RESEARCH ARTICLE

Phylogenetic diversity of nonmarine picocyanobacteria

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Abstract

We studied the phylogenetic diversity of nonmarine picocyanobacteria broadening the sequence data set with 43 new sequences of the 16S rRNA gene. The sequences were derived from monoclonal strains isolated from four volcanic high-altitude athalassohaline lakes in Mexico, five glacial ultraoligotrophic North Patagonian lakes and six Italian lakes of glacial, volcanic and morenic origin. The new sequences fall into a number of both novel and previously described clades within the phylogenetic tree of 16S rRNA gene. The new cluster of Lake Nahuel Huapi (North Patagonia) forms a sister clade to the subalpine cluster II and the marine *Synechococcus* subcluster 5.2. Our finding of the novel clade of 'halotolerants' close to the marine subcluster 5.3 (*Synechococcus* RCC307) constitutes an important demonstration that euryhaline and marine strains affiliate closely. The intriguing results obtained shed new light on the importance of the nonmarine halotolerants in the phylogenesis of picocyanobacteria.

Introduction

Picocyanobacteria are photosynthetic prokaryotes common in lakes and oceans and abundant across a wide spectrum of trophic conditions (Callieri *et al.*, 2012; Scanlan, 2012). The dominant genera of freshwater picocyanobacteria are *Cyanobium*, *Synechococcus* and *Cyanothece dianae/cedrorum* type (Komárek, 1996), while in the oceans, *Synechococcus* and *Prochlorococcus* dominate (Scanlan, 2012). In freshwaters, the most common genus is *Cyanobium* (1–2 μm long, 1 μm wide) often reported as *Synechococcus* type. Actually, *Synechococcus* (3–15 μm long and 1–3 μm wide) is rod-shaped and larger than *Cyanobium* (Callieri & Stockner, 2002). However, the lack of other morphological characteristics, beside size, fostered the study of phylogenetic lineage diversification to determine taxonomic relationships in the *Synechococcus* form-genus (Herdman *et al.*, 2001).

The genetic diversity and phylogeny of *Synechococcus* have been studied analysing the nucleotide sequences of the 16S rRNA gene, of the 16S-23S rRNA gene internal transcribed spacer (ITS-1) and of the *cpcBA* (phycocyanin operon) in freshwaters (e.g. Crosbie *et al.*, 2003; Ernst *et al.*, 2003; Jasser *et al.*, 2011) and in marine/brackish waters (e.g. Fuller *et al.*, 2003; Haverkamp *et al.*, 2009; Scanlan, 2012).

Bergey's Manual divides *Synechococcales* into five clusters (equivalent to genera) based on morphology, physiology and genetic traits (Herdman *et al.*, 2001). One striking characteristic that differentiate among clusters is salt tolerance: from *Cyanobium* cluster 1 (Rippka *et al.*, 2001) with, for example, *Cyanobium gracile* and PCC7009 not capable of growing in marine medium, to *Synechococcus* 5.1, which is distinctly marine, and several euryhaline strains (Herdman *et al.*, 2001). The ecological genomics of marine *Synechococcus* have been widely studied, and based on 16S rRNA gene sequences, this lineage divided into three subclusters: 5.1, 5.2 and 5.3 (Dufresne *et al.*, 2008; Scanlan *et al.*, 2009). Conversely in phylogenetic trees of nonmarine *Synechococcus,* the relationships among the lineages remain indeterminate (Crosbie *et al.*, 2003; Everroad & Wood, 2012) and often indicate the absence of a net genetic separation among freshwater and some marine *Synechococcus* strains. For example, *Synechococcus* strains isolated from brackish marshland or low-salinity pond appear in *C. gracile* cluster group A (Crosbie *et al.*, 2003) or even strains from Baltic Sea appear in Group B, A, I and in subalpine cluster II (subcluster 5.2) (Sánchez-Baracaldo *et al.*, 2008),

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indicating the proximity between freshwater and some marine *Synechococcus* strains.

