Phylogenetic diversity of freshwater picocyanobacteria

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RATIONALE

Picocyanobacteria are photosynthetic prokaryotes, common in lakes and oceans, and abundant across a wide spectrum of trophic conditions (Callieri et al 2012). The dominant genus of freshwater picocyanobacteria is *Synechococcus*. Analysis of 16S rRNA gene of freshwater *Synechococcus* showed its polyphyletic origin, requiring better insights in the present classification of the genus and possibly a revision (Crosbie et al 2003). The most updated picture of 16S rRNA phylogenetic tree of picocyanobacteria, including marine and freshwater strains, shows on the one side the well-defined marine *Prochlorococcus* and *Synechococcus* clade 5.1, on the other at least six freshwater clades represented by groups with high similarity. In between these two neatly defined groups are some borderline ones like the marine subclusters 5.2 and 5.3, Bornholm sea group, subalpine cluster II, and group I. In this study we tried to better resolve the *Synechococcus* phylogenetic tree enlarging the sequence data set of the borderline groups which could include not only the halotolerants but also picocyanobacteria from "extreme" lakes. Therefore we selected four volcanic high altitude athalassohaline lakes in Mexico as a source of non-marine halotolerant *Synechococcus*, five glacial ultraoligotrophic North Patagonian lakes as extreme ecosystems, and six Italian lakes of glacial, volcanic and morenic origin, with different trophic conditions. We tentatively used the maximal PSII quantum yield of dark adapted cultures, Fv/Fm, to define putative ecotypes.

