

Genetic diversity of cyanobacterial communities in Lake Kinneret (Israel) using 16S rRNA gene, *psbA* and *ntcA* sequence analyses

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ABSTRACT: The genetic diversity of cyanobacterial communities was studied at various depths in Lake Kinneret (Israel). Denaturing gradient gel electrophoresis (DGGE) of specific 16S rRNA gene PCR products showed significant differences in the cyanobacterial community structure between epi- and hypolimnetic waters. Sequences of clone libraries prepared from 16S rRNA gene PCR products from epi- and hypolimnion revealed the presence of at least 11 different groups of cyanobacteria. Clones related to the unicellular cyanobacteria (Chroococcales and picocyanobacteria) dominated the clone libraries from both depths. New primers to amplify the gene coding for the photosystem II reaction centre (*psbA*) and the nitrogen regulator gene (*ntcA*) of cyanobacteria were developed and used for further characterization of the cyanobacterial communities from the lake. Sequences of *psbA* amplicons clustered with those from 2 different groups of marine *Synechococcus* and Chroococcales. Cloned *ntcA* amplicons from the lake were closely related and did not cluster with sequences from cultured cyanobacteria or other environmental sequences from this gene. All the molecular markers analyzed here showed similarity to sequences from some groups of cyanobacteria in the lake and those so far found in marine habitats.

KEY WORDS: *Gloeocapsa* · Picophytoplankton · 16S rRNA gene · *psbA* · *ntcA*

INTRODUCTION

Cyanobacteria are distributed among a wide range of habitats including lakes, streams, oceans, soil, glaciers, deserts and hot springs. These oxygen-evolving microorganisms play a fundamental role in the global cycling of nitrogen, sulphur and carbon, affecting biogeochemical cycles in the past and present (Sanchez-Baracaldo et al. 2005). In Lake Kinneret (Israel), long-term records have shown an annual pattern of phytoplankton composition dominated by the dinoflagellate *Peridinium gatunense*, while cyanobacteria have been relatively minor contributors to the phytoplankton biomass in the lake. This changed in 1994 with the appearance of the filamentous nitrogen-fixing and toxin-producing cyanobacterium *Aphanizomenon ovalisporum* (Berman 1997, Hadas et al. 1999, Pollingher et al. 1998), continuing with blooms of *Cylindrospermopsis cuspidis* since 2003, considerably increasing the relative contribution of cyanobacteria to the total phytoplankton biomass in the lake (Zohary 2004).

Several groups of cyanobacteria have been recorded in the lake at different seasons. The occurrence of *Aphanizomenon ovalisporum* is mainly restricted to late summer into autumn (Walsby et al. 2003, Zohary 2004). Another group common in the historical records, *Microcystis* sp., appears in some years with relatively high abundances at the beginning and/or the end of winter and is correlated with changes in the annual bloom of *Peridinium gatunense* (Sukenic et al. 2002). Picocyanobacteria, unicellular cyanobacteria with sizes

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ranging from 0.2 to 2 μm (Callieri & Stockner 2002), are also a prominent component of the phytoplankton in Lake Kinneret (Malinsky-Rushansky et al. 1995). Microscopic observations during a period of 4 yr showed that the annual and vertical distribution of picocyanobacteria is correlated with temperature and light, and that this group appears at all depths throughout the year, with maximal abundances in late summer into early autumn in the epilimnion (Malinsky-Rushansky et al. 1995, 2002). Two of these picocyanobacteria, *Synechococcus* and *Prochlorococcus*, dominate the photoautotrophic picoplankton over vast areas in the oceans, having a great importance for global primary production (Partensky et al. 1999, Scanlan & West 2002).

Cyanobacteria have a high degree of morphological differentiation, which has been traditionally used for higher-level classification and taxonomy. However, several molecular analyses suggest that some of these morphologically defined groups do not have a monophyletic origin (Honda et al. 1999). Therefore, molecular approaches can contribute to improve our understanding of the dynamics of cyanobacterial communities in this subtropical lake. We selected 16S rRNA gene, *psbA* and *ntcA* genes as molecular markers. 16S rRNA gene sequences allow a rapid identification of complex cyanobacterial communities (Nübel et al. 1997), but may be inefficient to discriminate closely related species (Rocap et al. 2003). Therefore, the 2 functional genes *psbA* and *ntcA* were also included in this study. These 2 genes have been successfully used to characterize picocyanobacterial communities in the ocean (Lindell et al. 1998, 2002, Fuller et al. 2003, Zeidner et al. 2003, Penno et al. 2006) but not yet in freshwater.

