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Identification and toxigenic potential of a cyanobacterial strain (*Stigomena* sp.)

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Abstract

Cyanobacteria are well known for their production of a multitude of highly toxic substances. The genus *Stigomena* is regarded as good candidates for producing biologically active secondary metabolites, which are highly toxic to humans and other animals. The carcass of a dog was found at the shore of Lake Ali-Abad, Iran. Biomass from the discovery site appeared to be of cyanobacterial nature. We identified the strain as freshwater bloom-forming representative of the *Stigomena* genus on the basis of its 16S rRNA sequence. It carried the *mcyE* gene, as well as a potential cryptic *hassallidin* gene cluster. These results suggest that microcystin-induced liver damage may have significantly contributed to the death of the dog. This case is thus the first reported incident of potential microcystin intoxication in a dog in Iran.

Keywords: Cyanobacteria, *Stigomena* sp., Natural bioactive compound, Toxicity

Introduction

Mass occurrences of cyanobacteria, or so-called cyanobacterial blooms, are not only a significant problem in terms of water quality, but also pose a severe risk to human and animal health. They may contain potent hepato- and neurotoxic, as well as derma- and cytotoxic agents produced by strains of several cyanobacterial genera. Numerous cases of fatal animal poisonings, attributed to these toxins, have been reported around the globe over the past decades (1, 2). In freshwaters, blooms containing hepatoxic agents are more frequent than those containing neurotoxins (2). It has been documented that dogs, birds and young cattle have been killed by hepatoxins over the years, whereas as much as 40 cows suffered from fatal neurotoxin poisoning (1). In August 2011, a two-year old dog was found dead after being exposed to water of Lake Ali-Abad, Iran. The water that the dog ingested was mainly composed of green scum, assumed to be of cyanobacterial nature. Local authorities could neither draw conclusions upon the identity of the most dominant species nor determine whether or not it produces toxic metabolites. Hence, our laboratory was provided with an isolate to conduct. Evidence presented in this report suggests that the death of the dog is the first documented related intoxication in dogs in Iran.

Material and methods

Culture conditions, morphological characterisation, preparation of extracts

The strain was aseptically cultivated in 400 ml cultures of liquid media BG110 (Rippka et al. 1979) without $NaNO_3$ at 25°C under continuous illumination (8-12 µmol m^{-2} s^{-1}). One millilitre of culture was transferred to a sterile 1.5 ml microtube

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and stored at -20°C. The extract for the anatoxin analysis was prepared from 1 ml of frozen culture. For the preparation of methanol extracts, 236 mg of wet biomass were freeze-dried (Edwards lyophilisator). The dry cell mass was measured to be 7.3 mg**.** A microtube containing the latter amount of dried cells, 300 mg of glass beads (disruptor beads, 0.5 mm, Scientific Industries) and 1 ml of methanol (LC-MS grade, Fischer Scientific) was placed into a homogeniser (FastPrep®-24 instrument, MP Biome dicals) for 15 s at a speed of 6.5 ms^{-1}. The mixture was centrifuged as described above. The supernatant was stored refrigerated at 4°C until chemical analyses were performed. For the quantitation of microcystins, the supernatant was diluted 1 to 500 with methanol (LC-MS grade, Fischer Scientific).

Genomic DNA extraction

Genomic DNA was extracted utilising the E.Z.N.A.® SP Plant DNA kit (Omega Bio-tek).

16S rRNA gene-based identification

PCR amplification

In order to identify the unknown isolate on molecular level, a short fragment (approximately 420 bp) of its 16S rRNA gene was amplified utilising a cyanobacteria-specific primer set (3). As a positive control for the amplification procedure, genomic DNA of the cyanobacterium *Stigomena* sp. 202 was used, whereas sterile water was used as a negative control. The size of the products was estimated by comparison to marker DNA (λ/HinfIII + φx/HaeIII, Finnzymes). Products were purified using the Geneclean® Turbo kit (Qbiogene, MP Biomedicals) and their quantity was measured with a Nanadrop™ ND-1000 spectrophotometer (Thermo Scientific) and accounted for 14.9 ng/µl (260/280: 1.67, 230/260: 3.47).

Sequencing

Sequencing of the amplified 16S rRNA gene segment was performed by cycle sequencing. Reactions were prepared utilising the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies). Product DNA was denatured at 95°C for 2 min in a thermocycler (iCycler, Bio-Rad) and subsequently analysed with an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Life Technologies).