METHODS


Fig.1

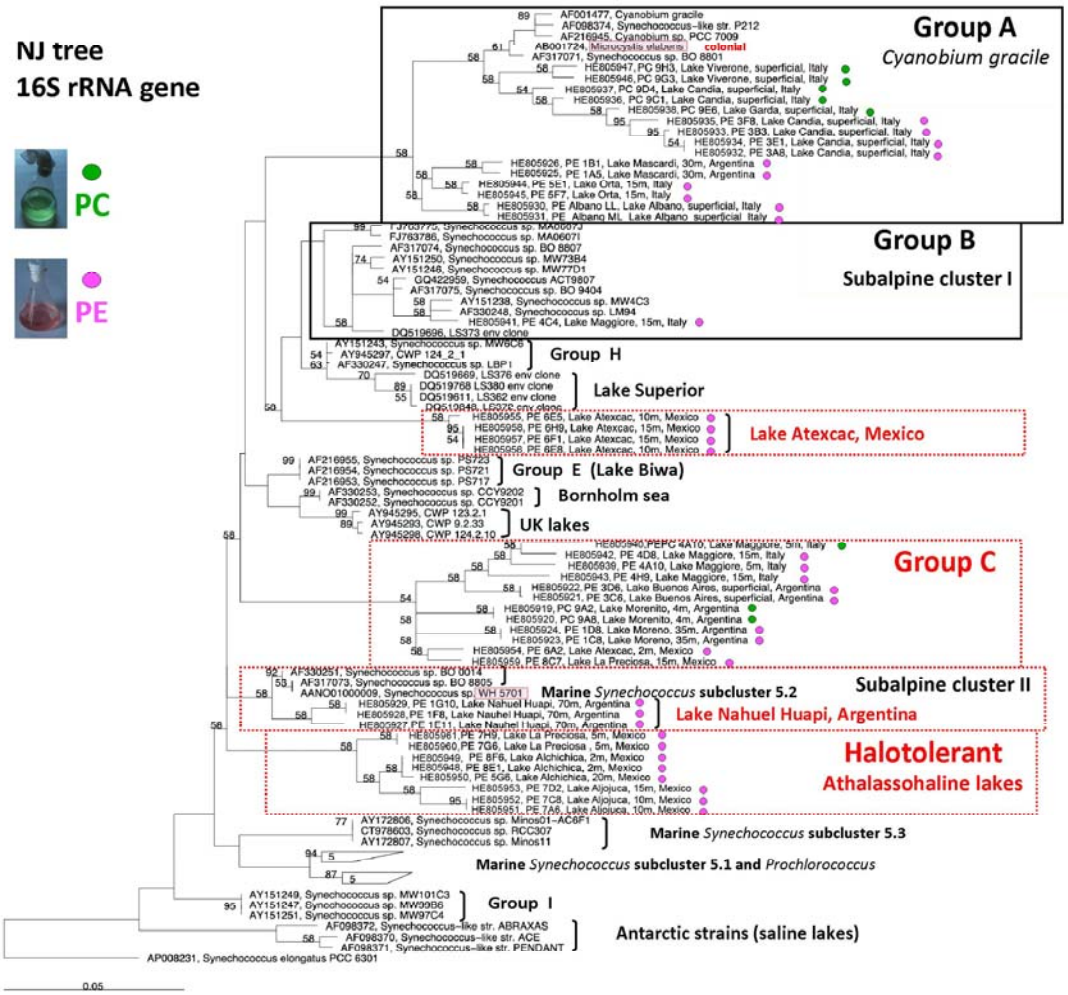
- **A total of 15 lakes** different for origin, thermal regime, maximum depth, trophic conditions, salinity and location (Argentina, Italy, and Mexico) were sampled at different depths (Tab.1). For most lakes the sample was gravity filtered through a 3 µm polycarbonate membrane and 5 replicates 3 ml were added to 3 ml BG11 medium at 18-20°C and kept at low light (10-15 µmol $m^{-2} s^{-1}$).
- **Purification** was performed by flow cytometric single-cell sorting on an InFlux V-GS flow cytometer (Becton Dickinson Inc.) equipped with a UV-laser (355 nm excitation wavelength, 60mW) and a blue laser (488 nm excitation wavelength, 200mW) 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 BG11 substrate per well. The plates were kept for two months at the same conditions as for the pre-cultures. A coloured well indicated the successful growth of a clonal culture.
- **To isolate DNA**, 1 ml culture was centrifuged, supernatant was decanted and pellet was suspended in 200 µl lysis buffer and stored overnight at -80°C. DNA was extracted from suspended pellet using the UltraClean® Microbial DNA Isolation Kit (MOBio Laboratories, Inc). Final elution was done into 80 µl of PCR water (Sigma- Aldrich) and the DNA quality was verified on 1% agarose gel in 1X TBE, stained with Gel RedTM (Biotium Inc. CA, USA) for 1h and visualized by UV transillumination. About 100 ng of single culture DNA was used for PCR amplification of 16S rRNA genes with Promega PCR Master Mix (2x). The primer set used were: 20bp forward primer 16S5'F (AGAGTTTGATCCTGGCTCAG) and 22bp reverse primer B23S5'R (CTTCGCCTCTGTGTGCCTAGGT) (Lepere 2000). Sequencing of the amplified fragments was carried out from purified PCR products by Macrogen Inc.
- **For phylogenetic analysis**, a total of 43 isolate sequences were used. Quality of sequences was checked with the software Geneious Pro 5.4.4 (Drummond 2011) and imported into the Database SSURef_104_SILVA (Pruesse 2007) with the ARB Software (Ludwig 2004; http://www.arb-home.de). For the alignment the ARB automatic alignment tool was used with manual refinement taking into account structural constraints with secondary structure tool of ARB editor. Sequences of about 750bp obtained from our isolates were aligned to selected reference sequences from the most representative *Synechococcus* isolated worldwide. *Synechococcus* PCC 6301 (former *Anacystis nidulans*) was used to root the Neighbour-joining tree, constructed with Jukes-Cantor correction and 10.000 bootstrap calculation. Pairwise nucleotide sequence identities were calculated using 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 665 bp and excluding sequences from putative co-isolates. The average percentages of identity ranged from 90.7 to 95.8%.
- **The maximal photosystem II (PSII) quantum yield, Fv/Fm,** of dark adapted cultures was measured with a Phyto-PAM fluorometer (Walz, Germany). Fv, the variable fluorescence, is Fm (the maximal fluorescence from fully reduced PSII) minus F (the intrinsic fluorescence from the antenna of fully oxidized PSII) (Gentry et al 1989). The Phyto-PAM (Optical Unit ED101-US) used in this study was equipped with Phyto-ML (LED 470, 520, 645 and 665 nm) and Phyto-AL Unit (37 actinic light LEDs peaking at 655 nm which permitted to reach up to ~1900 µmol $m^{-2} s^{-1}$).

RESULTS

The majority of the Italian isolates, both PC and PE, assembled inside the *Cyanobium gracile* cluster group A (Crosbie et al 2003). Only one L. Maggiore strain convened in group B, whereas many of our isolates clustered in a novel clade (group C) encompassing geographically widespread strains. Other strains displayed distinct habitat differentiation, like those isolated from high altitude tropical athalassohaline lakes in Mexico, which formed a well distinguished group of halotolerants near the marine subclusters 5.3 and 5.2 (*Synechococcus* WH 5701). Notably, *Synechococcus* strains from tropical lakes had the highest Fv/Fm, typical of ecotypes with high quantum conversion efficiency of PSII. *Synechocccus* strains isolated from the ultraoligotrophic, large and deep L. Nahuel Huapi (North Patagonia, Argentina) resulted as closely related to L. Constance subalpine cluster II (*Synechococcus* BO8805 and BO0014) and to marine subcluster 5.2, with high Fv/Fm.

