heterogeneity were estimated by tree-puzzle based on a quartet sampling plus neighbour-joining tree and the approximate likelihood function. We selected a model of among-site rate heterogeneity of substitutions that consisted of invariable rate estimated from data and eight variable rates with gamma distribution. Environmental sequence data have been submitted to the GenBank database, under accession numbers EU828639 to EU828650.

The assignment of the TRFs to phylogenetic entities was done by comparison with a computer-simulated restriction analysis of sequences obtained from GeneBank and cloning.

## RESULTS AND DISCUSSION

### *Amplification analysis of the 16S–23S ITS region of cyanobacterial strains*

Based on the variability of the 16S–23S ITS sequences and their utility as a distinctive character of the different genotypes of

*Prochlorococcus* and *Synechococcus* (Rocap et al. 2002), the aim of this study was to assess cyanobacterial diversity in the environment using their ITS sequences. For this study, we selected the most variable region of ITS (ITS-b in Fig.1), which is the main factor determining length and sequence variability of this sequence. The DGGE technique was used to distinguish PCR products for the ITS-b region obtained from six cloned sequences of *Prochlorococcus* and *Synechococcus* strains. These PCR products showed the expected variable lengths (340–548 bp). However, in all cases, more than one band for each cloned sequence was observed in the DGGE gel (Fig. 2A). This unexpected pattern was obtained even when the product of one cloned sequence was analyzed in a perpendicular DGGE with a gradient of 0–80 % (Fig. 2B). Essentially the same behaviour was observed with the other five tested sequences. Unexpected bands in studies with ITS sequences have been reported for other cyanobacterial strains, like for the genus *Microcystis*, due to the occurrence of multiple rRNA-ITS operons in one organism (Janse et al. 2003). This condition may produce artefacts in the PCR amplification of homologous sequences that lead to the formation of chimera and heteroduplexes (Jensen & Straus 1993, Kopczyński et al. 1994, Janse et al. 2003). However, that is not the case here because clones of ITS sequences were used as templates. In order to exclude some possible sources of multiple bands, we carried out controls and tested modifications in the procedure. No PCR products were obtained when only host *E. coli* DNA was used as template; this indicates that the formation of unexpected bands cannot be attributed to unspecific annealing of PCR primers to the host *E. coli* DNA. Modification of  $MgCl_2$  concentration (1–3  $\mu\text{M}$ ) and annealing temperature (57–60 °C) in the PCR reaction did not eliminate the multiple bands. Such bands were also obtained when, in the first PCR reactions, DINA primer pairs were replaced by M13 primer pairs, that target sequences of the vector in which the ITS region is cloned. The same bands were obtained when DNA extracted directly from cyanobacteria was used for the first PCR reaction with DINA primer pairs. A time increase in the extension temperature did not prevent the formation of unexpected bands

(Fig. 3A). When products of nested PCR were electrophoresed in 8 % polyacrylamide gels without a denaturing gradient, multiple bands were also observed in each cyanobacterial strain cloned sequence. In these electrophoresis conditions, the fast migrating bands correspond to the expected bands, in all cases. The re-amplification and re-run of the fast-migrating band, extracted from the polyacrylamide gel without denaturing gradient, always yielded more than one band per cloned sequenced (only result for *Synechococcus* strain WH8020 is shown in Fig. 3B). These results suggest an artefact associated with the PCR procedure. Moreover, the use of adjuvants, like DMSO (5 %) or betaine (5M), to improve amplification efficiency and specificity in the PCR reaction, did not eliminate the multiplicity of bands. The use of *Pfu* DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) with a buffer enhancer also failed to prevent the formation of unexpected bands.

