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Molecular characterization of cyanobacterial diversity in Lake Gregory, Sri Lanka*

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Abstract Eutrophication or the process of nutrient enrichment of stagnant waters due to excessive use of fertilizer is becoming a critical issue worldwide. Lake Gregory, an artificial lake situated in Nuwara Eliya, Sri Lanka was once a very attractive landscape feature and recreational area attracting large number of visitors. Rapid urbanization in surrounding areas and the consequent intensification of agricultural and industrial activities led to the enhancement of eutrophication and siltation in the lake. Present study was conducted to detect cyanobacterial diversity and their ability to produce hepatotoxic microcystins using polymerase chain reaction (PCR) based techniques.

Twenty five water samples (surface and bottom) were collected from the lake and total nitrogen and total carbon were estimated. Cyanobacterial cultures were grown in appropriate media and microscopic observations were used to determine the morphological diversity of cyanobacteria isolated from different sites. Genomic DNA was isolated and purified from cyanobacteria using Boom's method. DNA samples were analyzed by PCR with oligonucleotide primers for 16S rRNA gene and *mcyA* gene of the operon that encodes a microcystin synthetase. The 16S rRNA gene sequences reveled the presences of cyanobacteria belong to *Synechococcus* sp., *Microcystis aeruginosa*., *Calothrix* sp., *Leptolyngbya* sp., *Limnothrix* sp., order Oscillatoriales and order Chroococcales. The sequences obtained from this study were deposited in the database under the accession numbers [GenBank: GU368104 — GU368116]. PCR amplification of *mcyA* primers indicated the potential for toxin formation of isolated *M. aeruginosa* from Lake Gregory.

This preliminary study shows that the Lake Gregory is under the potential risk of cyanobacterial toxicity. Clearly more work is needed to extend this finding and clarify if other cyanobacterial isolates have genetic potential to produce microcystin since this lake is utilized for drinking water or recreation.

Keyword: Eutrophication; Booms method; PCR; Microcystin; *mcyA* gene

INTRODUCTION

Eutrophication or the process of nutrient enrichment of stagnant waters due to excessive use of fertilizer is becoming a critical issue worldwide. Increasing human population and the consequent intensification of agricultural and industrial activities along with deficient water management have led to the enhancement of eutrophication Environmental factors such as higher temperature, pH, low turbulence, high phosphorus (P) and nitrogen (N) inputs increase the development of planktonic cyanobacteria in lakes and reservoirs, leading to formation of surface blooms that may gather as scum (de Figueiredo, 2004).

Several studies have been conducted to asses problems associated with cyanobacterial blooms in freshwaters and their ecological consequences in recreational activities (Stewart et al., 2006a; Stone and Bress, 2007). Recently, cyanobacterial toxins or cyanotoxins have received wide attention from different scientific communities as well as the general public due to their potential health risk (Stone and Bress, 2007). The exposure to cyanotoxins can occur through oral ingestion, aspiration of water into the lungs, inhalation of mist and dermal contact (Stone and Bress, 2007). A range of illnesses associated with recreational exposure to cyanobacteria have been recorded including hay fever-like symptoms,

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pruritic skin rashes, gastro-intestinal symptoms and allergic responses (Stewart et al., 2006a). Also more serious acute illnesses, with symptoms such as severe headache, pneumonia, fever, myalgia, vertigo and blistering in the mouth have been documented (Stewart et al., 2006a, b). The first recorded human fatality related to recreational exposure to cyanobacteria was reported by US coroner that a teenage boy died as a result of accidentally ingesting of neurotoxic cyanotoxin from a golf course pond (Stewart, 2004).