Sequence analysis

The sequence was aligned with partial 16S rRNA sequences of 56 selected cyanobacterial taxa utilising ClustalW algorithm (4) as implemented in BioEdit Version 7.0 (5). Using the Phylip package (6), an evolutionary distance matrix was created. The created matrix was inferred according to the neighbour-joining method (7), where *Aquifex aeolicus* 16S rRNA sequence (GeneBank accession AJ309733) was used as outgroup. To prevent ingroup monophyly, 16S rRNA seuqnces of *Escherichia coli*, *Chloroflexus aurantiacus* and *Agrobacterium tumefaciens* were included into the alignment. The stability of the established relationships was tested by bootstrap analysis comprised of 1000 sampling repeats. The consensus bootstrap tree was visualised using TreeView (8).

Molecular detection of potential toxigenicity

To determine whether or not the unknown strain is a potential toxin producer, genomic DNA was amplified by conventional PCR using primers targeting a specific gene of the biosynthetic gene cluster of the toxin to be detected (Table 1 and 2). For each reaction (Table 2) 50 ng of genomic DNA was used as a template. As a positive control for each amplification, genomic DNA of selected cyanobacteria was used (Table 1, supplied by the University of Helsinki Cyanobacterial Culture Collection, UHCC), whereas sterile water was used as a negative control.

The PCR program A included the following phases: Initial denaturation at 95°C for 3 min, 30 cycles comprised of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and annealing at 72°C for 30 s, as well as final annealing phase at 72°C for 5 min. The reactions were allowed to cool at 4°C. The PCR program B was comprised of the same phases than program A, with the exception of the annealing phase being carried out at 60°C for 30 s. PCR program C was 94°C fir 3 minutes, 36 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, as well as 72°C for 10 min. The reactions were allowed to cool at 4°C.

Table 1. Primers used to amplify genes of the microcystin, nodularin, anatoxin and hassallidin as well as methylproline-containing compounds biosynthetic gene clusters.

Primer Pair (forward/reverse)	Gene	Genus	Toxin	Positive Control	Amplicon size (bp)	Reference
mcyEF2/12R	mcyE	Anabaena	Microcystin	Anabaena sp. 202	247	1
mcyEF2/R8	mcyE	Microcystis	Microcystin	Microcystis sp.205	247	1
mcyEF2/plaR3	mcyE	Planktothrix	Microcystin	Planktothrix sp. 49	249	1, 2
ndaF8452/8640	ndaF	Nodularia	Nodularin	Nodularia sp. HEM	188	3
anaC/anab	anaC	Anabaena	Anatoxin	Anabaena sp. 86	263	4
anaC-osc	anaC	Oscillatoria	Anatoxin	*Oscillatoria* sp. PCC 9029	216	4
hasA-F/R	hasA	nonspecific	Hassallidin	Anabaena sp. 90	920	5
nosF/R	nosF	nonspecific	Several[a]	Nostoc sp. UK1	1000	6

1, (17); 2, (10); 3, (18); 4, (10); 5, (13) 6, (19); a, amplifies compounds containing methylproline;

Table 2. PCR reactions for the amplification of the genes involved in toxin biosynthesis (Table 1) in a total volume of 20 µl.

Primer Pair (forward/reverse)	Forward primer (µM)	Reverse primer (µM)	dNTP[a] (µM)	Taq polymerase[b] (U)	Buffer[c]	Program
mcyEF2/12R	0.5	0.5	250	0.5	1x	A
mcyEF2/R8	0.5	0.5	250	0.5	1x	B
mcyEF2/plaR3	0.5	0.5	250	0.5	1x	A
ndaF8452/8640	0.35	0.35	65	1	1x	B
anaC/anab	0.5	0.5	200	0.5	1x	B
anaC-osc	0.5	0.5	200	0.5	1x	B
hasA-F/R	0.5	0.5	200	0.4	1x	C
nosF/R	0.5	0.5	200	0.4	1x	C
nosF/R	0.5	0.5	200	0.4	1x	C

a, dNTP mixture (Finnzymes); b, DyNAzyme™ II polymeras (Finnzymes); c, DyNAzyme™ PCR buffer (Finnzymes)

Amplification products were analysed by gel electrophoresis. The gels consisted of 1.5% (w/v) agarose (TopVision™, Fermentas) in 0.5 times TAE (Bio-Rad) stained with 0.10 µg ml^{-1} EtBr (Bio-Rad). Electrophoresis was carried out at 100 V for 30 min in 0.5 time TAE buffer (Bio-Rad). PCR products were visualised by UV light and documented using Kodak DC290 camera accompanied by the Kodak 1D v3.5.0 imaging software. The size of the products was estimated by comparison to marker DNA (λ/HinfIII + φx/HaeIII, Finnzymes).

