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Morphological and molecular analysis of bloom-forming Cyanobacteria in two eutrophic, shallow Mediterranean lakes

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ABSTRACT

We investigated the diversity of Cyanobacteria by microscopic observation and sequencing of cyanobacterial-specific amplified 16S rRNA genes in the water column of two shallow, eutrophic lakes (Doirani and Kastoria, northern Greece) during summer blooms. Previous phytoplankton studies in these lakes have shown that prolonged cyanobacterial blooms can occur, which are dominated by known toxic species, as well as other less known, co-occurring species. A total of 118 clones were sequenced which were grouped in 23 Cyanobacteria and 11 chloroplast-like phylotypes. Phylogenetic analysis revealed that each library included several unique phylotypes, as well as members of all common bloom-forming Cyanobacteria. Most of the phylotypes belonged to the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis-Raphidiopsis* group, *Limnothrix* and *Planktothrix*, comprising most of the diversity previously recognized by morphological observations in cyanobacterial morphospecies in these lakes. In addition, novel phylotypes belonging to the Chroococcales were recognized in both lakes. The structure of the cyanobacterial communities of the lakes were very similar, as revealed by the diversity index *H* (2.06 and 2.01 for *L. Doirani* and Kastoria, respectively) and LIBSHUFF analysis (XY_{12} *P*-value = 0.122 and YX_{12} *P*-value = 0.536), due to occurrence of groups of common phylotypes. This study gives an example for successful cyanobacterial bloom analysis by the combination of morphological and phylogenetic methods useful for monitoring cyanobacteria and water quality in freshwaters.

Introduction

Cyanobacteria are the dominant phototrophic component in many freshwater environments where they may form nuisance blooms (Chorus and Bartram 1999). The warm Mediterranean climate favours the extended duration of the cyanobacterial blooms in eutrophic freshwaters, which may start in spring and persist until December, or in hypertrophic lakes, may even be continuous throughout the year (Moustaka-Gouni et al. 2007). In Greece, common bloom-forming Cyanobacteria are mainly assigned to the genera *Microcystis* and *Anabaena*, followed by *Cylindrospermopsis* and *Aphanizomenon* (Vardaka et al. 2005) and *Limnothrix* (Moustaka-Gouni et al. 2007). In addition to the bloom-forming taxa, a wide range of less-known taxa are in some cases significant to the total cyanobacterial biomass. These "accompanying" species include filamentous (e.g. *Jaaginema*) and colonial (e.g. *Merismopedia*, *Snowella*) nanoplanktic Cyanobacteria (Vardaka et al. 2005), as well as, picocyanobacteria (e.g. *Synechococcus*) (Moustaka-Gouni et al. 2006). In lakes of Greece, such multi-species water blooms have been described (Vardaka et al. 2005).

The characterization of the bloom communities structure remains problematic because the cyanobacterial taxonomy of certain genera has not yet been resolved (Moustaka-Gouni et al. 2009). Although Cyanobacteria show higher morphological diversity than other bacterial groups, the discrimination at the genus or species level is often problematic. Indeed, morphological and morphometric features of many Cyanobacteria strains are influenced by the growth conditions, greatly complicating their identification (Anagnostidis and Komárek 1985, 1988). Cyanobacteria with the same genetic makeup may appear quite different under various physiological conditions as a result of differential gene expression (Castenholz and Norris 2005). Furthermore, some criteria such as the shape and structure of colonies (Rajaniemi-Wacklin et al. 2006) as well as the presence/absence of gas vesicles (Gkelis et al. 2005) can be altered in culture. Such limitations of phenotypic features have highlighted the requirement of more reliable methods and promoted the use of polyphasic approaches by combining morphological and molecular data (e.g. Gkelis et al. 2005;

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Table 1
Location and dates of the lakes sampled and summary of the clone libraries constructed from the water samples.

Lake (code)	Geographic coordinates		Sampling date	PCR cycles	Number of	
	(N)	(E)			Clones	Phylotypes
Doirani (ND2)	40°11′	22°45′	12 August 2004	24	69	29
Kastoria (NK2)	40°31′	21°18′	13 August 2003	8	49	18

Rajaniemi-Wacklin et al. 2006). Since the isolation of Cyanobacteria from environmental samples is not always successful, culture-independent identification of cyanobacteria is more promising in describing community structure. Molecular technologies based on 16S rRNA gene amplification are already widely employed for the analysis of natural samples (Eiler and Bertilsson 2004; Zwart et al. 2005; Junier et al. 2007).

The taxonomy of some of the potentially toxic cyanobacteria remains challenging (Gkelis et al. 2005) especially due to the co-occurrence of several different morphotypes (Moustaka-Gouni et al. 2009). The aim of the study was to monitor the occurring Cyanobacteria in two eutrophic, shallow Mediterranean lakes which have high cyanobacterial diversity in blooms (Vardaka et al. 2005), by comparing the diversity by morphological observation and phylogenetic analysis after PCR amplification of the 16S rRNA gene with cyanobacterial-specific primers.

Materials and methods

Samples were collected from Lakes Kastoria (summer bloom of 2003) and Doirani (summer bloom of 2004) (Table 1), located in northern Greece. For a detailed description of these lakes see Vardaka et al. (2005). Water samples were collected from the surface layer (0–1 m). Sub-samples were preserved with both Lugol's solution and formaldehyde. Water samples for 16S rRNA gene analysis were stored in polyethylene bottles and transferred to the laboratory (<5 h) under cool and dark conditions. Immediately upon return to the laboratory, 100–300 mL of lake water was filtered on a Whatman GF/C filter and the filter was stored at −20 °C. Although we initially planned to use polycarbonate 0.2 μm isopore filters, their use was not feasible due to extreme clogging and breakage of filamentous cyanobacteria after the first 10–20 mL of water.