Lake Kinneret, the main freshwater reservoir of Israel, is vulnerable to anthropogenic ecological deterioration. The cyanobacteria of the lake have been one of the most perturbed elements of the phytoplankton assembly in the past few years (Zohary 2004). The aim of the current study was to characterize the composition of cyanobacteria and specially picocyanobacteria in order to obtain insights on the recent fitness of this group in the lake and also to determine the effect of water column stratification on the assemblages observed.

MATERIALS AND METHODS

Site description. Lake Kinneret is 210 m below sea level and has mean and maximum depths of 24 and 42 m, respectively. It is a monomictic lake stratified from May to December, and mixing occurs between December and January. Samples were collected at

Stn F (35° 50' N and 35° 33' E with a maximum depth of 22 m) representing the pelagic area. At the sampling time (October 2004) the thermocline was at 16.5 m. Samples were taken from the surface (1 m) and above, in and below the thermocline (15, 16.5 and 20.7 m, respectively).

DNA extraction and PCR. Water samples were filtered through 0.2 μm pore size filters (Supor-200, PALL Life Sciences) and stored at -18°C for up to 3 d until DNA extraction with the UltraClean Soil DNA Kit (MoBio), following the manufacturer's guidelines. Concentration and quality of the DNA was checked by electrophoresis in 0.8% agarose gels stained with ethidium bromide.

For specific amplification of cyanobacterial 16S rRNA gene, PCR was carried out with the primers Cya106F/Cya781R (Nübel et al. 1997). For denaturing gradient gel electrophoresis (DGGE) a nested PCR with the primers P3 (GC-clamped) and P2 (Muyzer et al. 1993) was performed using the cyanobacterial 16S rRNA gene amplicons as templates. Prior to the second amplification, the templates were purified using the MiniElute PCR Purification Kit (Qiagen) and diluted 50 times in water. For nested PCR, a touchdown temperature program was used (Muyzer et al. 1993). In order to better characterize the populations of *Synechococcus* in the lake, new primers were designed to amplify the genes *psbA* and *ntcA*. The primers *psbA*86F (5'-TTT ATG TGG GTT GGT TCG G-3') and *psbA*980R (5'-TGA GCA TTA CGC TCG TGC-3') were used to amplify a fragment of 900 bp comprising almost the whole *psbA* gene, whereas the primers *ntcA*FP (5'-CCT GCY GAR AGG GTT TAT-3') and *ntcA*RP (5'-AAG DGC WAT YGG ATC AA-3') were used to amplify a fragment of 500 bp of the *ntcA* gene. The specificity of the primers was evaluated using BLAST and the tool PROBE MATCH in ARB (www.arb-home.de) using databases containing all available sequences from each of the genes deposited in GenBank. The primer combinations were also evaluated by PCR using genomic DNA from *Prochlorococcus marinus* SS120, *P. marinus* MED4, *Synechococcus* WH7803 and *Synechococcus* WH8102 (as positive controls); a collection of 9 strains isolated from Lake Kinneret (*Microcystis* sp. brown strain, *Microcystis* sp. green strain, *Synechococcus* CN, *Synechococcus* P41, *Aphanizomenon* sp., *Chroococcus* sp., and *Schizothrix* sp.); and *Synechocystis* sp., *Nostoc* sp. PCC 7120, *Thermosynechococcus elongatus* and *Gloeobacter* sp. PCR was set up in a total volume of 25 μl containing 1 to 2.5 ng DNA as template, 25 pmol of each primer, 1X PCR buffer (10 mM Tris-HCl; 15 mM MgCl_2 ; 5 mM KCl, pH 8.3), 10 pmol of each dNTP, 250 ng BSA (Sigma Aldrich), 1% formamide and 1 U of *Taq*