Sri Lanka is an island in the Indian Ocean endowed with rich water resources emanating from the central highlands that receive rain during the monsoons. Surface waters are carried radially from the central hills through 103 distinct river basins covering 90% of the Island. Its inland waters include man made lakes and ponds and marshes, constituting one of the highest densities in the world. Lake Gregory is a small man-made tank, located in city of Nuwara Eliya in the wet montane zone of Sri Lanka. The area of the lake is 0.4 km^2 with a perimeter of about 3.5 km (IUCN Sri Lanka and Central Environmental Authority, 2006). It is a very attractive landscape feature and recreational area attracting large number of visitors. A major tributary of this Lake is the Nanu Oya stream that originates from the Pidurutalagala Peak which is the highest peak in Sri Lanka. Lake Gregory receives an average annual rainfall of 2 000–2 500 mm, and a mean annual temperature of approximately 16°C. The underlying area of the Lake consists of highly crystalline charnockitic genesis rocks of Precambrian age (IUCN Sri Lanka and Central Environmental Authority, 2006). The Lake is surrounded on all sides by roads, and the surrounding area has been severely altered by commercial agriculture developments and tourist expansions. Intensive agricultural practices (pesticides and fertilizers), domestic and industrial effluents, cattle domestication and inadequate management of watersheds may be pointed out as the major causes of eutrophication of the lake. The heavy development of aquatic macrophytes (*Salivinia*, *Pistia*, *Nymphaea* spp.) and phytoplankton blooms are clear signs of the eutrophication of the lake Gregory.

The aim of the present study was to investigate the diversity of cyanobacteria in Lake Gregory with molecular techniques and to focus on the potential health risks associated with lake waters, through recreational activities.

MATERIAL AND METHOD

Sampling

Water samples and sediment samples were collected into sterilized brown glass containers from twenty five selected sites from Lake Gregory (Fig.1a and b). Sampling was conducted every four months at sites from May 2008 to April 2009. The collections were carried out at several points, both on the surface and down the water column. The samples were preserved in Lugol's iodine solution immediately after collection for phytoplankton analysis.

Isolation of cyanobacteria

For culture, samples were concentrated by centrifugation and the resulting pellet was serially diluted and inoculated onto appropriate media. Cultures were incubated at 28+/–2°C with fluorescent light at a 16:8-h D/L cycle.

Microscopic observation

Morphological classification of cyanobacteria was carried out using observable characters under a

Fig.1 The figures of sampling sites

compound light microscope (Olympus BH2); (400–1 000×). The taxonomic assignments of morphotypes were based on the descriptions of Holt et al., 1994.

N and C composition of water

Total nitrogen and total carbon of samples were estimated using TOC analyzer (analytikjena multi N/C 2100).

Genomic DNA isolation

DNA extractions were carried out for cultured samples and environmental samples using standard techniques and nucleic acids were purified by Boom's method (1990) using silica particles and guanidium isothiocyanate.

PCR amplification of 16S rRNA gene and sequencing

Genomic DNA extracted from both cultured and environmental samples was used for the amplification of 16S rRNA gene region, with the cyanobacteria-specific primers designed by Nübel et al. (1997), forward primer CYA359F (5′-GGGGAATYTTCC GCAATGGG-3′) and the reverse primer CYA781Ra (5′-GACTACTGGGGTATCTAATCCCATT-3′), or the reverse primer CYA781Rb (5′-GACTACAGGGG TATCTAATCCCTTT-3′). Each reaction contained 0.6 μmol/L of each primer, 0.1 mmol/L of each deoxynucleoside triphosphate, 5 μl of 10×PCR buffer (100 mmol/L Tris–HCl [pH=9.0], 15 mmol/L $MgCl_2$, 500 mmol/L KCl, 1% [v/v] Triton X -100), 0.5 U of Super *Taq* DNA polymerase (HT Biotechnology, Ltd., Cambridge, UK) and template DNA. The PCR assay was carried out in a Perkin-Elmer/Cetus DNA Thermal Cycler in a final volume of 50 μl with 50 μl of mineral oil, with 1 min annealing at 60°C. PCR products were carefully excised and purified with the genElute™ Gel Extraction Kit (SIGMA) according to the manufacturer's instructions and DNA sequencing was carried out by Macrogen Inc., South Korea using ABI 3730XL sequencers with the corresponding reverse primers.