Fresh and preserved samples were examined using an inverted microscope (Nikon ECLIPSE TE2000-S) with phase-contrast. Species were identified using Komárek and Anagnostidis (1999, 2005), Hindák and Moustaka (1988) and the classification system of Komárek and Anagnostidis (1989).

DNA was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's protocol after slicing the filters with a sterile scalpel. For 16S rRNA gene amplification, we used 0.5 μL of the DNA template and the cyano-specific primers CYA106f (5′-CGGACGGGTGAGTAACGCGTGA-3′) and an equimolar mixture of CYA781r (a) (5′-GACTACTGGGGTATCTAATCCCATT-3′) and CYA781r (b) (5′-GACTACAGGGGTATCTAATCCCTTT-3′) (Nübel et al. 1997). All amplification reactions (25 μL) contained 10–20 ng genomic DNA, determined with the NanoDrop ND-1000 (NanoDrop Technologies, USA), as template, each primer at a concentration of 0.5 μM and PCR buffer supplied with the Go-Taq polymerase (Promega, USA), dNTPs (200 μM) and $MgCl_2$ (1.5 μM). PCR conditions were similar to those described by Berger et al. (2006) for Cyanobacteria.

In order to decrease PCR bias related to high number of cycles and minimize the differences in the clone library representation between rare and abundant phylotypes, PCR cycle optimization was performed, i.e. each PCR was performed at the minimum number of cycles where a positive PCR signal occurred (Table 1). The amplicons were separated in a 1.2% (w/v) agarose gel electrophoresis run at 70 V for 45 min in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8) and visualized by UV transillumination after staining with ethidium bromide (0.5 μg mL^{-1}).

The PCR products were purified using the Montage purification kit (Millipore, USA) and the purified PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen, USA) with chemically competent cells according to the manufacturer's specifications. A maximum of 90 clones were randomly selected and checked for having the correct insert size (ca. 680 bp). All positive clones were grown in liquid LB medium with 50 μg mL^{-1} kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel, Germany). Sequence data were obtained by capillary electrophoresis (Macrogen Inc., Seoul, Korea) using the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primer M13F (5′-GTAAAACGACGGCCAG-3′). Each sequence read was approximately 850 bp. Each sequence was checked for chimeras using the CHIMERA-CHECK function of the Ribosomal Database Project II (Maidak et al. 2001). All sequences were compared with the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/) for the detection of closest relatives. Sequence data were compiled using the MEGA4 software (Tamura et al. 2007) and aligned with sequences obtained from the GenBank (www.ncbi.nlm.nih.gov) databases, using the ClustalX aligning utility. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in MEGA4 (Tamura et al. 2007). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per data set, each followed by tree bisection-reconnection topological rearrangements. The topology of the tree was based on neighbour-joining according to Jukes-Cantor. Bootstrapping under parsimony criteria was performed with 1000 replicates. Sequences of the unique phylotypes found in this study have GenBank accession numbers FJ204870–FJ204887 (L. Kastoria) and FJ204841–FJ204869 (L. Doirani).

Library clone coverage was estimated according to Kemp and Aller (2004). The Shannon-Wiener (H) and Shannon evenness (J) indices (Shannon and Weaver 1949; Pielou 1969) were estimated to take both abundance and richness of phylotypes into account (Hill et al. 2003). H was calculated as $H = -\sum p_i \log_e$ where p_i is the proportion of clones in the ith OTU (estimated using n_i/N) and $J = H/\ln S$, where S is the number of OTUs found in a sample. Only cyanobacterial and chloroplast-related clones were included in the H and J calculations. Statistical significance of differences in the composition of the two libraries was tested using the LIBSHUFF program (http://www.arches.uga.edu/~whitman/libshuff/html) (Singleton et al. 2001).

Results

A total of 15 cyanobacterial taxa were microscopically identified, of which six taxa belonged to the Chroococcales, five to the Oscillatoriales and four to the Nostocales (Table 2). Nostocales of the *Cylindrospermopsis*/*Raphidiopsis* group were the dominant organisms in both lakes (Moustaka-Gouni et al. 2009, 2010). The bloom-forming *Microcystis aeruginosa* was also abundant in Lake Kastoria. The filamentous *Limnothrix redekei*, *Planktothrix* cf. *agard-*

Table 2
Cyanobacterial taxa, their percent contribution to the total phytoplankton biomass and the percent contribution of eukaryotic phytoplankton to the total phytoplankton biomass identified in the water blooms of Lakes Doirani and Kastoria. Minus (−) indicates absence of taxon in the water sample, plus (+) indicates biomass <0.5% of the total phytoplankton biomass.

Organism	Biomass in lake (% of total)	
	Doirani	Kastoria
Cyanobacteria		
Aphanocapsa incerta	+	−
Chroococcus limneticus	−	+
Microcystis aeruginosa	+	22.7
Microcystis flos-aquae	+	−
Snowella lacustris	3.9	−
Synechococcus spp.	+	−
Limnothrix redekei	−	9.5
Jaaginema sp.	2.6	−
Oscillatoria sp.	−	+
Planktolyngbya circumcreta	+	−
Planktothrix cf. *agardhii*	6.4	−
Anabaena aphanizomenoides	−	+
Anabaena flos-aquae	3.0	−
Aphanizomenon issatshenkoi	8.1	−
Cylindrospermopsis raciborskii	10.2	62.8
Eukaryotic phytoplankton	65.7	4.9

hii, *Aphanizomenon issatschenkoi* and *Anabaena flos-aquae* were also identified.