Chemical analysis

Cyanobacterial cell extracts (see Section 2.1) were analysed by liquid chromatography mass spectrometry (LC-MS) using a Agilent 1100 Series LC/MSD Trap XCT Plus System (Agilent Technologies). Samples for anatoxin analysis were separated with a Cogent Diamond Hydride column (100 Å, 150 mm × 2 mm, particle size 4 µm, MicroSolv), whereas samples for microcystin quantitation where separated with a Luna C18(2) reverse phase column (100 Å, 150 mm × 2 mm, particle size 5 µm, Phenomenex) and samples for the analysis of other bioactive compounds were separated on a Luna C8(2) reverse phase column (100 Å, 150 mm × 2 mm, particle size 5 µm, Phenomenex) (Appendix 1 and 2).

Results

The isolate was identified as a filamentous cyanobacterial strain, with barrel-shaped vegetative cells forming a straight filament. Neither false branching nor a clear mucilaginous

sheath surrounding the cells was observed. Intercalary heterocysts were present. Thus the strain was classified as a member of the family Stigonemataceae. BLAST sequence similarity searches uncovered a sequence similarity of 100% between partial 16S rRNA gene sequences of the unknown strain and *Stigonema mamillosum* partial 16S rRNA gene sequence (Fig. 1).

PCR results confirmed potential toxigenicity of the unknown strain (Fig. 2). Both primer sets were specific for the genus *Stigomena*, delivering additional proof regarding the genus-level identity of the isolate. Other PCR reactions did not yield amplification products. The product ion spectra (MS2), retention times and ion masses of MC-LR (Fig. 1) and MC-RR were consistent with those of the measured standards. Besides microcystins, several other cyclic peptides were identified (Fig.3) (Table 3 and 4).