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MATERIALS AND METHODS

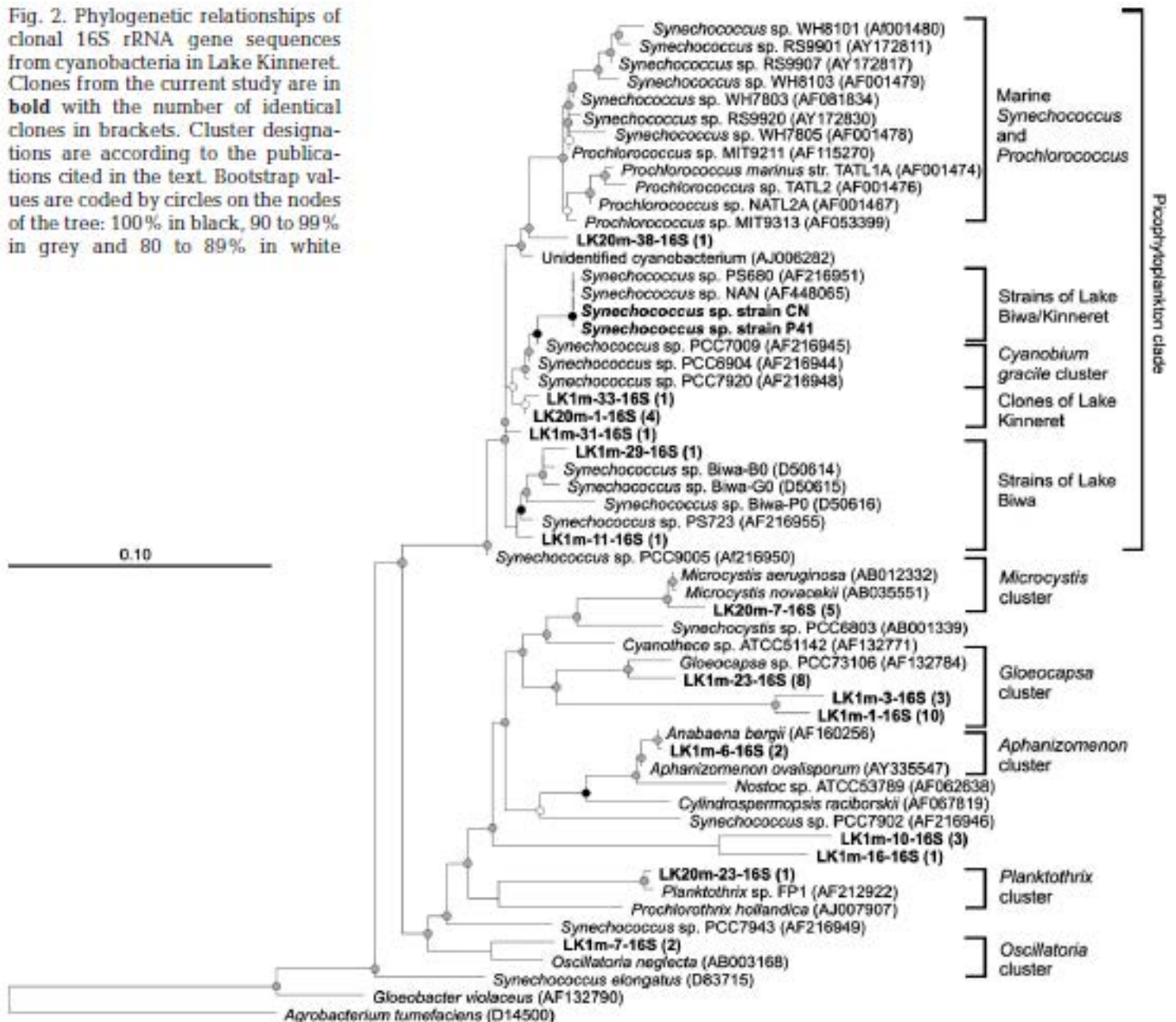
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Fig. 2. Phylogenetic relationships of clonal 16S rRNA gene sequences from cyanobacteria in Lake Kinneret. Clones from the current study are in **bold** with the number of identical clones in brackets. Cluster designations are according to the publications cited in the text. Bootstrap values are coded by circles on the nodes of the tree: 100% in black, 90 to 99% in grey and 80 to 89% in white



Comparison of sequences from the clones (Fig. 2) showed the existence of a more diverse assemblage of cyanobacteria than deduced from the DGGE. The majority of the clones grouped with colonial cyanobacteria, such as *Microcystis*, and unicellular, such as *Gloeocapsa* and *Synechococcus*, while others were related to *Aphanizomenon*, *Oscillatoria* and *Planktothrix*. This coincided with the regular microscopic observations of water samples from the lake (Zohary 2004). Seventeen out of 44 clones formed 2 separate clusters: (1) 13 clones distantly related to, but distinct from *Gloeocapsa*, and (2) 4 clones situated between the *Planktothrix/Oscillatoria* and *Microcystis/Gloeocapsa/Aphanizomenon* clusters.

Another group of 9 clones was spread among different clusters of the picophytoplankton clade sensu Urbach et al. (1998). Five of these clones formed an independent

group related to the *Cyanobium gracile* cluster. Two clones clustered together with isolates from Lake Biwa (Japan) at a lower bootstrap value (77%). One clone (LK1m-31-16S) was not related to any previous subcluster. The clone LK20m-38-16S was more similar to marine *Synechococcus* and *Prochlorococcus* spp. than to cultures from the freshwater lakes Biwa and Kinneret. This was supported by a high bootstrap value.