According to the Good's C estimator for the libraries clone coverage, an asymptotic curve close to or above 0.80 was reached for both lakes, while the ration of observed to predicted number of phylotypes was 56.7% and 73.7% for L. Doirani and Kastoria, respectively (Fig. 1). In total, 118 clones of 16S rDNAs were analysed which where attributed to 23 Cyanobacteria, 11 chloroplast-related and 13 other Bacteria phylotypes (Table S1, Figs. 2 and 3).

Chroococcales was the most diverse (13/23 phylotypes, 34/118 clones) group within the Cyanobacteria. Several phylotypes were

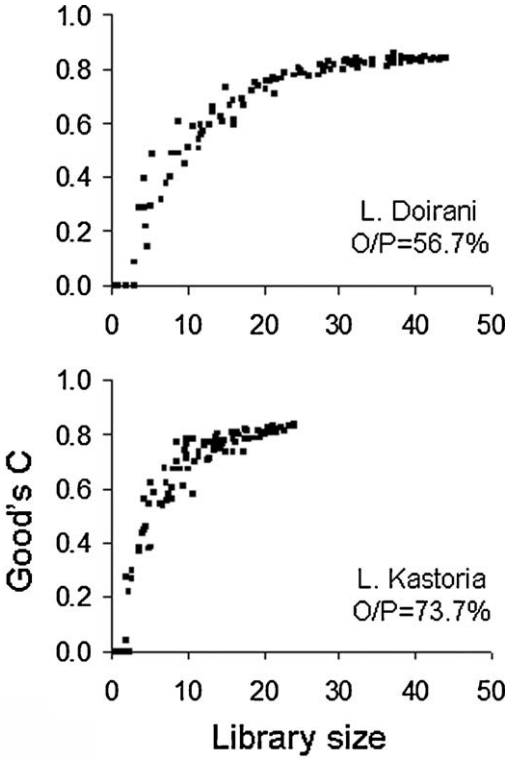

Fig. 1. Clone library coverage based on Good's C estimator and the ratio of observed (O) to the S_{Chao1} predicted (P) number of phylotypes from the water column of Lakes Doirani and Kastoria, Greece.

practically identical or very closely related to known species of the Chroococcales (Fig. 2 and Table S1). Phylotypes ND2-CYA-1-30, ND2-CYA-1-18, NK2-CYA-1-5 were very closely related to the same *Microcystis aeruginosa* strain. Phylotypes ND2-CYA-1-9 and ND2-CYA-1-11 were closely related to *Snowella litoralis* and *Pleurocapsa* sp. respectively. Phylotypes ND2-CYA-1-27 and ND2-CYA-4-1 were affiliated to a *Cyanobium/Synechococcus* monophyletic clade. Phylotypes ND2-CYA-4-34 and ND2-CYA-4-86 were grouped with *Chroococcus* and *Gleocapsa* strains. Finally, phylotypes NK2-CYA-1-14, NK2-CYA-3-3ame, ND2-CYA-3-15 and NK2-CYA-1-3-ame from both lakes, were not affiliated to any known species of the Chroococcales.

Nostocales (6/23 phylotypes, 22/118 clones) were also present in both lakes (Fig. 2 and Table S1). The L. Doirani phylotypes ND2-CYA-1-24, ND2-CYA-1-12 and ND2-CYA-1-3 had >98% similarity to *Anabaena smithii*, *Aphanizomenon flos-aquae* and *Aphanizomenon issatschenkoi*, respectively. Phylotypes ND2-CYA-4-32 and NK2-CYA-1-7 were clustered tightly together within the *Cylindrospermopsis raciborskii*/*Raphidiopsis mediterranea* clade. Phylotype NK2-CYA-1-18 was not closely related to any known taxa of the Nostocales.

Oscillatoriales phylotypes (4/23 phylotypes, 12/118 clones) were also found in both lakes (Fig. 2 and Table S1). Phylotypes NK2-CYA-1-13 and ND2-CYA-1-21 were >98% similar to *Limnothrix* sp. and *Planktothrix pseudagardhii*, respectively. Phylotype ND2-CYA-1-8 was grouped with *Cyanobacterium* and *Pseudanabaena* strains and NK2-CYA-2-14 was related to a *Phormidium* strain.

A distinct clade in the Cyanobacteria was formed including the chloroplast-related phylotypes, which occurred in both lakes (11/47 phylotypes, 29/118 clones) (Fig. 2 and Table S1). Phylotypes NK2-CYA-3-4, NK2-CYA-2-1 and ND2-CYA-4-27 were closely affiliated to plastids of *Cryptomonas curvata* and *Nitzschia frustulum*, respectively. The Lake Doirani phylotypes ND2-CYA-4-29-ame, ND2-CYA-1-22 and ND2-CYA-3-10 formed a distinct clade closely related to the *Aulacoseira ambigua* plastid but also to phylotypes retrieved for other lake ecosystems. A very distinct but well supported monophyletic group was revealed, containing phylotypes NK2-CYA-1-1, NK2-CYA-3-5 and ND2-CYA-1-7, related only with one phylotype from Lake Kinneret, Israel. Finally, ND2-CYA-4-83 was closely related to lake organic aggregates and ND2-CYA-3-11 was not related to any known chloroplast or phylotype from lake habitats.

The other Bacteria were dominated by uncultured Verrucomicrobia (10/13 phylotypes, 18/118 clones) (Fig. 3 and Table S1). In most cases, their closest relatives originated from other lakes during water blooms or when eutrophic conditions prevailed. The last three non-cyanobacterial singleton phylotypes were retrieved only from L. Kastoria and belonged to the Actinobacteria, the γ-Proteobacteria and the candidate division OP11 and were not related to lake habitats (Fig. 3). These microorganisms are not further discussed in this paper since they are not targeted with the primers used and their occurrence is probably underestimated due to the GF/C filtered used.