The most updated picture of the 16S rRNA gene phylogenetic tree of picocyanobacteria, including marine and freshwater strains, reported with spectral phenotypes (Everroad & Wood, 2012), confirms the indication by Dufresne *et al.* (2008) that *Synechococcus* 5.3 is not included in *Synechococcus* 5.1 but is its sister clade. The authors consider the nonmarine picocyanobacteria as closely related to *Cyanobium* strains and propose a monophyletic '*Cyanobium*-like' lineage. Nevertheless, Everroad & Wood (2012) recognize that 'the relationships of these lineages to one another remain ambiguous'. From this comes the need to enlarge the database with new sequences of isolated *Synechococcus* strains from a wider range of locations and environments to obtain a more realistic view of *Synechococcus* genus dispersal and evolution (Crosbie *et al.*, 2003).

In this study, we aimed to resolve better the *Synechococcus* phylogenetic tree enlarging the sequence data set of nonmarine picocyanobacteria including strains isolated from 'extreme' lakes. Therefore, we selected four high-altitude athalassohaline maar crater lakes in Mexico as a source for 'nonmarine halotolerant' *Synechococcus* (Macek *et al.*, 2009), five glacial ultraoligotrophic North Patagonian lakes as extreme ecosystems (Callieri *et al.*, 2007) and six Italian lakes of glacial, volcanic and morenic origin, with different trophic conditions (Callieri, 1996). Our objective was to create a new phylogenetic tree of nonmarine *Synechococcus* upgraded adding 43 new sequences derived from isolated picocyanobacteria and to refine the taxonomic relationships between nonmarine *Synechococcus* clades and *Synechococcus* 5.2 and 5.3.

Materials and methods

Sampling site description

Sampling for strain isolation has been performed in different years beginning from 2005 to gradually enrich the collection of the picocyanobacterial strains of CNR – ISE (Verbania, Italy). A total of 15 lakes different in origin, thermal regime, maximum depth, conductivity, trophic state and geographic area (Italy, Argentina and Mexico) were sampled, at different depths (Tables 1 and 2). The Italian lakes are located in northern Italy and cover deep glacial (Calderoni & Tartari, 2000; Salmaso *et al.*, 2007) and shallow morenic lakes (de Bernardi *et al.*, 1984), except one volcanic lake in central Italy (Elwood *et al.*, 2009). The Argentinean lakes include four deep glacial and ultraoligotrophic lakes and one shallow and small lake, all located in the North Andean Patagonia at 750 m a.s.l. (Morris *et al.*, 1995; Modenutti *et al.*, 1998). Athalassohaline maar crater lakes with different conductivity are situated in a tropical region in a high-altitude plateau (2340 m a.s.l.) within the endorheic Oriental Basin of central Mexico (Alcocer & Sarma, 2002). They range from freshwater to saline, but with a proportion of dissolved salts different from that of the sea.

Sample collection, isolation and purification of cyanobacterial strains

Samples for picocyanobacteria isolation were collected during spring/summer. The water was kept at the same temperature as of the sampling depth and processed in laboratory (or in field station) on the same day of sampling. For most lakes, the sample was gravity filtered through a 3-μm polycarbonate membrane, and 3 to 5 replicates of 3 mL were added to 3 mL BG-11 medium (Allen, 1968) in a glass scintillation vial (1 : 10 diluted or not, depending on the trophic state of the lake of origin). The vials were kept in thermostat, with the cap not completely closed to permit air flux, at 18–20 °C and low light (10–15 μmol photons m^{-2} s^{-1}). For the ultraoligotrophic lakes (particularly in Patagonia) to obtain growth, it was necessary to gravity filter on a 5-μm Nytex net; then, around 30 mL of the filtrate was concentrated on a 0.2-μm polycarbonate filter, which was gently washed in the vial with the diluted BG-11.

Where many picoeukaryotes were present, cycloheximide (final concentration: 0.3 mM) was added and kept for 3 days in the vial. Then, the cultures were transferred to a new BG-11 medium. The phycoerythrin (PE)-rich cultures required at least 2 months to begin their growth. Phycocyanin (PC)-rich strains were easier to isolate, and the growth was much more rapid than the PE-rich picocyanobacteria. Microscopic observation was routinely carried out to monitor growth, presence of eukaryotes and increase in bacteria number. All the work was carried out using sterile glassware and media to avoid excess bacterial growth, but the cultures were not axenic.