The sequences from 2 cultures of *Synechococcus* sp. (strain P41 and CN) previously isolated from Lake Kinneret (Malinsky-Rushansky et al. 1995) formed a homogenous subcluster together with *Synechococcus* sp. PS-680, isolated from Lake Biwa (Robertson et al. 2001), and *Synechococcus* sp. NAN, isolated from brackish marshlands in Japan (Fig. 2). A high bootstrap value supported this subcluster, which differed from any previously described subcluster within the

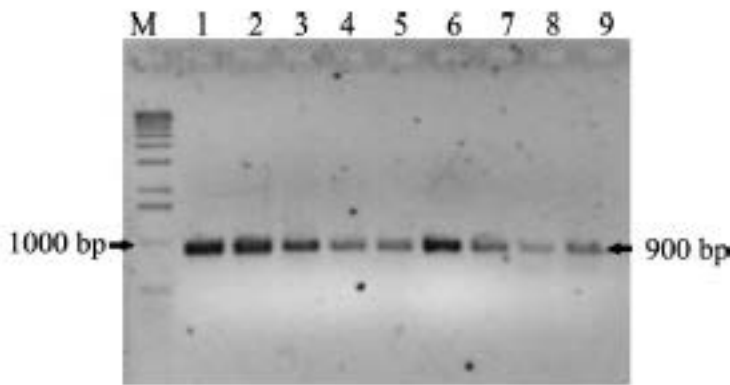


Fig. 3. Agarose gel with the *psbA* PCR products from (1) *Prochlorococcus marinus* SS120, (2) *P. marinus* MED4, (3) *Synechococcus* WH7803, (4) *Synechococcus* WH8102, (5) *Synechococcus* sp. strain CN, (6) *Synechococcus* sp. strain P41, (7) *Synechocystis* sp. PCC6803, (8) Lake Kinneret 1 m, and (9) Lake Kinneret 20.7 m. Arrow to left corresponds to 1000 bp in the 1 kb ladder (M). Arrow to right marks the PCR product of ca. 900 bp

picocyanobacteria clade (Urbach et al. 1998, Crosbie et al. 2003, Ernst et al. 2003).

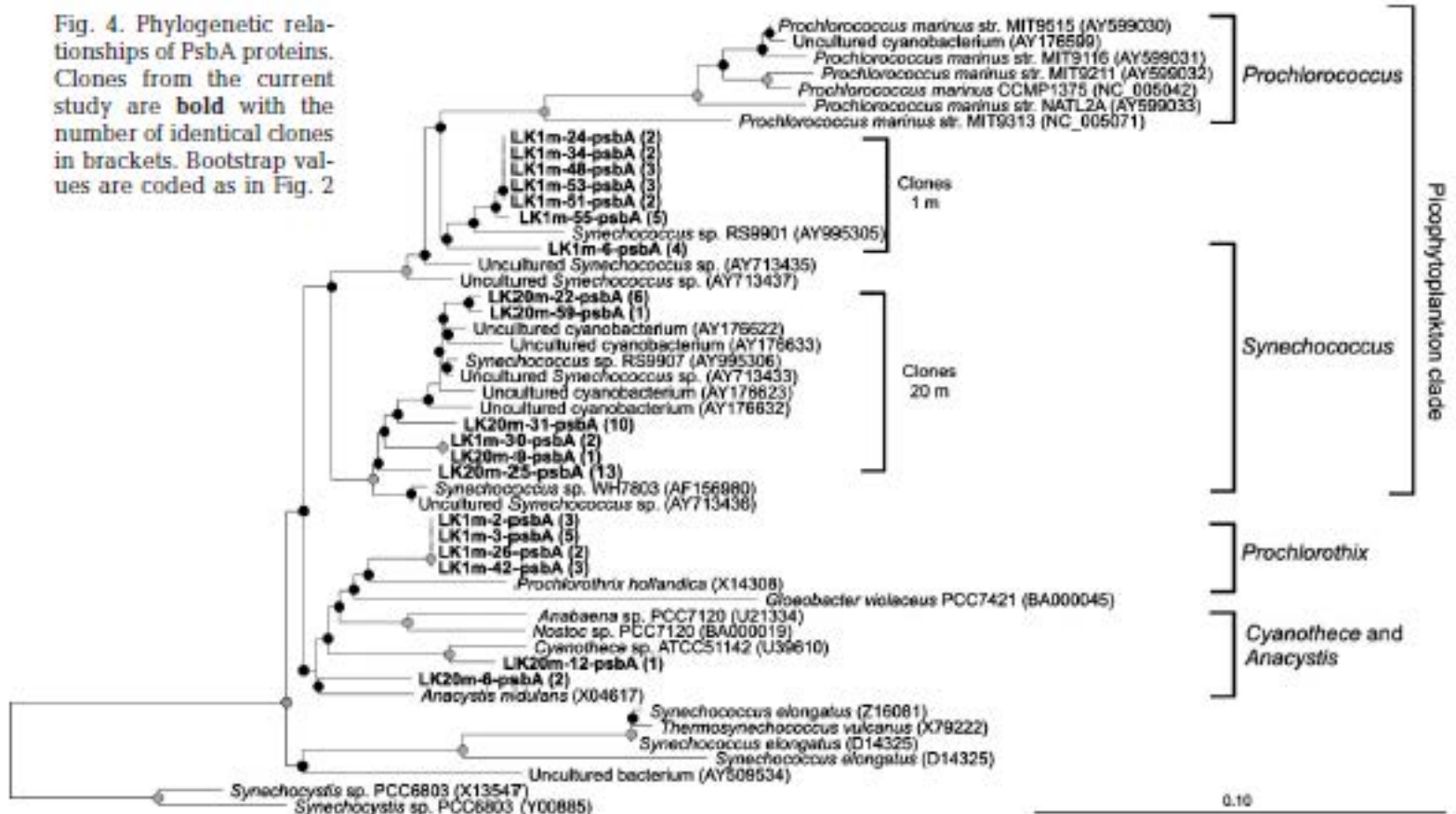
Community composition analyzed by *psbA*