The H diversity index was similar to both lakes, 2.06 and 2.01 for L. Doirani and L. Kastoria, respectively. Evenness was higher in L. Kastoria ($J = 0.84$) than in L. Doirani ($J = 0.66$). The LIBSHUFF analysis for the significance of the difference between the two clone libraries indicated that they were not significantly different (XY_{12} P-value = 0.122 and YX_{12} P-value = 0.536). For both the H index and LIBSHUFF analysis, only the Cyanobacteria phylotypes were considered.

Discussion

We compared the occurrence of Cyanobacteria during the summer water blooms of two shallow, eutrophic lakes (Lakes Doirani

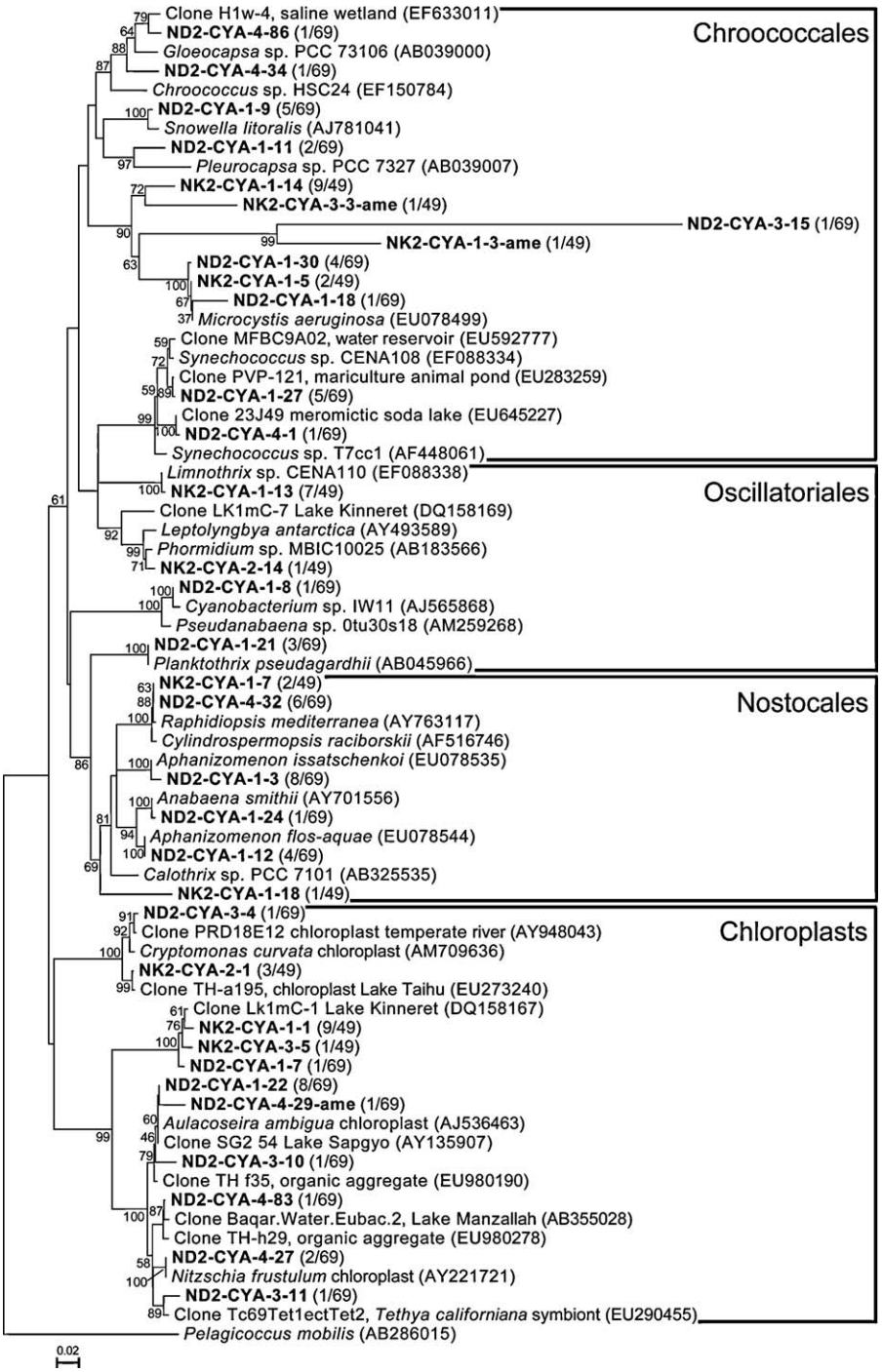

Fig. 2. Neighbour-joining tree showing phylogenetic relationships of cyanobacterial and chloroplast-like partial 16S rRNA genes from environmental sequences obtained from Lakes Doirani (ND2-CYA) and Kastoria (NK2-CYA), Greece. Numbers at nodes represent the bootstrap percentages from 1000 replicates. Values below 50% are not shown. Number of identical clones obtained for each phylotype and accession numbers for 16sRNA sequences obtained from databases are shown in parentheses. Bar indicates the number of substitutions per site. *Pelagicoccus maritima* was used as an outgroup.

and Kastoria, northern Greece), by standard morphological identification (light microscopy) and diversity through the amplification of the 16S rRNA genes with cyanobacterial specific primers. The water blooms of these systems are characterized by the co-occurrence of different morphospecies (Vardaka et al. 2005), several of them not firmly recognized by microscopic observation (Moustaka-Gouni et al. 2009).