Purification was performed on actively growing cultures that attained a well-defined colour of the prevalent pigment type. Flow cytometric single-cell sorting was carried out with an InFlux V-GS flow cytometer (Becton Dickinson Inc.) equipped with a UV laser (355 nm excitation wavelength, 60 mW) and a blue laser (488 nm excitation wavelength, 200 mW) as light sources. A defined interval of SSC vs. autofluorescence (530 nm) was selected and checked for sorting picocyanobacteria. From the events occurring within the selected interval, a single cell per well was sorted and directly inoculated in 96-well plates enriched with 100 μL of BG-11 substrate per well. Twelve replicates for each preculture were carried out. The plates were then kept for 2 months at the same conditions as the

Table 1. Main characteristics of the lakes of origin of isolates

Lake	Location	Description	Area (km^2)	Z_{max} (m)	$Z_{1\%}$ (m)	Conductivity (mS cm^{-1})	TP (μg L^{-1})
Mascardi	41°07′S 71°17′ W	Ultra-oligotrophic	39.2	218	28	0.06	3.6
Moreno	41°05′S 71°32′W	Ultra-oligotrophic	5.2	90	33	0.06	5.8
Buenos Aires	46°27′S 71°46′W	Ultra-oligotrophic	1850	586	n.a	n.a	n.a
Morenito	41°05′S 71°32′W	Oligotrophic	0.82	12	12	0.07	9.6
Nahuel Huapi	40°26′S 71°33′W	Ultra-oligotrophic	557	464	49	0.06	4.8
Maggiore	45°53′N 8°34′E	Oligotrophic	212	370	13	0.15	11
Garda	45°42′N 10°43′E	Mesotrophic	368	350	20	0.22	19
Orta	45°48′N 8°23′E	Oligotrophic	18.2	143	16	0.11	5
Candia	45°19′N 7°55′E	Eutrophic	1.35	8	5	0.11	32
Albano	41°44′N 12°40′E	Eutrophic	6.02	170	12	0.48	120
Viverone	45°25′N 8°02′E	Eutrophic	5.58	50	4	0.25	130
Alchichica	19°24′N 97°24′W	Mesotrophic Athalassohaline	1.81	63	25	12.3	27
Aljojuca	19°05′N 97°32′W	Athalassohaline	0.44	45	18	1.2	n.a.
Atexcac	19°20′N 97°27′W	Eutrophic Athalassohaline	0.29	32	22	13.0	81
La Preciosa	19°20′N 97°23′W	Eutrophic Athalassohaline	0.78	42	21	2.32	92

Z_{max}, maximum depth; $Z_{1\%}$, depth of 1% of surface irradiance; TP, average total phosphorus, measured at mixing for deep lakes and calculated as a mean over the water column for shallow lakes (mostly unpublished data); n.a., not available data.

precultures. The colour appearance in a well indicated the successful growth of a clonal culture.

DNA extraction, amplification and sequencing

To isolate DNA, 1 mL culture was centrifuged, supernatant was decanted and pellet was suspended in 200 μL lysis buffer (sucrose 0.75 M, TRIS-hydroxymethyl-aminomethane 50 mM, EDTA 40 mM, NaCl 400 mM). DNA was extracted from suspended pellet using the UltraClean® Microbial DNA Isolation kit (MOBio Laboratories, Inc., CA) according to manufacturer's instruction for maximum yields and few modifications in the centrifugation steps. Final elution was performed into 80 μL of water (Sigma-Aldrich, MO), and the DNA quality was verified on 1% agarose gel in 1× TBE, stained with Gel RedTM (Biotium, Inc., CA) for 1 h and visualized by UV transillumination. DNA quantification was performed with Qubit® fluorometer (Life Technologies Ltd, UK) according to manufacturer's instructions.

About 100 ng of single culture DNA was used for PCR amplification of 16S rRNA genes with Promega PCR 2× master mix. The primers used were as follows: the 20-bp forward primer 16S5′F (AGAGTTTGATCCTGGCTCAG) and 22-bp reverse primer B23S5′R (CTTCGCCTCTGTGT GCCTAGGT), targeting positions 8-27 and 30-52 of the 16S and 23S rRNA genes of *Synechococcus* PCC6301, respectively (Wilmotte *et al.*, 1993; Lepere *et al.*, 2000). The thermocycling (Gene Cycler, Bio-Rad, CA) program consisted of the following condition: 5 min at 95 °C, 30 cycles of 45 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and a final elongation step of 10 min at 72 °C. The PCR products were visualized on 1% (w/v) agarose gel in 1× TBE and stained for 1 h in Gel RedTM. Sequencing was carried out from purified PCR products by Macrogen Inc. (Seoul, Korea).