Using the sequences of the gene coding for protein D1 of the photosystem II reaction centre (*psbA*) available from *Synechococcus* and *Prochlorococcus*, we designed new PCR primers suitable for the amplifica-

tion and cloning of this gene. These primers were evaluated by PCR with genomic DNA from *P. marinus* SS120, *P. marinus* MED4, *Synechococcus* sp. strains WH7803, WH8102, CN, and P41, *Synechocystis* sp. PCC6803, and the environmental samples from Lake Kinneret (Fig. 3). In all the cases a PCR product of ca. 900 bp was obtained, coinciding with the expected size. The evaluation of the primers with PROBE MATCH and BLAST showed that both primers matched at up to 3 mismatches with all the sequences included in our database, suggesting that the primers could have a broad amplification spectrum. This was evaluated by using a collection of strains from Lake Kinneret and other cyanobacterial species. A PCR product of the expected size was obtained with all the strains except for *Microcystis* sp. brown strain, *Aphanizomenon* sp., *Chroococcus* sp., and *Schizothrix* sp. (data not shown), which were likely to be neglected in the environmental samples.

The PCR products from the samples taken from 1 and 20.7 m were cloned and sequenced. The majority of the *psbA* clones (54 out of 71) clustered together with sequences from marine *Synechococcus* and *Prochlorococcus* (Fig. 4) and split into 2 groups: (1) sequences from 1 m and (2) from 20.7 m. Clonal sequences from 1 m and *Synechococcus* sp. RS9907 formed a separate cluster between *Prochlorococcus* and *Synechococcus* spp., supported by a high bootstrap value. Most sequences from 20.7 m (31 out of 34)

Fig. 4. Phylogenetic relationships of *PsbA* proteins. Clones from the current study are bold with the number of identical clones in brackets. Bootstrap values are coded as in Fig. 2



and 3 clones from 1 m clustered with marine isolates of *Synechococcus* and uncultured cyanobacteria from marine habitats.

Thirteen clones from 1 m formed a separate cluster distantly related to *Prochlorothrix hollandica*, a filamentous cyanobacterium from freshwater. Three sequences from 20 m grouped within the freshwater cyanobacteria but without close association to any of the groups.

Community composition analyzed by *ntcA*

As the sequence conservation of *ntcA* in different cyanobacteria is low, only sequences from the genome of *Prochlorococcus* were used to design PCR primers to amplify the *ntcA* gene. When using these primers with genomic DNA from *P. marinus* SS120, *P. marinus* MED4, *Synechococcus* sp. strains WH7803, WH8102, CN and P41, and the samples from Lake Kinneret, PCR products of different size (between 500 and 300 bp) were obtained (Fig. 5). The PCR product from both strains of *Prochlorococcus* was ca. 500 bp, and from *Synechococcus* WH7803 and WH8102 ca. 300 bp. Only one of the *Synechococcus* strains isolated from the lake (CN) gave a product >300 bp with the *ntcA* primers. More than 1 band was detected with the 2 samples from the lake. The evaluation of the primers with PROBE MATCH and BLAST showed that they only matched sequences from marine *Prochlorococcus* and *Synechococcus*. This was confirmed by the experimental evaluation in which PCR products of the expected size were obtained from the *Prochlorococcus* spp. only. However, unspecific amplifications were observed with *Microcystis* sp. brown strain, *Schizothrix* sp., *Synecho-*