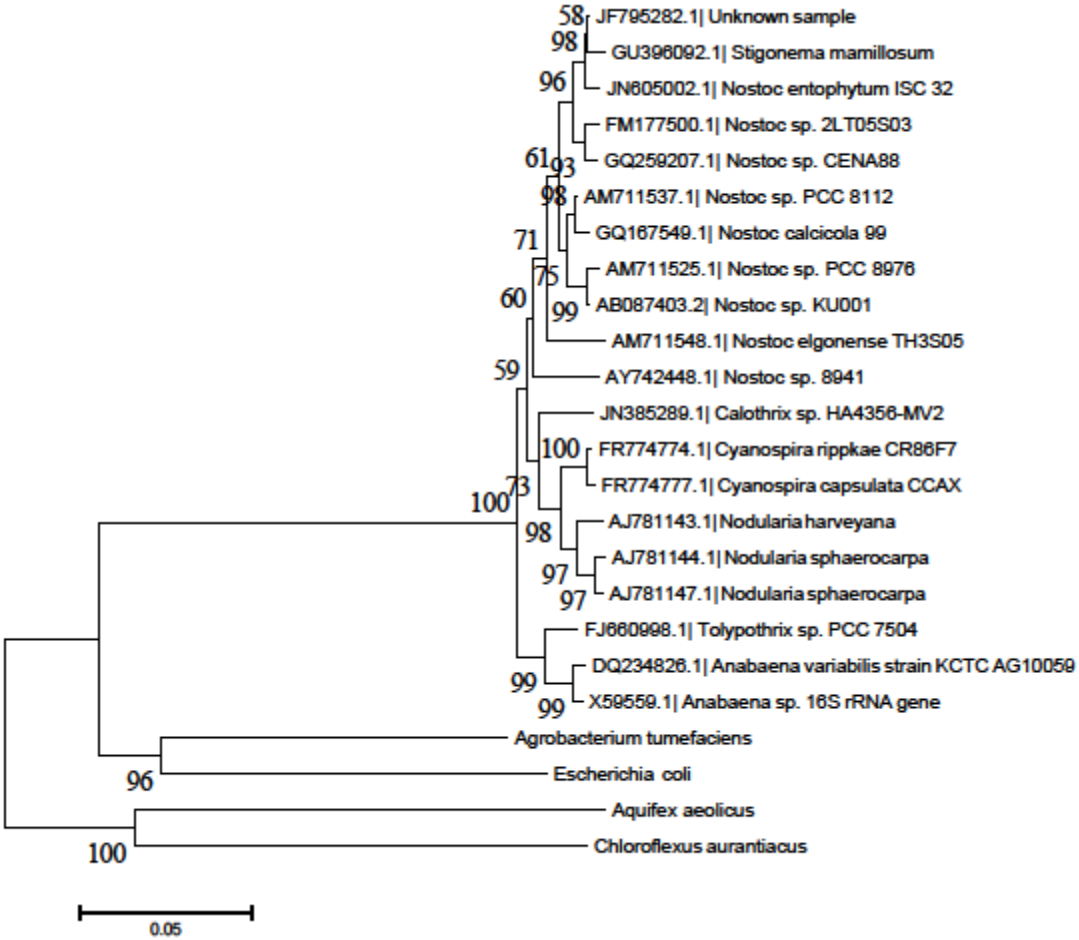

Figure 1. Consensus bootstrap tree on the basis of neighbour-joined distances of 1,299 bp long 16S rRNA gene sequences. It illustrates the phylogenetic position of the unknown strain (Sample 8) in relation to other cyanobacterial taxa. *A. aeolieus, C. aurantiacus, A. tumefaciens* and *E. coli* represent outgroup species.

Discussion

Our results show the isolated strain produces hepatotoxic microcystins. We have identified the strain as *Stigomena* species, highly similar to the freshwater strain *Stigonema mamillosum* that was isolated in 1986 from Lake Ali-Abad, Iran. Freshwater *Stigonema* species are predominant in toxic or non-toxic blooms of some lakes (9) and known for their potential production of microcystins (10, 11). Besides microcystins, we have discovered that the isolated strain produces a broad variety of other secondary metabolites, a common feature of freshwater cyanobacteria (12). Interestingly, the cyclic lipopeptide hassallidin was

not detected to be produced by the isoalted *Stigonema* strain. A positive amplification of the *hasA* indicated potential production. It appears that the strain carries a cryptic biosynthetic gene cluster, containing at least one non-functional gene. This finding resembles the discovery of Vestola et al. (13), who discovered a cryptic hassallidin gene cluster, inactivated by a frameshift mutation of the *hasV* gene, in the highly similar strain *Stigonema mamillosum.* Cryptic biosynthetic gene clusters may increase the adaptive potential of microbial strains by allowing them to activate production via mutation within a few generations (14).

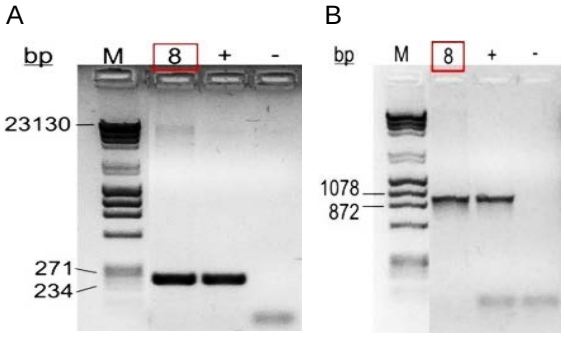

Figure 2. Visualised *mcyE* **(A)** and *hasA* **(B)** gene amplification products of the unknown strain (8). The products obtained were of the same size as the expected amplicon: 247 bp for *mcyE* (A) and 920 for *hasA*.

Among the microcystins discovered, was the highly potent variant MC-LR (median lethal dose [LD_{50}] of 50 µg per kg of mice) was found (2). The other microcystin variants detected were less potent, nonetheless still known to exert significant toxicity: The LD_{50} value of MC-RR accounts for 600 μgkg^{-1}, 250 μgkg^{-1} for [D-Asp3]MC-RR and 160-300 μgkg^{-1} for [D-Asp3] MC-LR (2). Anabaenopeptins have been reported to cause a relaxation of noradrenaline-induced contraction of aortic preparions in rats and anabeanopeptolides have been documented to cause inhibition of the serine protease chymotrypsin (12). Their effect on whole mammalian organisms remains, however, unknown. Furthermore, the toxicity of Anacylamide A10 remains to be evaluated (15).
Considering previous cases of mammalian poisonings (1) in addition to the high potency of the MC-LR variant, it is likely that the dog suffered from microcystin intoxication. This assumption is also in accordance with pathologic findings reported by the local authorities. Their findings revealed hepatocellular necrosis, indicating that the dog has suffered from liver damage and potential liver failure. The amount of MC-LR in ng per mg dry mass is significant (Table 3). However, the amount per liquid culture medium only accounted for 0.04 µg. Considering the LD_{50} value of the compound (50 $\mu g\ kg^{-1}$) and the dog's weight of 35 kg, the dog must have ingested extensive

Table 3. Molecular-related ions (*m/z*), corresponding retention times, peak areas and amounts of detected Microcystin variants from the analyzed isolate.