Phylogenetic analyses

For the phylogenetic analysis, a total of 43 isolate sequences were used. Quality check of sequences and contig

Table 2. List of the strains isolated in this study as they appear in the ML tree. Lake of origin, lake type and country of location are reported together with the prevalent phycobiliprotein of the strain, the bp length and accession number of the 16S rRNA gene sequences

Accession number	bp length	Lake of origin	Depth (m)	Lake type	Country	Prevalent phycobiliprotein	Strain name
HE805936	765	Candia	0.5	Morenic	Italy	PC	9C1
HE805937	765	Candia	0.5	Morenic	Italy	PC	9D4
HE805938	700	Garda	5	Glacial	Italy	PC	9E6
HE805934	765	Candia	0.5	Morenic	Italy	PE	3E1
HE805932	725	Candia	0.5	Morenic	Italy	PE	3A8
HE805933	765	Candia	0.5	Morenic	Italy	PE	3B3
HE805935	764	Candia	0.5	Morenic	Italy	PE	3F8
HE805946	758	Viverone	0.5	Morenic	Italy	PC	9G3
HE805947	757	Viverone	0.5	Morenic	Italy	PC	9H3
HE805925	702	Mascardi	30	Glacial	Argentina	PE	1A5
HE805926	702	Mascardi	30	Glacial	Argentina	PE	1B1
HE805930	661	Albano	0.5	Volcanic	Italy	PE	LL
HE805931	662	Albano	0.5	Volcanic	Italy	PE	ML
HE805944	749	Orta	15	Glacial	Italy	PE	5E1
HE805945	749	Orta	15	Glacial	Italy	PE	5F7
HE805941	745	Maggiore	15	Glacial	Italy	PE	4C4
HE805956	757	Atexcac	10	Volcanic	Mexico	PE	6E8
HE805957	757	Atexcac	15	Volcanic	Mexico	PE	6F1
HE805958	757	Atexcac	15	Volcanic	Mexico	PE	6H9
HE805955	703	Atexcac	10	Volcanic	Mexico	PE	6E5
HE805942	745	Maggiore	15	Glacial	Italy	PE	4D8
HE805943	746	Maggiore	15	Glacial	Italy	PE	4H9
HE805939	745	Maggiore	5	Glacial	Italy	PE	4A10
HE805940	758	Maggiore	5	Glacial	Italy	PE/PC	4A10
HE805921	745	Buenos Aires	0.5	Glacial	Argentina	PE	3C6
HE805922	744	Buenos Aires	0.5	Glacial	Argentina	PE	3D6
HE805954	712	Atexcac	2	Volcanic	Mexico	PE	6A2
HE805959	788	La Preciosa	15	Volcanic	Mexico	PE	8C7
HE805923	761	Moreno	35	Glacial	Argentina	PE	1C8
HE805924	761	Moreno	35	Glacial	Argentina	PE	1D8
HE805919	761	Morenito	4	Glacial	Argentina	PC	9A2
HE805920	761	Morenito	4	Glacial	Argentina	PC	9A8
HE805960	752	La Preciosa	5	Volcanic	Mexico	PE	7G6
HE805961	783	La Preciosa	5	Volcanic	Mexico	PE	7H9
HE805948	794	Alchichica	2	Volcanic	Mexico	PE	8E1
HE805949	794	Alchichica	2	Volcanic	Mexico	PE	8F6
HE805950	663	Alchichica	20	Volcanic	Mexico	PE	5G6
HE805952	820	Aljojuca	10	Volcanic	Mexico	PE	7C8
HE805951	820	Aljojuca	10	Volcanic	Mexico	PE	7A6
HE805953	820	Aljojuca	15	Volcanic	Mexico	PE	7D2
HE805928	789	Nahuel Huapi	70	Glacial	Argentina	PE	1F8
HE805929	789	Nahuel Huapi	70	Glacial	Argentina	PE	1G10
HE805927	789	Nahuel Huapi	70	Glacial	Argentina	PE	1E11