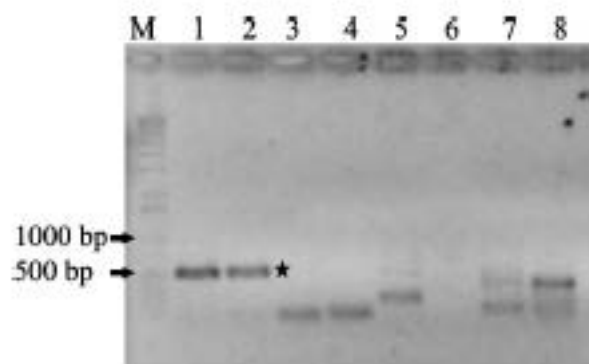


Fig. 5. Agarose gel showing the *ntcA* PCR products from (1) *Prochlorococcus marinus* SS120, (2) *P. marinus* MED4, (3) *Synechococcus* WH7803, (4) *Synechococcus* WH8102, (5) *Synechococcus* sp. strain CN, (6) *Synechococcus* sp. strain P41, (7) Lake Kinneret 1 m, (8) and Lake Kinneret 20.7 m. On the left side of the gel the bands corresponding to 1000 and 500 bp in the 1 kb ladder (M) are marked by arrows. The bands with the expected size of ca. 500 bp obtained from both strains of *Prochlorococcus* are marked by a star

cystis sp., *Nostoc* sp. PCC 7120, *Thermosynechococcus elongatus* and *Gloeobacter* sp. (data not shown). Therefore, only the PCR products of ca. 500 bp from *P. marinus* MED4 and from samples of the lake (Fig. 5) were cloned and sequenced.

The *ntcA* sequence from *Prochlorococcus marinus* MED4 was identical with the one already deposited in GenBank (Acc. No AY122321). The cloning of the sample from 20.7 m was very inefficient. In the phylogenetic tree (Fig. 6), all 28 *ntcA* sequences from 1 m formed an independent cluster supported by high bootstrap values. This cluster with only clonal sequences from lake Kinneret was separated from cultures that had been isolated from marine or freshwater ecosystems and from environmental sequences from the Red Sea (Penno et al. 2006).