NJ tree
16S rRNA gene

PC
PE



Tab.1. List of the isolated strains

	GenBank acc.no.	bp lenght	Lake of origin	Depth m	Lake type	Country	Prev. phycob.	Fv/Fm 645nm
Group A	HE805947	758	Viverone	0.5	morenic	Italy	PC	0.21
	HE805946	758	Viverone	0.5	morenic	Italy	PC	0.21
	HE805937	766	Candia	0.5	morenic	Italy	PC	0.19
	HE805936	766	Candia	0.5	morenic	Italy	PC	0.10
	HE805938	701	Garda	5	glacial	Italy	PC	0.10
	HE805935	766	Candia	0.5	morenic	Italy	PE	0.42
	HE805933	766	Candia	0.5	morenic	Italy	PE	0.45
	HE805934	766	Candia	0.5	morenic	Italy	PE	0.36
	HE805932	726	Candia	0.5	morenic	Italy	PE	0.45
	HE805926	702	Mascardi	30	glacial	Arg.	PE	0.19
	HE805925	702	Mascardi	30	glacial	Arg.	PE	0.28
	HE805944	749	Orta	15	glacial	Italy	PE	0.40
	HE805945	749	Orta	15	glacial	Italy	PE	0.47
	HE805930	662	Albano	0.5	volcanic	Italy	PE	0.49
	HE805931	662	Albano	0.5	volcanic	Italy	PE	0.25
Group B	HE805941	746	Maggiore	15	glacial	Italy	PE	0.29
Lake Atexcac	HE805955	703	Atexcac	10	volcanic	Mex.	PE	0.38
	HE805958	757	Atexcac	15	volcanic	Mex.	PE	0.50
	HE805957	757	Atexcac	15	volcanic	Mex.	PE	0.21
	HE805956	757	Atexcac	10	volcanic	Mex.	PE	0.44
Group C	HE805940	758	Maggiore	5	glacial	Italy	PE/PC	0.42
	HE805942	746	Maggiore	15	glacial	Italy	PE	0.42
	HE805939	746	Maggiore	5	glacial	Italy	PE	
	HE805943	746	Maggiore	15	glacial	Italy	PE	0.39
	HE805922	745	B.Aires	0.5	glacial	Arg.	PE	0.15
	HE805921	745	B.Aires	0.5	glacial	Arg.	PE	0.13
	HE805919	761	Morenito	4	glacial	Arg.	PC	0.22
	HE805920	761	Morenito	4	glacial	Arg.	PC	
	HE805924	761	Moreno	35	glacial	Arg.	PE	0.15
	HE805923	761	Moreno	35	glacial	Arg.	PE	0.11
	HE805954	712	Atexcac	2	volcanic	Mex.	PE	0.51
	HE805959	791	La Preciosa	15	volcanic	Mex.	PE	0.43
Subalpine cluster II	HE805929	789	N.Huapi	70	glacial	Arg.	PE	0.43
	HE805928	789	N.Huapi	70	glacial	Arg.	PE	0.40
	HE805927	789	N.Huapi	70	glacial	Arg.	PE	0.52
Halotolerant athalassohaline	HE805961	783	La Preciosa	5	volcanic	Mex.	PE	0.37
	HE805960	753	La Preciosa	5	volcanic	Mex.	PE	0.54
	HE805949	794	Alchichica	2	volcanic	Mex.	PE	0.30
	HE805948	794	Alchichica	2	volcanic	Mex.	PE	0.46
	HE805950	663	Alchichica	20	volcanic	Mex.	PE	0.40
	HE805953	820	Aljojuca	15	volcanic	Mex.	PE	0.39
	HE805952	820	Aljojuca	10	volcanic	Mex.	PE	0.62
	HE805951	820	Aljojuca	10	volcanic	Mex.	PE	0.51

CONCLUSIONS

The phylogenetic analysis of our new isolates provides more clear evidence of the non-marine nature of group A as well as of the phylogenetic relationship between marine and freshwater halotolerant *Synechococcus* strains. The indication of the Fv/Fm as "signature" of putative ecotypes should be taken into consideration and examined something closely.

References

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