In both lakes, there was a good agreement between the two approaches (Table 2 and Fig. 2). The found organisms have been reported in previous phytoplankton studies in L. Doirani and Kastoria (Temponeras et al. 2000; Vardaka et al. 2005). Members of the most abundant genera identified by microscopic observations, i.e. *Cylindrospermopsis*, *Microcystis*, *Aphanizomenon*, *Anabaena*, *Snowella*, *Limnothrix*, *Planktothrix* and *Synechococcus*, were also found in the clone libraries in high relative abundances. This is attributed to the satisfactory clone coverage for both samples, as revealed by the curvilinear patterns of the Good's C estimator, the high ratios of observed to predicted number of phylotypes (Fig. 1) and also the fact that both libraries contained high numbers of singleton phylotypes. This indicates that at least the

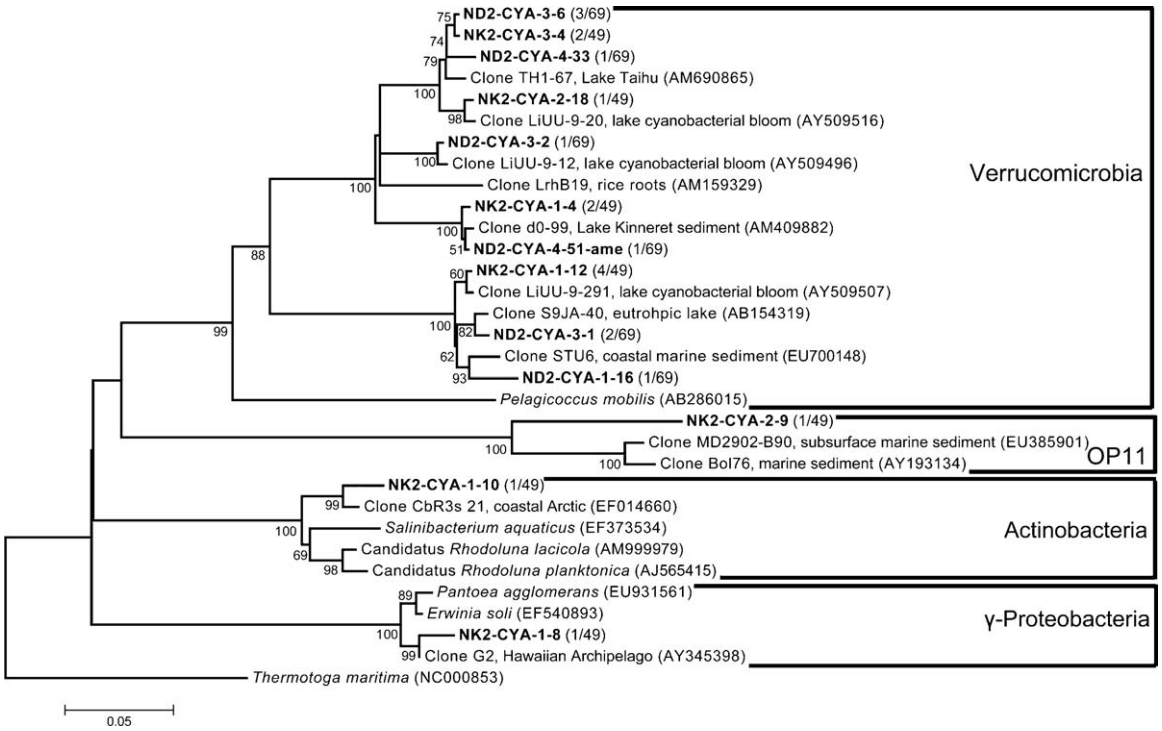

Fig. 3. Neighbour-joining tree showing phylogenetic relationships of non-cyanobacterial partial 16S rRNA genes from environmental sequences obtained from Lakes Doirani (ND2-CYA) and Kastoria (NK2-CYA), Greece. Numbers at nodes represent the bootstrap percentages from 1000 replicates. Values below 50% are not shown. Number of identical clones obtained for each phylotype and accession numbers for 16sRNA sequences obtained from databases are shown in parentheses. Bar indicates the number of substitutions per site. *Thermotoga maritima* was used as an outgroup.

most abundant Cyanobacteria have been revealed. In addition, the satisfactory clone coverage allowed the use of the *H* index for our samples (Hill et al. 2003). However, the clone libraries revealed new phylotypes, representing species that cannot be recognized morphologically.

Chroococcales was the most diverse cyanobacterial group, dominated by clones related to cosmopolitan Cyanobacteria well known to form and dominate lake water blooms worldwide, such as *Microcystis aeruginosa* (Sivonen and Jones 1999), and other non-bloom forming coccal species commonly found in eutrophic temperate waters, such as *Snowella* spp. (Rajaniemi-Wacklin et al. 2006). The Chroococcales do not form a phylogenetically coherent clade. In addition, apart from the genera *Microcystis* and *Cyanobium*, the vast majority of the available sequences suggests that it is a polyphyletic group (Castenholtz 2001).

Synechococcus-like picocyanobacteria have been recorded in Lake Kastoria (Moustaka-Gouni et al. 2006). *Synechococcus* is a genus defined principally on the basis of morphological traits, including both marine and freshwater strains, and it is clearly polyphyletic (Wilmotte and Herdman 2001). Among the freshwater species included in the picophytoplankton clade sensu Urbach et al. (1998), several sub-clusters have been defined based on sequence analyses of 16S rRNA gene, from cultures isolated from different lakes in Europe (Crosbie et al. 2003; Sanchez-Baracaldo et al. 2008).