PCR amplification of mcy A gene

DNA amplification was performed for the *mcy* A gene using the self designed forward primer McyAF19 (5′-AACATCCAGCAGTTGAGCAAGC-3′) and the reverse primer McyAR47 (5′-CTCCC TCTAAAACCCGCAGTAAG-3'). Reaction mixtures contained 0.4 μmol/L of each primer, 0.1 mmol/L of each deoxynucleoside triphosphate, 10 μl of 10×PCR buffer (50 mmol/L Tris –HCl [pH 8.0], 25 mmol/L $MgCl_2$, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 50% glycerol, 1% [v/v] Triton X –100), 1.0 U of *Taq* DNA polymerase (Promega., Madison, WI) and template DNA. The standard DNA used was the DNA extracted from *Microcystis aeruginosa* (PCC 7941). Amplifications were carried out in 100 μl volumes in a Perkin-Elmer/ Cetus DNA Thermal Cycler with 35 incubation cycles with annealing 1 min at 56°C. Aliquots of the resulting ~594 bp sequences were electrophoresed in 1.5% agarose gels containing 10 μg/ml ethidium bromide and documented through Polaroid instant camera.

Nucleotide accession numbers

The sequences obtained from this study were deposited in the database under the accession numbers [GenBank: GU368104 — GU368116].

RESULT

The samples from 25 sites were subjected to microscopic examination and the cyanobacterial species observed were recorded. *Microcystis* species was dominant in the water column throughout the lake. The second highest species recorded was *Limnothrix* sp. which was comparatively very low in numbers. Also *Synechocystis* sp., *Synechococcus* sp., *Chroococcus* sp., Order: Chroococcales cyanobacterium, *Lyngbya* sp., *Leptolyngbya* sp., *Pseudoanabaena* sp., *Anabaena* sp. and *Calothrix* sp., were observed in water samples collected from Lake Gregory. Thus, based on microscopic observation of cultured isolates from different sites of the lake, the following cyanobacterial species were tentatively identified; *Microcystis* sp. *Synechococcus* sp., *Synechocystis* sp., Chroococcales cyanobacterium, *Leptolyngbya* sp., *Limnothrix* sp., *Pseudanabaena* sp. and *Calothrix* sp., (Fig.2).

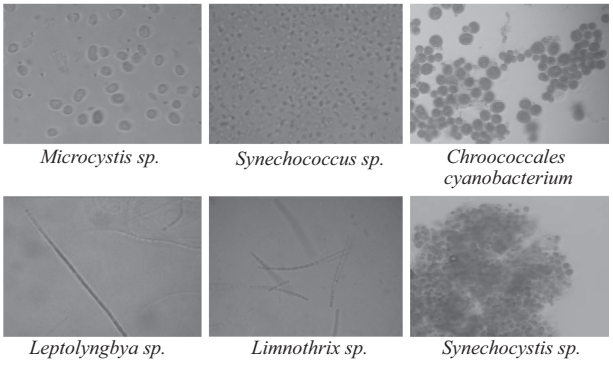

Fig.2 Micrographs of cultured isolates

Species of *Synechocystis*, *Lyngbya*, *Anabaena*, *Synechococcus* and *Leptolyngbya* were recorded from bottom (~3.5 m) of the water column, although *Pseudoanabaena* sp. and *Calothrix* sp. were observed in surface of the water body. *Limnothrix* sp., Order Chroococcales cyanobacteria, *Chroococcus* sp. and *Microcystis aeruginosa* were found in both surface and bottom of the water body.

Total nitrogen and total carbon concentrations were measured for the water samples collected from both surface and bottom of the water body (Fig.3). Data obtained from the present study revealed that the total carbon (TC) concentration was higher than the total nitrogen (TN) concentration in the lake in 4 visits. However, bottom TC and TN concentrations were higher than the surface concentrations.