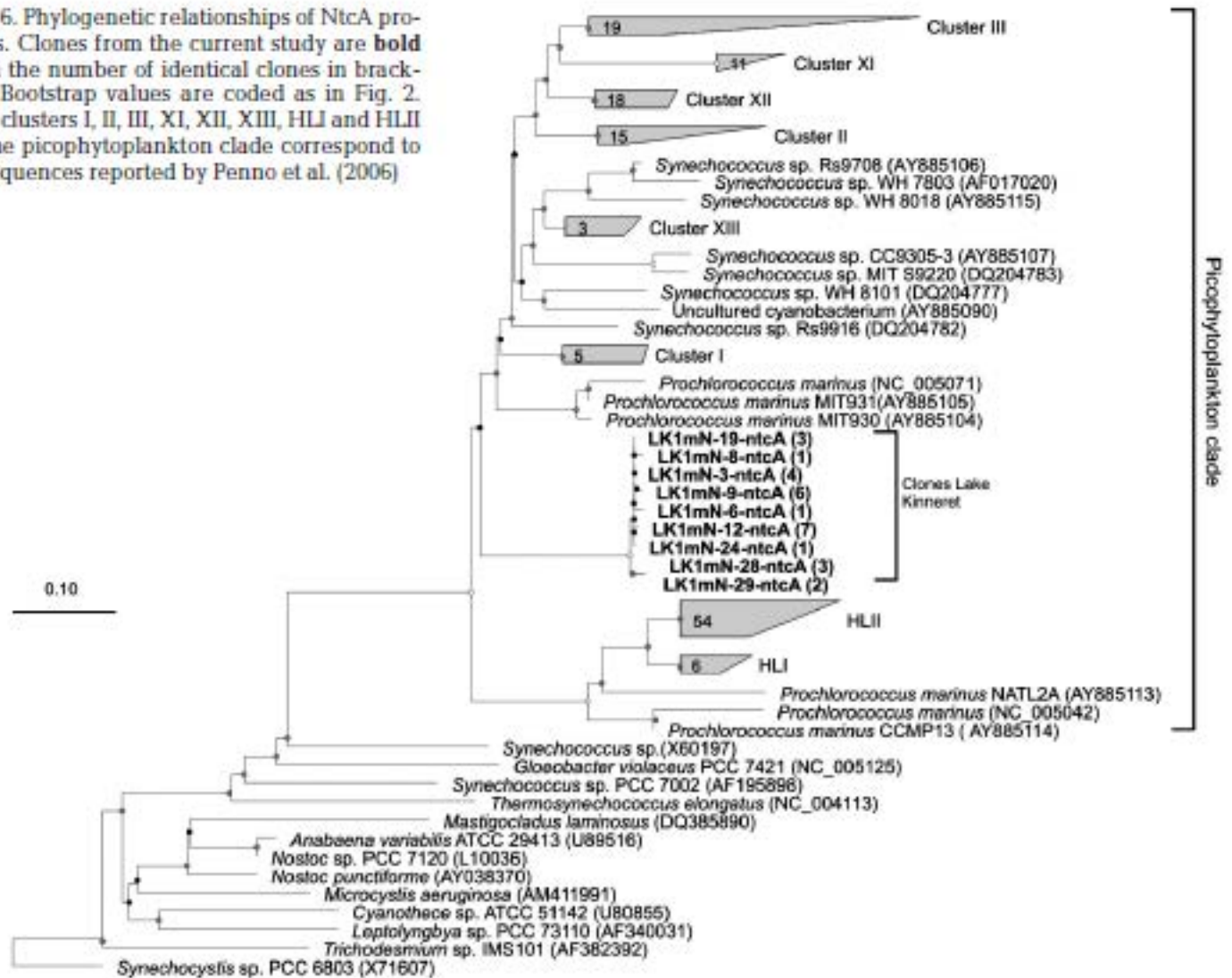
DISCUSSION

The long-term records of phytoplankton composition in Lake Kinneret offer a unique opportunity for monitoring ecosystem changes induced by human-driven perturbations and management. Recent revision of the historical records suggested evidences of ecosystem deterioration (Zohary 2004). One of the groups that has changed significantly during the last 12 years is cyanobacteria, including the toxin-producing and nitrogen-fixing species.

The clonal sequences obtained from 2 different depths in Lake Kinneret account for most of the groups reported in the regular microscopic examinations of water samples from the lake (Zohary 2004). Clone libraries in the epi- and hypolimnion of the lake were dominated by clones related to the Chroococcales, which are reported to be one of the dominant species at the epilimnion during stable summer stratification due to their capability to grow at higher temperatures and low nutrients (Zohary 2004). Within this group, clones distantly related to *Gloeocapsa* were the most frequent (21) in the libraries and might represent a new yet uncultured group. *Gloeocapsa* spp. have been reported in some extreme environments (Fourcans et al. 2004, Vincent et al. 2004); however, little information is known on the ecological role and the worldwide distribution of this genus. In Lake Kinneret, *Gloeocapsa* spp. have been observed in the microscopic analysis in the benthos but not as part of planktonic communities (Zohary pers. comm.). Therefore, the identity and the role of these abundant cyanobacteria that are genetically related to *Gloeocapsa* remains to be addressed.

Picocyanobacteria of the *Synechococcus* type have been recorded in Lake Kinneret in a long-term study (Malinsky-Rushansky et al. 1995). *Synechococcus* is a

Fig. 6. Phylogenetic relationships of NtcA proteins. Clones from the current study are **bold** with the number of identical clones in brackets. Bootstrap values are coded as in Fig. 2. The clusters I, II, III, XI, XII, XIII, HLI and HLII in the picophytoplankton clade correspond to sequences reported by Penno et al. (2006)



genus defined principally on the basis of morphological traits, including both marine and freshwater strains, and it is clearly polyphyletic. Based on 16S rRNA gene sequences, isolates from this genus have been affiliated with several independent clusters of cyanobacteria (Honda et al. 1999, Robertson et al. 2001). One of these clusters corresponds to the picophytoplankton clade sensu Urbach et al. (1998), which includes marine *Synechococcus* spp., all species from *Prochlorococcus* and 2 *Microcystis* strains. Two cultures isolated from the lake (Malinsky-Rushansky et al. 1995) were included in this cluster together with 9 clones from the 16S rRNA gene library.

Among the freshwater species included in the picophytoplankton clade, several subclusters have been defined based on sequence analyses of 16S rRNA gene, the intergenic spacer between 16S and 23S rDNA (ITS1), and part of the phycocyanin operon (*cpcBA*), from cultures isolated from different lakes in Japan (Robertson et al. 2001) and Europe (Crosbie et al. 2003, Ernst et al. 2003). The *Synechococcus* strains isolated from Lake Kinneret and the clones from the libraries analyzed in the current study were related

to strains from Lake Biwa (Japan). The separation of these subclusters, containing so far only strains from the non-European lakes Biwa and Kinneret, would support the hypothesis of worldwide distribution of picocyanobacteria belonging to the picophytoplankton clade (Crosbie et al. 2003), with the common speciation of specific groups in similar environments.