Since the presence of other sites for primer pairs annealing into the cloned sequence, or in the host *E. coli* DNA, was discarded, the results obtained with and without a denaturing gradient strongly suggest the formation of incomplete single-stranded amplicons. This is due to double-stranded nucleic acid fragments (> 50 bp) generally having higher migration velocity than their single-stranded counterparts in polyacrylamide gel electrophoresis (Ravnk-Glavac et al. 1994, Sainz et al. 1994). Incorrect re-annealing of single strands in complex samples, or differences in primer binding energy, can generate PCR-chimera as heteroduplexes, or partially single stranded amplicons (Polz & Cavanaugh 1998, Qiu et al. 2001). The multiple band pattern observed in this work after re-amplification would be produced by heteroduplex artefacts (Ferris & Ward 1997). However, heteroduplexes are usually generated when mixtures of homologous genes are used as a template (Qiu et al. 2001, Daffonchio et al. 2003).

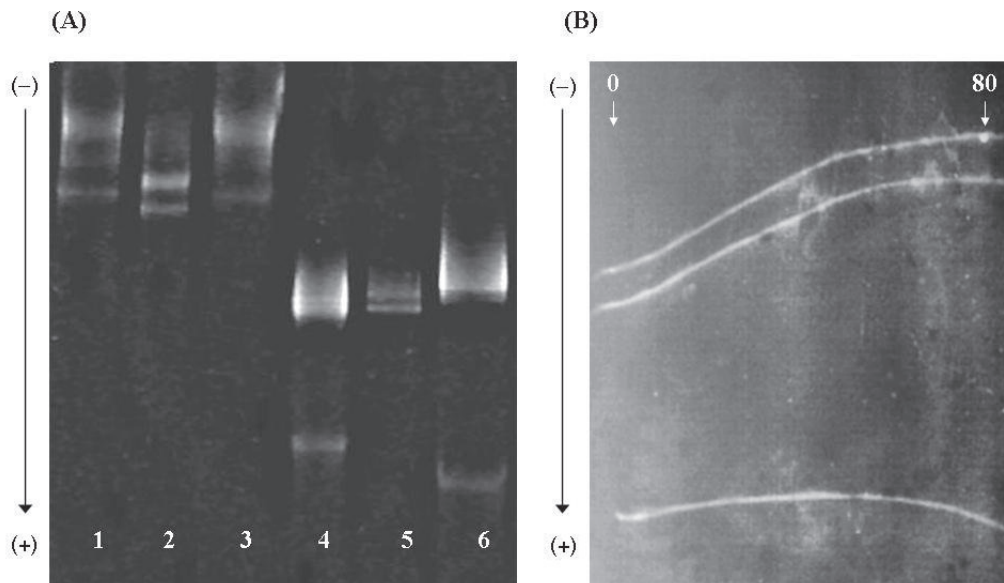


Fig. 2: (A) DGGE of ITS-b region PCR products from six cloned sequences of cyanobacterial strains: *Prochlorococcus* MED4 (lane 1), *Prochlorococcus* SS120 (lane 2), *Prochlorococcus* SB (lane 3), *Synechococcus* WH8103 (lane 4), *Synechococcus* WH7803 (lane 5) and *Synechococcus* WH8020 (lane 6). Electrophoresis conditions: 200 V, 5 h, 60 °C. (B) Perpendicular DGGE (0-80 %) of the ITS-b region PCR product from *Synechococcus* WH8103. Electrophoresis conditions: 200 V, 10 h, 60 °C.

(A) DGGE de productos de PCR de la región ITS-b de seis secuencias clonadas de las cepas de cianobacterias: *Prochlorococcus* MED4 (carril 1), *Prochlorococcus* SS120 (carril 2), *Prochlorococcus* SB (carril 3), *Synechococcus* WH8103 (carril 4), *Synechococcus* WH7803 (carril 5) y *Synechococcus* WH8020 (carril 6). Condiciones de electroforesis: 200 V, 5 h, 60 °C. (B) DGGE perpendicular (0-80 %) de producto de PCR de la región ITS-b de *Synechococcus* WH8103. Condiciones de electroforesis: 200 V, 10 h, 60 °C.