The *Microcystis*-like phylotypes of this study fell in two subclades, one grouped with a *M. aeruginosa* strain isolated from Portugal (Valério et al. 2009) and one separate sub-clade not containing any known cultured strain. The high diversity of *Microcystis* species complex is known at the morphological (Komárek and Anagnostidis 1999), genetic (Wilson et al. 2005; Yoshida et al. 2008) and physiological (i.e. microcystin producing) level (Lyra et al. 2001), within and across habitats.

Sequences of algal plastids phylogenetically belong to the Chroococcales since they are considered to be of cyanobacterial origin (Sagan 1967) and, thus, it is possible that they represent true cyanobacterial phylotypes. However, species of the genera *Cryptomonas*, *Aulacoseira* and *Nitzschia* were present in both lakes during our study period (M. Moustaka-Gouni, unpublished data), suggesting that the three clades containing phylotypes related to these algae represent their chloroplasts. The rest of the chloroplast-like phylotypes which were not related to any known plastid sequences could be the plastids of other algae we observed in the lakes, such as the chlorophytes *Oocystis*, *Crucigenia*, the diatoms *Rhizosolenia*, *Acanthoceras* and the cryptyophyte *Rhodomonas minuta*.

Within the Oscillatoriales, the L. Kastoria phylotype NK2-CYA-1-13 shared 99% 16S rDNA gene similarity with *Limnothrix* sp. CENA 110, isolated from Brazil (Furtado et al. 2009) and with the *Limnothrix redekei* strains previously isolated from L. Kastoria (Gkelis et al. 2005). Phylotype ND2-CYA-1-8 clustered tightly together with the strain IW11 isolated from Lake Loosdrecht, The Netherlands, of the *Limnothrix/Pseudanabaena* cluster (Zwart et al. 2005). The IW11 strain, morphologically very similar to *L. redekei*, was the most divergent isolate in this cluster sharing only 94% similarity with *L. redekei* (Zwart et al. 2005) and thus belonging to another species or even another genus. It is possible, therefore, that our ND2-CYA-1-8 clone corresponds to the closely related *Jaaginema* sp. identified microscopically in L. Doirani water sample, for which no 16S rDNA sequences are yet available. In L. Doirani, the microscopically observed *Planktothrix* cf. *agardhii* population seems to correspond to the *Planktothrix pseudagardhii* phylotype clustering tightly together with the *Planktothrix pseudagardhii* T19-6′-8 strain (Suda et al. 2002). This discrepancy is important in monitoring practices, since *P. agardhii* is well known to produce microcystins (Sivonen 1990) but no such evidence has been obtained for the morphologically identical *P. pseudagradhii* (Suda et al. 2002).

Most of the Nostocalean phylotypes included sequences from L. Doirani and were associated with species known to dominate

in L. Doirani and other lakes of Greece, such as *Aphanizomenon flos-aquae*, *Aphanizomenon issatschenkoi* and *Cylindrospermopsis raciborskii* (Temponeras et al. 2000; Vardaka et al. 2005), which we identified microscopically in our samples. One phylotype from L. Kastoria and one from L. Doirani clustered tightly together with Chinese strain *R. mediterranea* HB2 (Li et al. 2008) and *Cylindrospermopsis raciborskii* Florida I (Neilan et al. 2003). These phylotypes are practically identical to clone NK2-532 clone from L. Kastoria, confirming previous hypothesis that *Raphidiopsis* and *Cylindrospermopsis* are practically the same genus (Moustaka-Gouni et al. 2009).

We used the LIBSHUFF analysis for testing the significance of the difference between the two clone libraries. It is assumed that significantly different libraries have been derived from communities of different composition (Singleton et al. 2001). This analysis showed that the composition of the cyanobacterial (including the chloroplast-related) phylotypes L. Doirani and Kastoria is similar. Indeed, the two lakes share five groups of common phylotypes (Figs. 2 and 3) with each group containing two or three phylotypes and in some cases these phylotypes have considerable relative abundance. This resemblance in community structure is reflected in the highly similar values of the *H* diversity index.

In conclusion, our study showed that in the case of water blooms with co-occurring cyanobacterial species, the use of cyanobacterial specific primers for the PCR amplification of cyanobacterial 16S rRNA genes corroborates well with standard microscopic estimations. However, the 16S rRNA approach provides concomitantly information on potentially toxic species and a more thorough taxonomic description of the bloom community by unraveling either rare phylotypes or phylotypes that can be attributed to morphotypes which cannot be securely recognized by morphological observations. This by no means undermines the usefulness of microscopic analysis. Biological features like different morphotypes, cell counting of the same morphospecies for the estimation of biovolume, separation of intact (viable) vs. destroyed (non-viable) cells and life-cycle stages remain the most informative advantages of direct microscopic observation of a cyanobacterial bloom (e.g. Moustaka-Gouni et al. 2009). In addition, microscopy can be coupled with other techniques in order to provide a more realistic tool for discriminating individual organisms and their features (for a brief review see Orphan 2009) and such combined approaches should be included in future studies of lake cyanobacterial dynamics.

The multilocus approach, i.e. the combined use of genes with phylogenetic information (mostly 16S rRNA) and functional genes (e.g. Gugger et al. 2005; Thomazeau et al. 2010), is one the most successful examples of the application of the sequencing progress of the last decade. Such efforts are expected to benefit further by the use of pyrosequencing by using multiple tags (e.g. Anderson et al. 2010). Particularly for the case of cyanobacterial blooms, we believe that the metagenomic approach might be the most informative (see Pope and Patel 2008). However, such data, along with the limited cyanobacterial genomes available (61, with half of them belonging to the order of Chroococcales) are scarce and subsequently limit our knowledge on the ecological role, potential applications and insights on how to prevent the toxic ones, of these organisms. The above methodologies and their numerous combination allow for customization of monitoring programs of cyanobacteria in freshwaters even at the site-specific level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.limno.2010.10.003.