assemblage was performed with the software Geneious Pro 5.4.4 (Drummond *et al.*, 2011). Sequences were imported into the Database SSURef_104_SILVA (Pruesse *et al.*, 2007) with the ARB Software (Ludwig *et al.*, 2004; http://www.arb-home.de). The ARB Editor software was used for the alignment of the isolates' sequences with reference species or group consensus by means of the Integrated Aligner Panel. Manual refinement was carried out taking into account structural constraints with the secondary structure tool of the ARB Editor and considering 52 selected reference sequences from the most representative *Synechococcus* isolated worldwide. Maximum-likelihood trees (ML), neighbour-joining (NJ, DNADIST and Jukes & Cantor correction) and maximum-parsimony (MP, DNAPARS, PHYLIP package) were calculated with the ARB software tree package (NJ and MP not shown) using the bacterial position variability filter by parsimony (Ludwig & Klenk, 2001), the 660-bp-long sequences and *Synechococcus* PCC

6301 (former *Anacystis nidulans*) as the outgroup (Crosbie *et al.*, 2003). In particular, the ML tree was constructed with the RAxML program (version 7.0.3, Stamatakis *et al.*, 2005) with GTR + Γ nucleotide substitution model.

Pairwise nucleotide sequence identities were calculated using the software MegaBLAST for highly similar sequences in the BLASTN Suite (BLASTN 2,2,26+) (Zhang *et al.*, 2000). Average values were calculated considering an alignment of 660 bp and excluding sequences from putative co-isolates. Sequence accession numbers (HE805919-HE805961) are reported in Table 2 together with sequence length, strain isolation source (depth, lake and country) and prevalent pigment.

Results

Pairwise similarity analysis of the 16S rRNA gene sequence of each new isolate against the others ranged from 90.7% to 95.7%; overall, the mean pairwise difference between all the 43 strains was 5.9%. Pairwise similarity analyses were conducted also between our isolates and reference sequences based on grouping result in ML tree. Phylogenetic trees were calculated using ML, NJ and MP method, the former being presented in Fig. 1. In general, the trees showed highly similar structures between each other as well as with previously published trees of nonmarine picocyanobacteria. In particular, the nomenclature used for cluster designation in the ML tree was based on Crosbie *et al.* (2003).

Fifteen sequences from our isolates grouped within the *C. gracile* group (group A) with moderately supported bootstrap values in all the trees (78% in ML, 68% in NJ and 72% in MP). These isolates were PE- and PC-rich strains isolated from shallow eutrophic lakes and PE-rich strains from deep lakes from widely separated locations, namely Lake Candia, Viverone, Garda, Orta and Albano in Italy and Lake Mascardi in Argentina. In particular, in the ML tree, these 15 sequences formed a sister clade (96% bootstrap) to the *C. gracile* group that includes *Microcystis elabens* (reclassified as a species of *Aphanothece*, Komárek & Anagnostidis, 1999) as well as Arctic isolates. In addition, in the MP tree, the five PC-rich isolates fell into the *C. gracile* group and they separated from the remaining 10 PE-rich isolates. Finally, the mean value of pairwise sequence comparison between our isolates and the reference sequences contained in group A was 95.8%.

Our ML tree confirmed the presence in group B subalpine cluster I of strains isolated from Lake Constance (BO8807, BO9404) (Ernst *et al.*, 2003), Lake Mondsee (MW73B4) (Crosbie *et al.*, 2003) and Lake Maggiore (LM94) (Ernst *et al.*, 2003) with poorly supported bootstrap values (58% in ML, 59% in NJ and 61% in MP). Interestingly, our new strain PE 4C4, isolated at 15 m from Lake Maggiore, fell into group B, near LM94, a strain isolated 10 years ago from the same lake (Ernst *et al.*, 2003). Group M, containing strains isolated from the Mazurian lakes (MA0607J, MA0607I) (Jasser *et al.*, 2011), was included in group B in the ML tree (56% bootstrap) but appears as separated in the NJ and MP trees (99% and 90% bootstrap, respectively).

A new group of sequences from the Mexican Lake Atexcac was formed; this group was distant from the other Mexican saline lakes and although well supported by bootstrap (78% in ML, 58% in NJ and 78% in MP) with a mean pairwise similarity of 93.5%. The majority of isolate sequences from athalassohaline crater lakes of the tropical high-altitude Mexican plateau, namely Lake Alchichica, Aljojuca, La Preciosa, formed a cluster of 'halotolerants' with a mean similarity of 95.9%. This group showed moderately supported bootstrap at the node in ML (76%) and NJ (60%) trees and it was divided into two sister clades with 90% and 71% of bootstrap each in MP. With respect to reference sequences of *Synechococcus*, this monophyletic group of halotolerants was sister to *Synechococcus* 5.3 in a single poorly supported clade (58% bootstrap in ML, not resolved in NJ and MP).