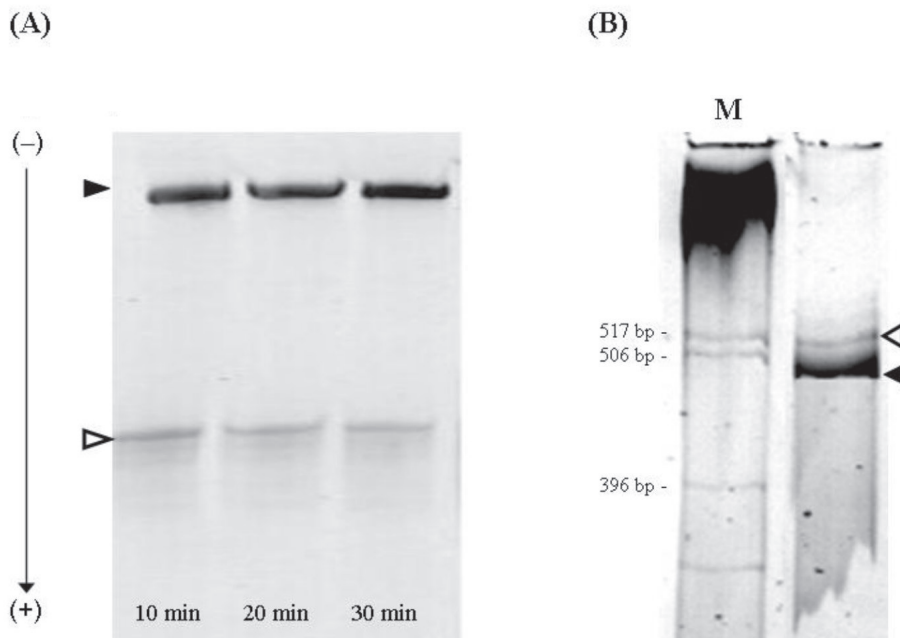


Another explanation of these results would be that the GC content of DINA primer pairs (55 and 66 % of GC content in reverse and forward primer pairs, respectively) could lead to incomplete amplification and generate single-stranded DNA (Polz & Cavanaugh 1998).

#### Secondary structure modelling of the ITS-b region

PCR amplification of 16S rRNA genes from single and mixed templates can form partially single-stranded amplicons as a result of template secondary structures (Jensen & Straus 1993, Ernst et al. 2003). In order to explore such a possibility, we used a computational tool based on thermodynamic methods to predict folding and melting temperatures of the secondary structure of the ITS-b sequences (Mathews et al. 1999, Zuker 2003).

At the annealing temperature (61 °C), the formation of three to four hairpin loops was predicted for the ITS-b region in *Prochlorococcus* strains, and four to eight for *Synechococcus* strains. Due to the high melting temperature of some hairpin loops (ranging from 77.4 to 77.7 °C for *Prochlorococcus* and from 74.6 to 79.8 °C for *Synechococcus*), most of these hairpins can still be present during the extension temperature (72 °C). The unexpected bands reported in this work may correspond to short PCR products or single-stranded PCR products generated by the effect of these transient secondary structures on the Taq polymerase activity. A blockage of the enzyme may take place, which would avoid the complete synthesis of the complementary strand (Polz & Cavanaugh 1998, Qiu et al. 2001).



**Fig. 3:** (A) DGGE of the ITS-b region PCR product from *Synechococcus* WH8020 amplified using different times for the elongation step: 10 min (lane 1), 20 min (lane 2) and 30 min (lane 3). (B) Polyacrylamide gel (8 %) electrophoresis of the reamplification and re-run of the fast migrating band of the ITS-b region PCR product from *Synechococcus* WH8020, (M) - 1 kb DNA ladder. Filled triangle corresponds to the expected size amplicon. Open triangle indicates an unexpected band.