In order to assess the genetic diversity of cyanobacteria, partial sequencing was carried out for the 16S rRNA gene. The resulted sequences were analyzed using the BLAST program in GenBank (Altschul et al., 1990) for alignment to database sequences and to confirm that the origin of the generated sequence was cyanobacterial. The 16S rDNA gene sequences were then compared to each other and to other sequences available in GenBank. The obtained sequences showed similarity to previously reported cyanobacterial sequences ranging from 75% to 99% (Table 1).

DNA extracted from cultured isolate of *M. aeruginosa* N11 (GU368108) yielded the unique fragment of about 594 bp, using the microcystin synthetase gene *mcyA* forward primer McyAF19 and the reverse primer McyAR47 (Fig.4).

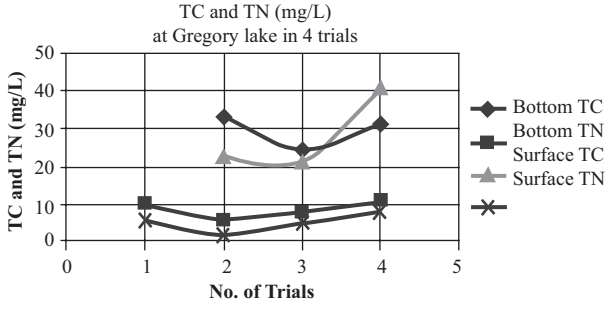

Fig.3 Total carbon and total nitrogen concentration of the Lake Gregory in four visits

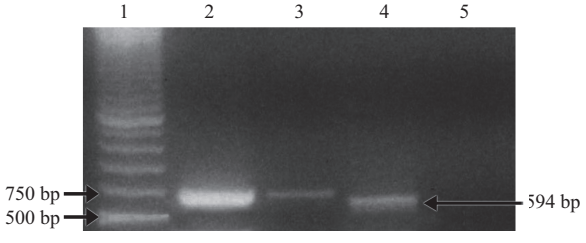

Fig.4 PCR amplification of the may a gene with DNA extracted from *M. aeruginosa* N11 (GU368108) Lane 1, DNA marker; Lane 2, *M. aeruginosa* BL1 (EF0S1239); Lane 3, *M. aeruginosa* (PCC7491); Lane 4, *M. aeruginosa* N11 (GU368108); Lane 5, negative control

Table 1 Nucleotide sequence accession numbers of the partial sequences of 16S rRNA gene of cyanobacterial isolates and highest similarities of the sequence analysis in GenBank by BLASTN

Sample name	Accession number	Organism	16S rRNA highest match NCBI	Similarity (%)
N2a	GU368104	Unicellular cyanobacterium N2a	Uncultured cyanobacterium clone CYAB24 (FJ774057.1)	82
N2b	GU368105	*Synechococcus* sp. N2b	*Synechococcus* sp. LS0501 (DQ526414.1)	97
N14	GU368106	*Synechocystis* sp. N14	*Synechocystis* PCC6308 (AB039001.1)	97
N1a	GU368107	Chroococcales cyanobacterium N1a	Uncultured Chroococcales cyanobacterium clone 3b/p2b6 (EF160022.1)	91
N11	GU368108	*Microcystis aeruginosa* N11	*Microcystis aeruginosa* PCC 7820 (DQ786006.1)	98
N16	GU368109	Unicellular cyanobacterium N16	Uncultured cyanobacterium clone CYAVE59 (FJ774037.1)	75
N1b	GU368110	*Calothrix* sp. N1b	*Calothrix* sp. KVSF5 (EU022730.1)	98
N2c	GU368111	*Leptolyngbya* sp. N2c	*Leptolyngbya* sp. PCC 73110 (AM398786.1)	98
na1	GU368112	*Limnothrix* sp. na1	*Limnothrix redekei* LMECYA 145 (EU078512.1)	99
na3	GU368113	*Pseudanabaena* sp. na3	Uncultured *Pseudanabaena* sp. isolate DGGE gel band DL17-13 (EU878146.1)	99
na5	GU368114	*Synechococcus* sp. na5	*Synechococcus* sp. PCC 8916 (AF448071.1)	99
na7	GU368115	*Calothrix* sp. na7	*Calothrix* sp. KVSF5 (EU022730.1)	98
na12	GU368116	Uncultured cyanobacterium na12	*Synechococcus* sp. PCC 8916 (AF448071.1)	94