Sequences from PE-rich strains from worldwide distributed lakes (Maggiore, Buenos Aires, Moreno, La Preciosa. Atexcac) as well as 2 PC-rich isolates from Lake Morenito clustered in a novel clade poorly supported by bootstrap in ML tree (58% vs. 55% in NJ and < 50% in MP); pairwise comparison returned a 93.7 similarity percentage between those isolates.

Marine *Synechococcus* subcluster 5.2 fell into the subalpine cluster II as previously reported by other authors (Sánchez-Baracaldo *et al.*, 2008; Everroad & Wood, 2012) separated from the marine *Synechococcus* subcluster 5.3, group I and the Antarctic strains. It is noteworthy that sequences of Lake Nahuel Huapi, Argentina, clustered together with bootstrap of 58% in ML (95% in MP and 68% NJ), distant from other isolates of Argentinean lakes and formed a sister clade with subcluster 5.2 with moderately supported bootstrap values (78%, 60% and 85% in ML, NJ and MP, respectively). Pairwise similarity between Lake Nahuel Huapi isolates and 5.2 subcluster sequences was 98.8%.

Discussion

The phylogeny of nonmarine *Synechococcus* picocyanobacteria was investigated and increased in resolution thanks to the addition of 43 new isolate sequences. The new strains were isolated from several lakes characterized by different trophic conditions and salinity. Their genetic affiliation ascribed part of them to already established