(A) DGGE del producto de PCR de la región ITS-b de *Synechococcus* WH8020 amplificado utilizando diferentes tiempos de elongación: 10 min (carril 1), 20 min (carril 2) y 30 min (carril 3). (B) Electroforesis en gel de poliacrilamida (8 %) de la re-amplificación de la banda de migración más rápida del producto de PCR de la región ITS-b de *Synechococcus* WH8020, (M) - Marcador de peso de ADN 1 kb. Triángulo lleno corresponde al tamaño del amplicón esperado. Triángulo abierto indica una banda inesperada.

*Analysis of the 16S-23S ITS region of cyanobacterial strains using T-RFLP*

The results of the previous sections indicate that the complete fragment of the ITS-b sequence cannot be used to assess diversity, because it would lead to an over estimation. An *in silico* analysis of T-RFs of ITS-b sequences, using *AluI*, suggested that complete amplicons can generate T-RFs that allow the assessment of diversity and the discrimination among genotypes. T-RFs can be assigned to genotypes as follows: 80 to 104 bp and 144 to 157 bp correspond to *Synechococcus*; 120 to 134 bp correspond to *Prochlorococcus* HL ecotype and 136 to 142 bp correspond to *Prochlorococcus* LL ecotype. Based on this observation, we decided to evaluate if the T-RFLP approach is a useful tool to assess cyanobacterial diversity. The results obtained with the standard T-RFLP method showed the presence of several unexpected T-RFs, in addition to the theoretical T-RF, in both ITS regions (Table 1). The ITS-a region sequences produced more unexpected signals than the ITS-b region. Most of the

unexpected signals were shorter than the theoretical T-RFs. An incomplete digestion of amplicons would produce longer, rather than shorter T-RFs. Interestingly, electropherograms of undigested PCR products of the ITS region yielded similar anomalous results to those obtained with DGGE, i.e., more than one band/peak from cloned sequences. It is known that single-stranded and partially single-stranded fragments show slower migration than double-stranded fragments, both in polyacrylamide gels and in capillary electrophoresis (Wenz 1994, Atha et al. 2001). On the other hand, Type II restriction enzymes preferentially cleave transiently-formed secondary structures in single-stranded DNA composed of two recognition sequences with two fold rotational symmetry (Nishigaki et al. 1985). Therefore, it is possible to suggest that smaller than expected T-RFs (or pseudo T-RFs) are produced by DNA cleavage at transiently formed recognition sites. It has been proposed that the use of a lower number of PCR cycles may decrease the extent of pseudo T-RF formation (Polz & Cavanaugh 1998, Egert & Friedrich 2003).

TABLE 1

T-RFs obtained with the standard and the modified T-RFLP using primer pairs for the ITS-a and ITS-b regions

T-RFs obtenidos con el método de T-RFLP estándar y modificado usando pares de partidores para las regiones de ITS-a y ITS-b