References

Anagnostidis, K., Komárek, J., 1985. Modern approach to the classification system of cyanophytes. 1. Introduction. Arch. Hydrobiol. Suppl. 71, 291–302.
Anagnostidis, K., Komárek, J., 1988. Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. Arch. Hydrobiol. Suppl. 80, 327–472.
Anderson, A., Riemann, L., Bertilsson, S., 2010. Pyrosequencing reveals contrasting seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. ISME J. 4, 171–181.
Berger, C., Ba, N., Gügger, M., Bouvy, M., Rusconi, F., Coutè, M., Troussellier, M., Bernard, C., 2006. Seasonal dynamics and toxicity of *Cylindrospermopsis raciborskii* in Lake Guiers (Senegal, West Africa). FEMS Microbiol. Ecol. 57, 355–366.
Castenholtz, R.W., 2001. Phylum BX. Cyanobacteria. Oxygenic photosynthetic bacteria. In: Boone, D.R., Castenholtz, R.W., Garrity, G.M. (Eds.), Bergey's Manual of Systematic Bacteriology. The Archaea and the Deeply Branching and Phototrophic Bacteria, vol. 1, 2nd ed. Springer, New York, pp. 473–599.
Castenholz, R.W., Norris, T.B., 2005. Revisionary concepts of species in the cyanobacteria and their applications. Algol. Stud. 117, 53–69.
Chorus, I., Bartram, J., 1999. Toxic Cyanobacteria in Water. World Health Organization, 1st ed. E. and F.N. Spon, London and New York.
Crosbie, N.D., Pockl, M., Weisse, T., 2003. Dispersal and phylogenetic diversity of nonmarine picocyanobacteria, inferred from 16S rRNA gene and cpcBA-intergenic spacer sequence analyses. Appl. Environ. Microbiol. 69, 5716–5721.
Eiler, A., Bertilsson, S., 2004. Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. Environ. Microbiol. 6, 1228–1243.
Furtado, A.L.F.F., Calijuri, M.C., Lorenzi, A.S., Honda, R.Y., Genuário, D.B., Fiore, M.F., 2009. Morphological and molecular characterization of cyanobacteria from a Brazilian facultative wastewater stabilization pond and evaluation of microcystin production. Hydrobiologia 627, 195–209.
Gkelis, S., Rajaniemi, P., Vardaka, E., Moustaka-Gouni, M., Lanaras, T., Sivonen, K., 2005. *Limnothrix redekei* (Van Goor) Meffert (Cyanobacteria) strains from Lake Kastoria, Greece form a separate phylogenetic group. Microb. Ecol. 49, 176–182.
Gugger, M., Le Berre, B., Dufour, P., Bernard, C., Humbert, J.-F., 2005. Genetic diversity of Cylindrospermopsis strains (Cyanobacteria) isolated from four continents. Appl. Environ. Microbiol. 71, 1097–1100.
Hill, T.C.J., Walsh, K.A., Harris, J.A., Moffet, B.F., 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiol. Ecol. 43, 1–11.
Hindák, F., Moustaka, M., 1988. Planktic cyanophytes of Lake Volvi, Greece. Arch. Hydrobiol. Suppl. 80, 497–528.
Junier, P., Witzel, K.-P., Hadas, O., 2007. Genetic diversity of cyanobacterial communities in Lake Kinneret (Israel) using 16S rRNA gene, psbA and ntcA sequence analyses. Aquat. Microb. Ecol. 49, 233–241.
Kemp, P.F., Aller, J.Y., 2004. Estimating prokaryotic diversity: when are 16S rDNA libraries large enough? Limnol. Oceanogr. Meth. 2, 114–125.
Komárek, J., Anagnostidis, K., 1989. Modern approach to the classification system of cyanophytes, 4 Nostocales. Arch. Hydrobiol. Suppl. 82 (Algol. Stud.) 56, 247–345.
Komárek, J., Anagnostidis, K., 1999. Cyanoprokaryota, 1. Teil: Chroococcales. Süßwasserflora Mitteleur. Band 19 (1), 270–323.
Komárek, J., Anagnostidis, K., 2005. Cyanoprokaryota, 2. Teil: Oscillatoriales. Süßwasserfl. Mitteleur. Band 19, 1–759.
Li, R., Wilhelm, S.W., Carmichael, W.W., Watanabe, M.M., 2008. Polyphasic characterization of water bloom forming Raphidiopsis species (cyanobacteria) from central China. Harmful Algae 7, 146–153.
Lyra, C., Suomalainen, S., Gügger, M., Vézie, C., Sundman, P., Paulin, L., Sivonen, K., 2001. Molecular characterization of planktic cyanobacteria of Anabaena, Aphanizomenon, Microcystis and Planktothrix genera. Int. J. Syst. Evol. Microbiol. 51, 513–526.
Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker Jr., C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M., 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. 29, 173–174.
Moustaka-Gouni, M., Kormas, K.A., Vardaka, E., Katsiapi, M., Gkelis, S., 2009. Raphidiopsis mediterranea SKUJA represents non-heterocytous life-cycle stages of *Cylindrospermopsis raciborskii* (WOLOSZYNSKA) SEENAYYA et SUBBA RAJU in Lake Kastoria (Greece), its type locality: evidence by morphological and phylogenetic analysis. Harmful Algae 8, 864–872.
Moustaka-Gouni, M., Kormas, K.A., Polykarpou, P., Gkelis, S., Bobori, D.C., Vardaka, E., 2010. Polyphasic evaluation of *Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* in a Mediterranean lake. J. Plankton Res. 32, 927–936.
Moustaka-Gouni, M., Vardaka, E., Michaloudi, E., Kormas, K.A., Tryfon, E., Mihalatou, H., Gkelis, S., Lanaras, T., 2006. Plankton food web structure in a eutrophic polymictic lake with a history of toxic cyanobacterial blooms. Limnol. Oceanogr. 51, 715–727.