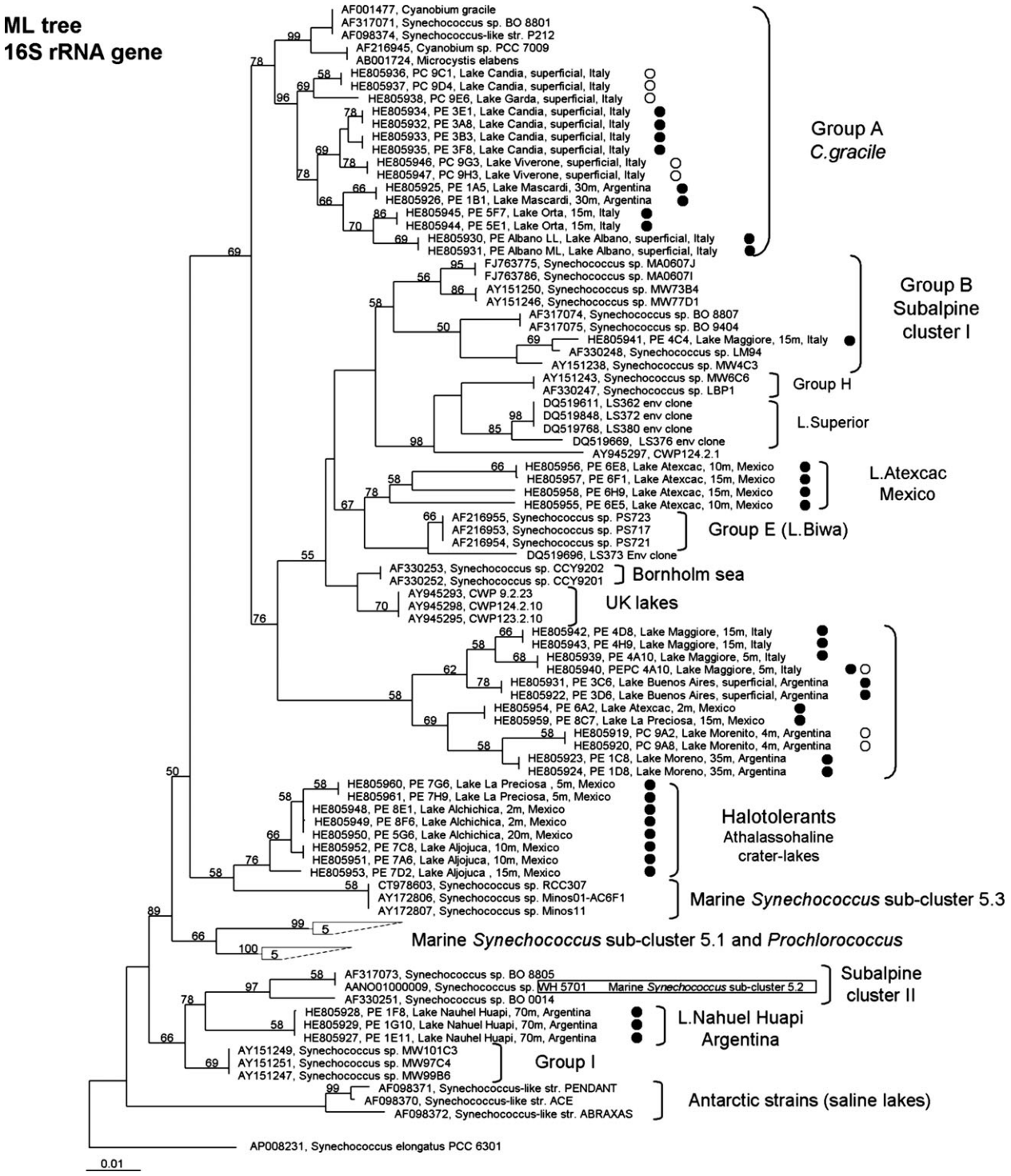

Fig. 1. ML tree inferred from 16S rRNA gene sequences of 43 isolated strains from lakes in Italy, Mexico and Argentina, including other taxa selected to obtain a good representation of the Syn/Pro clade and avoiding redundancy (95 strains in total) (Crosbie *et al.*, 2003; Ivanikova *et al.*, 2007; Sánchez-Baracaldo *et al.*, 2008; Jasser *et al.*, 2011). For the new isolates (marked with a circle), terminal branches display accession number, pigment type (●, PE-rich; ○, PC-rich), name of the strain, lake of origin, depth and location. Bootstrap values (10 000 replicates) are shown at nodes (values < 50% are not shown). The outgroup was *Synechococcus* PCC6301 (formerly *Anacystis nidulans*).

groups, while a number of strains formed new clades in the phylogenetic tree of 16S rRNA gene (Fig. 1).

The maar crater lakes are an example of transient habitats from freshwater to athalassohaline, including lakes with a conductivity only slightly higher than freshwater and others where it is similar to marine environments, despite the different salt composition (e.g. Lake Atexcac). While four of our isolates from Lake Atexcac (2 m depth) formed a separated group with respect to the other halotolerants, thus displaying a distinct habitat differentiation, the presence in this lake of a strain belonging to another clade formed by PE- and PC-rich *Synechococcus,* coming from widely separated locations, seems to negate the isolation of this lake.

Interestingly, the other strains isolated from most of athalassohaline lakes formed a monophyletic clade, sister to marine *Synechococcus* 5.3, and here named 'halotolerants'. Recently, the subcluster 5.3, consisting of strains RCC307, Minos 11, Minos 01 and BL3 (Scanlan, 2012), has been found incongruent with a placement as part of the 5.1 clade and recognized rather as sister to *Synechococcus* 5.1 (Dufresne *et al.*, 2008; Everroad & Wood, 2012). In three different phylogenetic trees of nonmarine *Synechococcus* (Crosbie *et al.*, 2003; Sánchez-Baracaldo *et al.*, 2008; Jasser *et al.*, 2011), the subcluster 5.3 never appeared, probably because no halotolerant strains from freshwater habitats (athalassohaline lakes) have been isolated in the past. Therefore, the identification of the monophyletic clade of the 'Halotolerants', sister to marine subcluster 5.3, constitutes an important reconstruction of the phylogenetic evolution of *Synechococcus*. Actually, the proximity of typically marine to freshwater strains was found in the subcluster 5.2 where *Synechococcus* WH5701 (Herdman *et al.*, 2001) falls together with BO8805 (Lake Constance, Germany) (Sánchez-Baracaldo *et al.*, 2008; Everroad & Wood, 2012) characteristically assigned to subalpine cluster II (Crosbie *et al.*, 2003).

We observed a contiguity of Lake Nahuel Huapi strains to two well-studied strains from Lake Constance (BO8805 and BO0014, Becker *et al.*, 2004; Ivanikova *et al.*, 2007). The strain BO0014, isolated from periphyton in Lake Constance, was assigned to subalpine group II; BO8805, isolated in the pelagic zone of Lake Constance, was also found in the periphyton in the littoral zone of the lake (artificial tiles) (Becker *et al.*, 2004). This versatility to live in the pelagic environment as well as to grow in periphytic consortia is an indication of *Synechococcus* adaptation to colonization of different ecological niches and can explain its global dispersal.