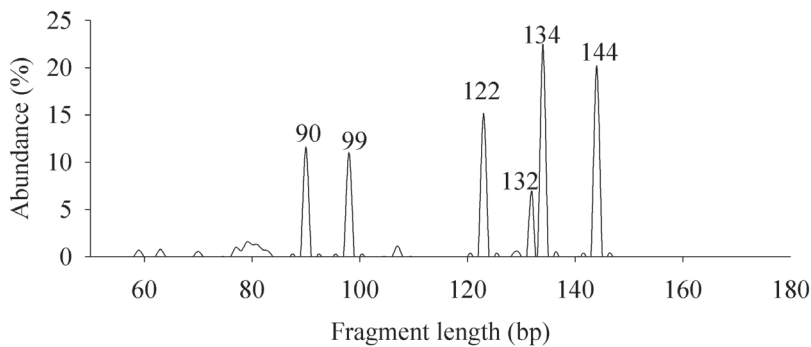
Strains	<i>AluI</i> ITS-a region T-RF length (bp)		
	Theoretical	Observed	
		Standard method	Modified method
<i>Prochlorococcus</i> med4	148	143 (68 %)	144 (63 %), 91 (24 %)
<i>Prochlorococcus</i> ss120	148	145 (73.5 %), 227 (11.3 %)	144 (76 %), 91 (13 %)
<i>Prochlorococcus</i> SB	150	67 (10.7 %), 89 (10.8 %), 146 (51.5 %)	148 (74 %)
<i>Synechococcus</i> WH7803	100	78 (17 %), 96 (76 %)	97 (81.5 %)
<i>Synechococcus</i> WH8103	213	90 (13 %), 102 (44.6 %), 208 (18.1 %), 481 (16.4 %)	209 (36 %), 90 (74 %)
<i>Synechococcus</i> WH8020	107	102 (78 %)	103 (76 %), 92 (14 %)
	<i>AluI</i> ITS-b region T-RF length (bp)		
	Theoretical	Observed	
		Standard method	Modified method
<i>Prochlorococcus</i> med4	135	132 (63 %)	133 (88.5 %)
<i>Prochlorococcus</i> ss120	140	139 (56.3 %), 141 (24.6 %)	141 (87 %)
<i>Prochlorococcus</i> SB	133	69 (12.3 %), 79 (27.5 %), 131 (43 %)	133 (90 %)
<i>Synechococcus</i> WH7803	101	67(15.5 %), 97 (36 %), 141 (46.6 %)	99 (72 %)
<i>Synechococcus</i> WH8103	151	148 (37.2 %), 421 (15.2 %)	148 (100 %)
<i>Synechococcus</i> WH8020	144	145 (80 %)	143 (83 %)

To avoid the detection of pseudo T-RFs, we modified the T-RFLP procedure by adding labelled primers in the last two PCR cycles. In such a way only complete T-RFs should be marked. This modified method significantly reduced the number of pseudo T-RFs per ribotype detected in the T-RFLP profiles (Table 1). However, the modified method only eliminated unexpected peaks in the T-RFLP profiles from the ITS-b region (Table 1). These results are probably due to differences in the stability of secondary structures. The positions of the T-RFs with the modified procedure using the ITS-b region, allowed clear distinction between *Prochlorococcus* ecotypes and *Synechococcus* (Fig. 4).

#### *Picocyanobacteria diversity in natural marine waters*

The results obtained from the environmental sample with the modified T-RFLP approach, and cloning and sequencing of the ITS sequences showed a high phylogenetic diversity of picocyanobacteria at 20 m depth. The T-RFLP showed 6 T-RF's assignable peaks, corresponding to at least 5 cyanobacterial clades (Fig. 5A and 5B); while cloning and sequencing showed 11 operational taxonomix units present in six separated phylogenetic clades (Fig. 5C). The genus *Synechococcus* was the most abundant, with clade IV as the main representative, followed

(A)



(B)

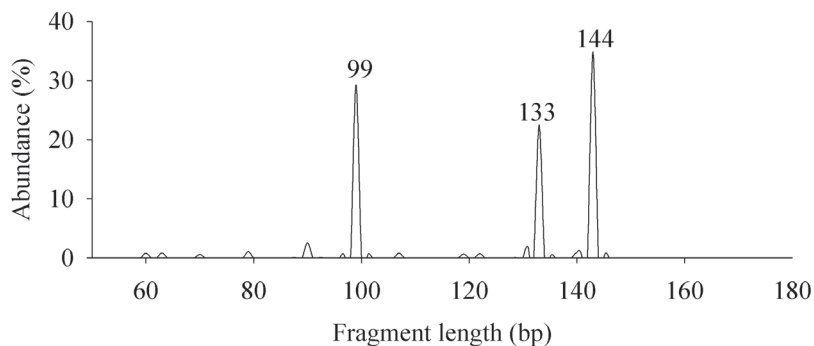


Fig. 4: Comparison between the standard (A), and the modified (B) T-RFLP, using primer pairs for the ITS-b region, for a mixture of the cloned sequences from *Prochlorococcus* MED4 (133 bp), *Synechococcus* WH7803 (99 bp) and *Synechococcus* WH8020 (144 bp).