Moustaka-Gouni, M., Vardaka, E., Tryfon, E., 2007. Phytoplankton species succession in a shallow Mediterranean lake (L. Kastoria, Greece): steady-state dominance of *Limnothrix redekei*, *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*. Hydrobiologia 575, 129–140.

Neilan, B.A., Saker, M.L., Fastner, J., Törökné, A., Burns, B.P., 2003. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. Mol. Ecol. 12, 133–140.

Nübel, U., Garcia-Pichel, F., Muyzer, G., 1997. PCR primers to amplify 16S rRNA genes from Cyanobacteria. Appl. Environ. Microbiol. 63, 3327–3332.

Orphan, V.J., 2009. Methods for unveiling cryptic microbial partnerships in nature. Curr. Opin. Microbiol. 12, 231–237.

Pielou, E.C., 1969. An Introduction to Mathematical Ecology. Wiley, New York.

Pope, P., Patel, B.K.C., 2008. Metagenomic analysis of a freshwater toxic cyanobacteria bloom. FEMS Microbiol. Ecol. 64, 9–27.

Rajaniemi-Wacklin, P., Rantala, A., Mugnai, M.A., Turicchia, S., Ventura, S., Komárková, J., Lepistö, L., Sivonen, K., 2006. Correspodence between phylogeny and morphology of Snowella spp. and *Woronichinia naegeliana*, cyanobacteria commonly occurring in lakes. J. Phycol. 42, 226–232.

Sagan, L., 1967. On the origin of mitosing cells. J. Theor. Biol. 14, 225–274.

Sanchez-Baracaldo, P., Handley, B.A., Hayes, P.K., 2008. Picocyanobacterial community structure of freshwater lakes and the Baltic Sea revealed by phylogenetic analyses and clade-specific quantitative PCR. Microbiology 154, 3347–3357.

Shannon, C.E., Weaver, W., 1949. The Mathematical Theory of Communication. University of Illinois Press, Urbana.

Singleton, D.R., Furlong, M.A., Rathbun, S.L., Whitman, W.B., 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. 67, 4374–4376.

Sivonen, K., 1990. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl. Environ. Microbiol. 56, 2658–2666.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water. World Health Organization. E and FN Spon, London and New York, pp. 41–110.

Suda, S., Watanabe, M.M., Otsuka, S., Mahakahant, A., Yongmanitchai, W., Nopartnaraporn, N., Liu, Y., Day, J.G., 2002. Taxonomic revision of water-bloom-forming species of oscillatorioid cyanobacteria. Int. J. Syst. Evol. Microbiol. 52, 1577–1595.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Temponeras, M., Kristiansen, J., Moustaka-Gouni, M., 2000. Seasonal variation in phytoplankton composition and physical–chemical features of the shallow Lake Doirani, Macedonia, Greece. Hydrobiologia 424, 109–122.

Thomazeau, S., Houdan-Fourmont, A., Couté, A., Duval, C., 2010. The contribution of sub-saharan African strains to the phylogeny of Cyanobacteria: focusing on the Nostocaceae (Nostocales, Cyanobacteria). J. Phycol. 46, 564–579.

Urbach, E., Scanlan, D.J., Distel, D.L., Waterbury, J.B., Chisholm, S.W., 1998. Rapid diversification of marine picophytoplankton with dissimilar light-harvesting structures inferred from sequences of Prochlorococcus and Synechococcus (Cyanobacteria). J. Mol. Evol. 48, 723–739.

Vardaka, E., Moustaka-Gouni, M., Cook, C.M., Lanaras, T., 2005. Cyanobacterial blooms and water quality in Greek freshwaters. J. Appl. Phycol. 17, 391–401.

Valério, E., Chambel, L., Paulino, S., Faria, N., Pereira, P., Tenreiro, R., 2009. Molecular identification, typing and traceability of cyanobacteria from freshwater reservoirs. Microbiology 155, 642–656.

Wilmotte, A., Herdman, M., 2001. Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In: Boone, D.R., Castenholtz, R.W., Garrity, G.M. (Eds.), Bergey's Manual of Systematic Bacteriology. The Archaea and the Deeply Branching and Phototrophic Bacteria, vol. 1, 2nd ed. Springer, New York, pp. 487–493.

Wilson, A.E., Sarnelle, O., Neilan, B.A., Salmon, T.P., Gehringer, M.M., Hay, M.E., 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: implications for harmful algal blooms. Appl. Environ. Microbiol. 71, 6126–6133.

Yoshida, M., Yoshida, T., Satomi, M., Takashima, Y., Hosoda, N., Hiroishi, S., 2008. Intra-specific phenotypic and genotypic variation in toxic cyanobacterial Microcystis strains. J. Appl. Microbiol. 105, 407–415.

Zwart, G., Kamst-van Agterveld, M.P., van der Werff-Staverman, I., Hagen, F., Hoogveld, H.L., Gons, H.J., 2005. Molecular characterization of cyanobacterial diversity in a shallow eutrophic lake. Environ. Microbiol. 7, 365–377.