DISCUSSION

The work presented here was initiated as a first attempt to characterize the cyanobacterial composition of the Lake Gregory, Sri Lanka using phylogenetic approach together with morphological data.

The major problems in applying morphological criteria in cyanobacterial classification arise from morphological features that vary with environmental conditions (Willmotte and Golubic, 1991). Sometimes microscopy and enrichment cultures have limited use since distinct species of cyanobacteria can share similar morphological features and some strains have been missing during cultivation (Ferris et al., 1996). Due to inherent plasticity of morphological features commonly used in traditional bacterial systematics, molecular and microcellular data can offer more reliable criteria for phylogeny and taxonomy.

The difficulties often encountered in both isolation and subsequent purification of cyanobacteria greatly affected studying these organisms. During the study most of the strains were cultured in BG11 medium. However, *Lyngbya* and *Anabaena* species did not grow after purification, due to nutrient deficiency, inhibitory factors within the culture medium or variation of light intensity. There have been various reports that describe the difficulty in growing some cyanobacterial strains as pure cultures (Fujishiro et al., 2004). *Dermocarpa* spp. grew much better in association with *Scytonema* spp. than by themselves when stirred and sparged with 0.3% CO_2 in air (de Chazal et al., 1992).

Some strains also change their morphology during laboratory cultivation (Rajaniemi et al., 2005). This may due to decreases the nutrients during the growth, variation of light intensity, degree of aeration, etc (Zapomělová et al., 2008). *Limnothrix* strains no longer showed polar gas vacuoles. In our study, *Calothrix* strains lost their filamentous nature and grew as unicellular. These morphological features are critical for the morphological identification of these genera. Changes in phenotype during laboratory cultivation seem to be common among cyanobacteria: *Microcystis* (Mlouka et al., 2004), *Aphanizomenon* (Gugger et al., 2002), *Nodularia* (Lehtimäki et al., 2000), *Merismopedia* (Palinska et al., 1996) and *Planktothrix* (Beard et al., 2002). The altered phenotype could make incorrect microscopic identification of the strains. The *Limnothrix* isolates without polar gas vacuoles could not be separated from the other thin filamentous cyanobacteria (Gkelis et al., 2005). Amended growth conditions could allow the re-appearance of these features for the strains.

The high levels of nitrogen in the bottom of the water body were associated with increases the presence of *Synechocystis*, *Lyngbya*, *Synechococcus* and *Leptolyngbya* species who were non heterocyst formers. Further heterocyst forming species such as *Calothrix* inhabited surface of the lake exhibiting their ability to tolerate low levels of nitrogen concentrations. Therefore, TC and TN play a significant role in cyanobacterial distribution in Lake Grgory.

Cyanobacteria grow best in non turbulent, warm rivers, lakes and reservoirs (Hoehn and Long, 1999). *Microcystis* occasionally forms a bloom, or dense aggregation of cells, that floats on the surface of the water forming a thick layer or 'mat'. Blooms usually occur during the warmest months of the year, especially when the water contains an over abundance of nitrogen (N) and phosphorus (P). Excessive P most often provides the stimulus for cyanobacterial blooms, especially if the total N to total P concentration ratio is less than 10 (Hoehn and Long, 1999).