Comparación entre el método estándar de T-RFLP (A) y el modificado (B), utilizando el par de partidores para la región del ITS-b, para una mezcla de las secuencias clonadas de *Prochlorococcus* MED4, *Synechococcus* WH7803 y *Synechococcus* WH8020.

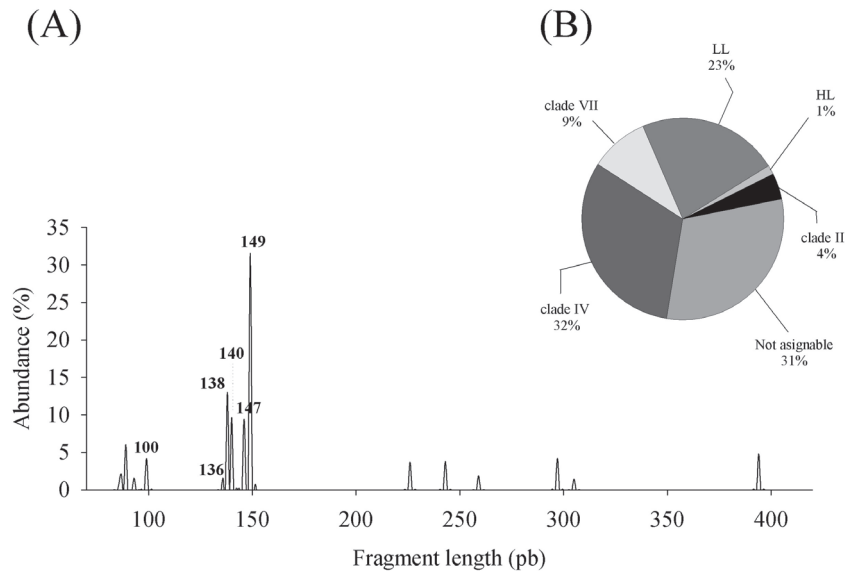


Fig. 5: (A) T-RFLP profile of PCR-amplified ITS fragment from environmental sample. (B) Relative abundance of operational taxonomic units with phylogenetic assignment to *Synechococcus* and *Prochlorococcus* ecotypes. (C) Phylogenetic analysis of ITS sequences. Tree based on the maximum-likelihood principle and quartet puzzling using ITS amplicons (368 - 682 bp). Numbers correspond to respective sizes of T-RFs after *in silico* analysis with the restriction enzyme *AluI*. The scale bar represents 0.1 substitution per positions.

(A) Perfil de T-RFLP de fragmentos del ITS de muestra ambiental. (B) Abundancia relativa de unidades taxonómicas operacionales con asignaciones para ecotipos de *Synechococcus* y *Prochlorococcus*. (C) Análisis filogenético de secuencias de ITS: Arbol basado en el principio de máxima verosimilitud y "quartet puzzling" usando amplicones de ITS (368 - 682 pb). Números corresponden al tamaño respectivo del fragmento después de un análisis *in silico* con la enzima de restricción *AluI*.

by clade VII. Meanwhile, *Prochlorococcus* also appears with a significant contribution to the picophytoplankton, with representatives of the clades HL and LL, the latter being the most abundant within this genus.

This is the first report of cyanobacterial genetic diversity for the costal upwelling zone off northern Chile. Results showed the presence of different clades of *Synechococcus* and *Prochlorococcus* in these waters. Previous reports using zeaxanthin pigment as an indicator of cyanobacteria, particularly of *Synechococcus*, and divinil chl-a and chl-b as an indicator of *Prochlorococcus*, suggested the presence of mixed assemblages of these cyanobacteria in the region (Stuart et al. 2004). A recent report of cyanobacterial diversity obtained by dot blot at farther south (latitude ~35° S), showed the presence of at least five clades (*Prochlorococcus* clade LL and *Synechococcus* clades I, IV, V/VI/VII, III) (Zwirgmaier et al. 2008), consistent with our results, except for clade I which was not found in our work.