Variant	[M-H]$^{-a}$	Rt^{b} (min)	Peak area	S/N^{c}	C (ng/mg)^{d}	C (ng/ml)^{e}
MC-RR	1036	12.9	1807627	39.7	375.5	9.6
[D-Asp3] MC-RR	1022	12.3	134968	5.0	51.2	1.3
MC-LR	993	18.3	16776403	381.8	1713.0	44.5
[D-Asp3] MC-LR	979	18.3	3677611	97.9	686.0	17.8

a, Deprotonated ion mass, b, Retention time; c, Signal to noise ratio; d, MC concentration per mg dry weight; e, MC concentration per mg liquid culture; f, D-Asp: D-aspartic acid;

Table 4. Molecular related ions (m/z) and retention times of the detected cyclic peptides from the isolated strain.

Compound	Peptide Category	[M+H]$^{+}$
Anabaenopeptilide A	Cyclic depsipeptide	935
Anabaenopeptilide B		956
Anabaenopeptin A	Cyclic ureido peptide	845
Anabaenopeptin B		838
Anabaenopeptin C		810
Anacyclamide A10	Cyanobactin	1035

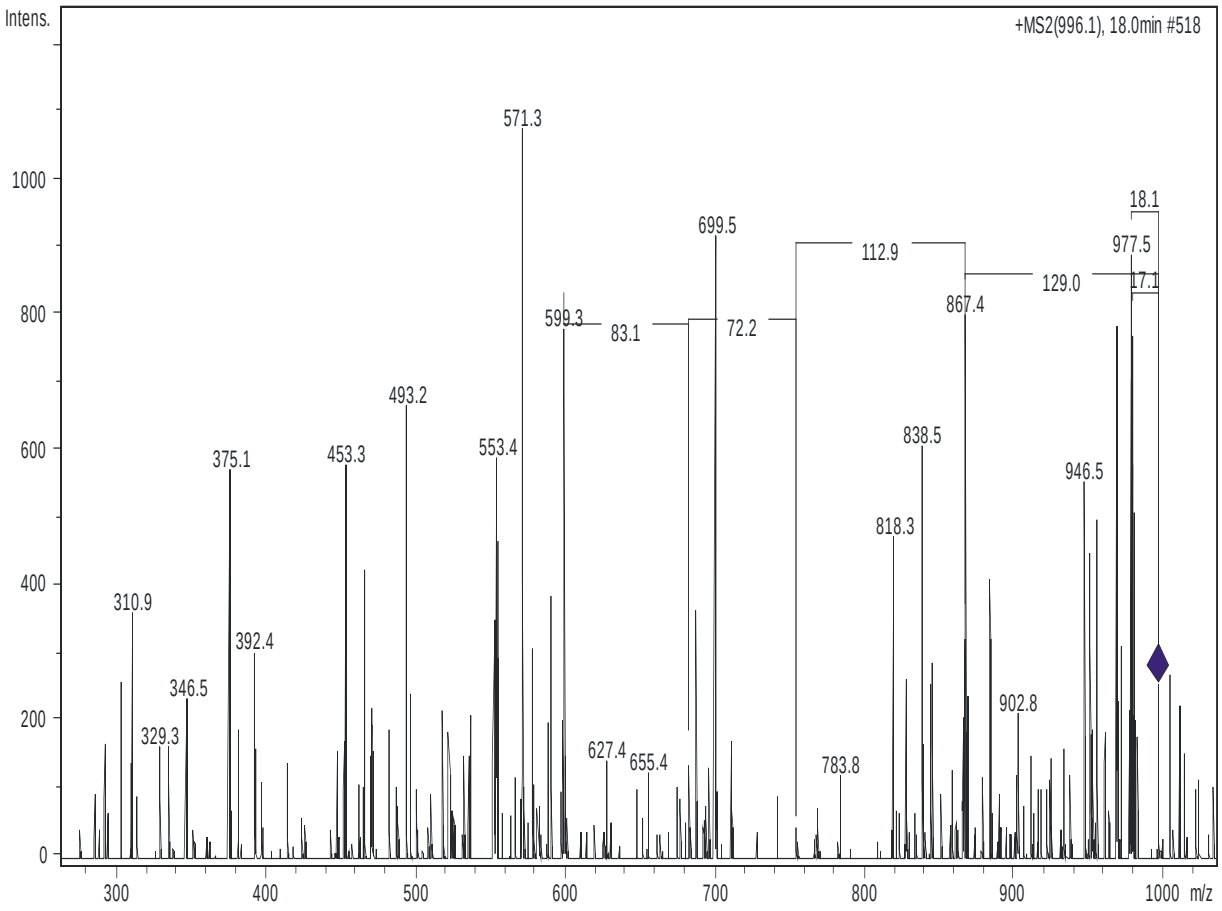

Figure 3. Product ion spectrum of protonated MC-LR (*m/z* 996). Characteristic neutral losses of the parent ion (PI) corresponding to PI - 129 mass unites = *m/z* 867 **(A)** A-113 mass units **(B)** B - 72 mass units **(C)** and C- 83 = *m/z* 599 mass units, are indicated with scale lines.

amounts of bloom water (around 43 litres) to be intoxicated lethally. This represents a highly unlikely scenario. There is thus a reason to assume that also other factors influenced the lethal outcome of this incident. It remains, however, the first reported case of potential microcystin intoxication of a dog in Iran. Further chemical analysis of organ tissue, as performed for example by Simola et al. (16), would deliver final proof for what the data gathered in this report suggests.

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References

1. Sivonen,K. (2009) Cyanobacterial Toxins. In Schaechter,M. (ed), *Encyclopedia of Microbiology*. Elsevier, pp. 290-307.
2. Sivonen,K. and Jones,G. (1999) Cyanobacterial toxins. In Chorus,I., Bartram,J. (eds), *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring, and management*. E & FN Spon, pp. 41-111.