In this study, 16S rRNA gene was used for identification and characterization of the cyanobacterial community composition because it is ubiquitous, function is conserved, the gene is easy to sequence and a large database is available for sequence alignment and identification. Sequence analysis of the 16S rRNA subunit can provide a much higher degree of resolution among cyanobacterial taxa than either morphological or chemical traits (Wilmontte et al., 1994). Recent studies on the 16S rRNA gene sequences have extended the knowledge of the phylogenetic relationship among some prokaryotic strains and in identification of other cyanophytes (Ishida et al., 1997). The 16S rRNA gene is especially valuable for phylogenetic studies due to the presence of highly conserved regions as well as some variable regions (Řeháková et al., 2007). 16S rRNA sequences data have been used to study phylogenetic relationships of cyanobacteria in all taxonomic level (Wilmotte et al., 1992; Wilmotte and Herdman, 2001). Additionally, the 16S rRNA gene has been useful in identifying and classifying strains that belong to a single clade (Otsuka et al., 1998). The strains which have clear differences in their 16S rRNA can be categorized into distinct species, but no determination can be made for strains that are genetically similar (>97.5%) in this gene (Řeháková et al., 2007).

The closest sequence similarity of the excised and sequenced PCR bands revealed 13 different phylotypes using NCBI BLASTN (Table 1). All of the sequences obtained were of cyanobacterial origin, confirming the specificity of the primers described by Nübel et al. (1997). According to the closest sequence similarity to cultured strains using BLASTN (Table 1) the cyanobacterial phylotypes were represented by seven genera, one filamentous heterocystous (*Calothrix* sp.), three filamentous non-heterocystous (*Leptolyngbya* sp., *Limnothrix* sp., *Pseudanabaena* sp.) and three unicellular (*Synechococcus*, *Microcystis aeruginosa*, *Synechosystis* sp.).

Unicellular cyanobacterium N16 (GU368109) isolated from Lake Gregory demonstrated 75% sequence similarity to uncultured cyanobacterium clone CYAVE59 (FJ774037) but could not be assigned into exact genera identified from water column of Lake Marathon, Greece. Chroococcales cyanobacterium N1a isolated from Lake Gregory showed morphologically similar characters to *Synechocystis* sp. But its 16S partial sequence assigned this species only into order Chroococcales and was unable to be characterized up to genus level. Chroococcales cyanobacterium N1a showed only 91% sequence similarity to previously identified uncultured Chroococcales cyanobacterium clone 3b/p2b6, from intertidal microbial mat of Tanzania. The utility of the 16S rDNA sequence was limited in definition of genera with order Chroococcales. *Synechocystis* PCC6308 was recorded from Pasture Culture Collection and *Synechocystis* sp. N14 isolated from Lake Gregory showed 97% sequence similarity. This suggests that these isolates represent one genotype.

PCR amplification of *mcyA* primers indicated the potential for toxin formation of isolated *M. aeruginosa* from the lake Gregory. *McyA* is essential in the biosynthesis of microcystin to incorporate N-methyl-dehydro-alanin and L-alanin into the cyclic peptide (Tillett and Neilan, 2000). Detection of microcystin producing cynobacteria using molecular markers may have great use in routine analysis of aquatic ecosystems. According to our results Lake Gregory is under the potential risk of cyanobacterial toxicity. Clearly more work is needed to extend this finding and clarify if other cyanobacterial isolates have genetic potential to produce microcystin since this water body is utilized by human being. Also, it is well known that *M. aeruginosa* coexistence with toxic and non-toxic strains and microcystin production is affected by various environmental factors such as water temperature, pH, intensity of solar radiation, dissolved oxygen and CO_2 availability (Grobbelaar et al., 2004). Therefore, analytical methods should be carried out on water samples to confirm the toxicity of cyanobacteria present in the lake.

This preliminary study shows that further work for detection cyanobacterial diversity and their potential microcystin producing ability in Lake Gregory is important to protect public health where surface water is used for recreational activities.

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