Light is known to be an important factor in niche differentiation in cyanobacteria. Even when *Prochlorococcus* ecotypes have a characteristic niche-partitioning in the water column (West & Scanlan 1999), physiological studies of cultured strains have demonstrated that HL- and LL-adapted strains are able to grow under a range of irradiances (Partensky et al. 1993, Moore et al. 1995, Partensky et al. 1997, Moore et al. 1998). Therefore, the co-occurrence of *Prochlorococcus* ecotypes (HL and LL), detected by T-RFLP and cloning can be attributed to vertical mixing (Palenik 1994, Ferris & Palenik 1998, Moore et al. 1998, Urbach & Chisholm 1998, Bouman et al. 2006). In the case of *Synechococcus*, some, but not all, of the clades possess distinguishing physiological characters that render them ecologically distinct. As an example, members of clades I, III and IV are chromatically adaptive, altering the relative ratio of their accessory pigments according to the spectral quality of the light under which they are growing (Palenik 2001, Everroad et al. 2006).

(C)

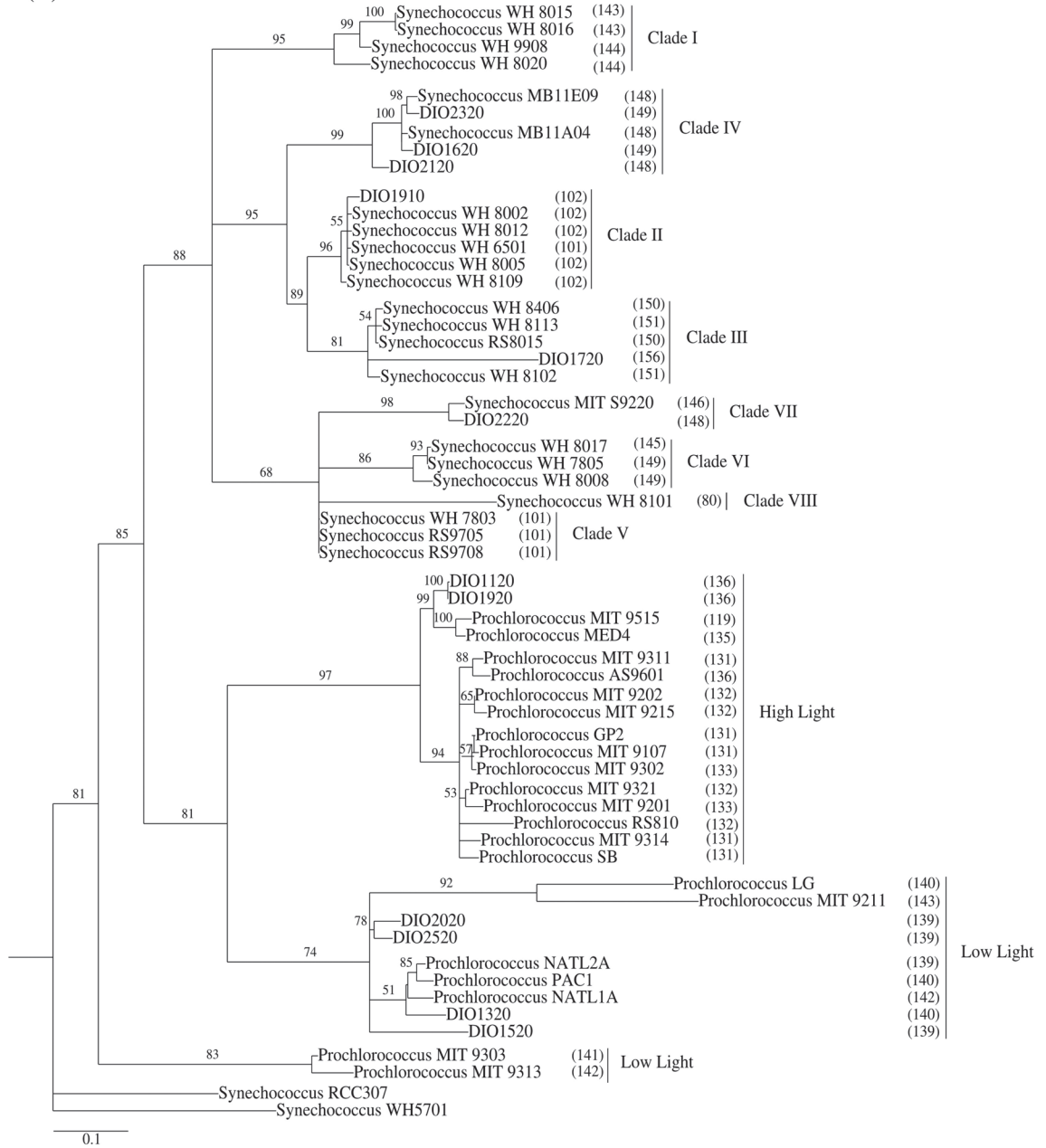


Fig. 5: (C)

In this case, this ability to adapt chromatically to better compete in changing environmental conditions prevent the detection of a clear niche differentiation by light as in *Prochlorococcus* ecotypes HL and LL. Based on the spatial distribution patterns of *Synechococcus*, a recent report showed that the distribution of clade IV is restricted to subtropical areas above 30° N and below 30° S

(Zwirgmaier et al. 2008). The detection of this clade at 20° S reported here can be explained as a result of the Humboldt Current System, which transport colder waters from the south. The detection of clade VII in our study, on the other hand, is consistent with their cosmopolitan nature (Zwirgmaier et al. 2008).

Despite that our modified T-RFLP method can discriminate cultured strains and ITS