The gene coding for protein D1 of the photosystem II reaction centre (*psbA*) has been used to study picophytoplankton diversity in marine environments (Zeidner et al. 2003). The topology observed for the cyanobacterial *PsbA* tree (Fig. 4) of our study was similar to the one previously reported for marine environments (Zeidner et al. 2003). Although the results from 16S rRNA gene and *psbA* clone libraries might not be comparable because of the differences in the primer specificity, some links between both markers can be found. For example, the majority of the *psbA* sequences from Lake Kinneret were grouped together with sequences from cultures of marine *Synechococcus* sp. (strain RS9901 and RS9907), which are placed into the picophytoplankton clade (Fuller et al. 2003). For those *psbA* sequences that are more similar to freshwater

cyanobacteria, this link is less clear because this molecular marker has, up to now, not been used to analyze cyanobacterial communities in freshwater.

In *Prochlorococcus* the development of specific ecotypes under conditions of high- (HL) or low-light intensity (LL) has been studied. These ecotypes are differentially distributed within the stratified oceanic water column (Moore et al. 1998, Rocap et al. 2003). The clonal sequences of *psbA* from 1 and 20.7 m in Lake Kinneret were separated, suggesting a possible separation of ecotypes of *Synechococcus* in the lake, a phenomenon that must be further explored. The separation of ecotypes adapted to different niches has been also observed in *Prochlorococcus* (Johnson et al. 2006, Zinser et al. 2006); however, in the case of *Prochlorococcus* this observation is based on 16S rRNA gene sequences. Therefore, it is surprising that the separation of 2 types of clonal sequences observed here by *psbA* is not observed in the 16S rRNA gene libraries. One possible reason to explain this difference is that the 16S rRNA gene approach does not amplify all the genotypes that were observed when applying the *psbA* marker.

The transcriptional activator NtcA, which is involved in the regulation of the N-response, has been found in all cyanobacteria studied so far (Herrero et al. 2001). Some attempts have been made to use the gene of this protein as a molecular marker to study cyanobacterial communities in the environment (Penno et al. 2006). Although the NtcA protein has high identity in different species, 2 groups of protein sequences have been described (Herrero et al. 2001). One group includes the unicellular marine organisms *Synechococcus* and *Prochlorococcus* spp., and the other includes heterocyst formers and unicellular freshwater cyanobacteria. In the phylogenetic reconstruction (Fig. 6) these 2 groups separated, but the clonal sequences from Lake Kinneret formed an additional independent group related to clonal sequences obtained from the Red Sea (Penno et al. 2006). Since the *ntcA* sequences from this study are the only environmental sequences from freshwater environments available, it cannot be determined if they represent the same population of cyanobacteria that has been detected by 16S rRNA gene and *psbA* analyses. More information, especially from environmental studies, is required to clarify this point.

When comparing results from independent genes, it should be noted that the different primer sets used in the current study possessed different specificities that were obviously reflected in the topology of the phylogenies from the different genes and the number of clusters detected by each of the approaches (lower diversity of *psbA* and *ntcA* compared to 16S rRNA gene). Additionally, the *psbA* and *ntcA* sequences in the current study are the first of these functional

markers reported from freshwater environments. Therefore, further studies with environmental samples are needed to better define the *psbA* and *ntcA* clusters observed. Attention should also be paid to the effect of horizontal gene transfer (HGT) of photosynthesis genes, including *psbA* by cyanophages, infecting *Synechococcus* and *Prochlorococcus* (Sullivan et al. 2003, Lindell et al. 2004, Zeidner et al. 2005) over the phylogeny of this gene in relation to other markers analyzed.

The similarity of some of the 16S rRNA gene, *psbA* and *ntcA* sequences from Lake Kinneret to those from marine environments raises the question of the origin of these populations. Lake Kinneret represents a unique case of limnological and geochemical evolution (Hazan et al. 2005); during the Miocene, a seawater arm covered the subsiding rift valley and formed inland water bodies with high salinity. The formation of Lake Kinneret involved a rapid transition from a saline to a freshwater lake (Hurwitz et al. 2000), and some of the cyanobacterial populations from Lake Kinneret could be remnants from this ancient time. However, in order to verify this hypothesis, it is essential to have additional information from other freshwater lakes with a long continental geological history, in which, according to this hypothesis, the cyanobacterial community must be clearly differentiated from marine genotypes.

Acknowledgements. This research was supported by G.I.F. (German-Israeli Foundation) Grant No. I-711-83.8/2001 and BSF (Binational Science Foundation) Grant No. 2002-206. Samples were taken during the German Israeli Minerva School in October 2004. We thank the Yigal Allon Kinneret Limnological Laboratory personnel for their assistance during the sampling, Dr. W. Hess for providing genomic DNA from picocyanobacterial cultures, and Dr. Hess, Dr. A. Post, Dr. T. Zohary and Dr. S. Becker for their valuable comments on earlier versions of this study.

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