PE strains 1E11, 1G10 and 1F8 isolated from the ultraoligotrophic Lake Nahuel Huapi (North Patagonia, Argentina) clustered together as a sister clade to subalpine cluster II (central Europe) demonstrating the high widespread dispersal of this clade, which includes strains from subalpine and subandine lakes. If this group can be considered sister to group I, it is still ambiguous, but for the first time here a number of strains from the Southern and Northern Hemisphere and from marine and freshwater environments are analysed together. Antarctic strains (PENDANT and ACE) are well supported as a separate group (bootstrap of 99%) with a mean pairwise similarity of 98.9%.

Our line of evidence on the proximity of the marine subcluster 5.2 and freshwater strains is well supported by previous studies (Sánchez-Baracaldo *et al.*, 2008; Everroad & Wood, 2012). A close relation of Baltic Sea *Synechococcus* with freshwater strains was observed, not surprisingly considering the low salinity of the Baltic. The two strains B9803 (from Baltic Sea) and BO8805 (from Lake Constance) fall in the subcluster 5.2 (Sánchez-Baracaldo *et al.*, 2008; : Everroad & Wood, 2012).

In the study of the phylogenesis of nonmarine picocyanobacteria, different authors found endemic *Synechococcus* strains as well as cosmopolitan ones: in Lake Superior (Ivanikova *et al.*, 2007), in Mazurian lakes (Jasser *et al.*, 2011) and in Mondsee (Crosbie *et al.*, 2003). Different authors (Crosbie *et al.*, 2003; Felföldi *et al.*, 2011) emphasize the high dispersive potential of microorganisms and recommend to be cautious on the geographic restriction of novel clades, possibly due to under-sampling. As a matter of fact, we found Lake Superior as distinct group confirming previous findings (Ivanikova *et al.*, 2007), whereas the two Mazurian lake sequences inserted in our analyses were included in group B, in our ML tree.

The majority of the isolates from Italian lakes, both PE- and PC-rich, assembled inside the *C. gracile* cluster group A, forming a well-resolved subcluster. We are aware that our new isolates are separated by a considerable distance from some of the *C. gracile* cluster sequences. Nevertheless, we propose here to admit our new isolates into group A as they shared on average 95.8% identity in 16S rRNA gene sequences with *Cyanobium* group and the enlarged group has a 78% bootstrap. We recognize two subclusters in group A: one is the previously defined group A (e.g. Crosbie *et al.*, 2003) with *C. gracile*, PCC7009, BO8801 and also with some PC-rich Arctic strains (e.g. P212) isolated from the Bylot Island Lake (Vincent *et al.*, 2000) and the other, formed with 96% of bootstrap, is composed by strains isolated in shallow eutrophic lakes both PE- and PC-rich, and derived from deep lakes.

Microcystis elabens is a colonial form now reclassified as a species of *Aphanothece* in the same family as *Synechococcus* (Komárek & Anagnostidis, 1999; Vincent *et al.*, 2000) included in group A. It is interesting to note that one of the new strains of the enlarged group A (Lake Albano) is a single-cell *Synechococcus,* which showed the tendency

towards microcolony formation under ultraviolet radiation in laboratory experiments (Callieri *et al.*, 2011). This capability was not evident in other strains selected from other phylogenetic groups. Further studies are in progress (C. Callieri, pers. commun.) to understand whether there is a one factor over the others that induce microcolony formation and whether specific genes are involved in this process.

In conclusion, our research sheds new light on the global distribution and evolution of picocyanobacteria, enriching the phylogenetic tree with 43 new sequences from established cultures. The finding of new clades, affiliated closely to marine subcluster 5.2 and 5.3, demonstrates the importance of investigating both transition environments with different degree of salinity and ultraoligotrophic Southern Hemisphere lakes. As at present an accurate interpretation of the evolution of the freshwater *Synechococccus* lineage is still far from being achieved, the better resolution of the phylogenetic analysis of *Synechococccus* here proposed can constitute an ideal base for alternative hypotheses on *Synechococcus* phylogeny.

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