sequences from environmental samples, showing good agreement with the computer-simulated restriction analysis of cloned sequences (Fig. 5B and 5C), some caveats are worth mentioning. Some T-RFLP peaks observed in the sample using a 0.5 % threshold that have a low abundance (< 5 %) could not be phylogenetic assigned, whereas all the peaks with a high abundance (> 5 %) could be phylogenetically identified (Fig. 5A). However, some of the assigned peaks have abundances lower than 5 % (clade II and clade HL). Using this approach, we were able to assign a 69 % of the total fluorescence. However, if the threshold is set at 5 %, which served to remove any bias caused by the amount of PCR product while having a minor effect on the overall area of the remaining T-RFs (Sait et al. 2003, Rees et al. 2004), the T-RFs assignation of this environmental sample goes up to 91.3 % of total fluorescence. Using these conditions the operational taxonomic units are less abundant, as clade II and clade HL, could not be included in the analysis.

It is well known that quantification of the relative abundance of organisms by PCR-based methods presents many uncertainties. In order to have an estimate for the possible PCR biases, we compared the results obtained by T-RFLP with those obtained by flow cytometry (a technique that gives absolute concentrations). All relative peak abundances assignable from T-RFLP (using both thresholds) were grouped into the two genera of cyanobacteria, since individual clades cannot be distinguished by flow cytometry. The relative abundance of *Prochlorococcus* and *Synechococcus* obtained by both techniques was similar (34.8 and 65.2 % by T-RFLP, 30.6 and 69.4 % by flow cytometry, respectively).

In summary, T-RFLP analysis of the ITS region can be used to assess the genetic diversity of *Prochlorococcus* and *Synechococcus* in the marine environment. Application of this approach in natural waters reveal the presence of several lineages of these picocyanobacteria in the coastal upwelling waters off northern Chile.

#### ACKNOWLEDGEMENTS

This study was supported by the FONDAP grants 1501-0007/COPAS Program-2 and 1501-

0001/CASEB Program-7, the Millennium Nucleus for Microbial Ecology and Environmental Microbiology and Biotechnology grant P/04-007-F, and the grants Marine Genomics-CONICYT and FUNDACION ANDES C-13851.

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Associate Editor: Iván Gómez

Received November 12, 2007; accepted June 7, 2008