3. Nübel,U., Garcia-Pichel,F. and Muyzer,G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied Environmental Microbiology*, **63**, 3327-3332.
4. Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.

Nucleic acids research, **22**, 4673-80.

5. Hall,T. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*, **41**, 95-98.
6. Felsenstein,J. (2005) PHYLIP (Phylogeny Inference Package).
7. Saitou,N. and Nei,M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, **4**, 406-25.
8. Page,R.D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Computer applications in the biosciences : CABIOS*, **12**, 357-8.
9. Sivonen K, Niemelä SI, Niemi RM, Lepistö L, Luoma TH & Räsänen LA. (1990) Toxic cyanobacteria (blue-green algae) in Finnish fresh and coastal waters. *Hydrobiologia*, **190**, 267-275.
10. Rantala,A., Rajaniemi-Wacklin,P., Lyra,C., Lepistö,L., Rintala,J., Mankiewicz-Boczek,J. and Sivonen,K. (2006) Detection of microcystin-producing cyanobacteria in Finnish lakes with genus-specific microcystin synthetase gene E (mcyE) PCR and associations with environmental factors. *Applied and environmental microbiology*, **72**, 6101-10.
11. Sivonen,K., Namikoshi,M., Evans,W., Carmichael,W., Sun,F., Rouhiainen,L., Luukkainen,R. and Rinehart,K. Isolation and Characterization of a Variety of Microcystins from Seven Strains of the Cyanobacterial Genus Anabaena. *Applied and Environmental Microbiology*, **58**, 2495-2500.
12. Harada,K.-I. (2004) Production of secondary metabolites by freshwater cyanobacteria. *Chemical & pharmaceutical bulletin*, **52**, 889-99.
13. J,V., J,J. and Fewer DP, Shishido T, Wahlsten M, Wang H,R.L.& S.K.U. The non-ribosomal biosynthesis of novel variants of the antifungal glycolipopeptide hassallidin, produced by several cyanobacterial genera.
14. Hall,B., Yokoyama,S. and Calhoun,D. (1983) Role of cryptic genes in microbial evolution. *Molecular biology and evolution*, **1**, 109-124.
15. Leikoski,N., Fewer,D.P., Jokela,J., Wahlsten,M., Rouhiainen,L. and Sivonen,K. (2010) Highly diverse cyanobactins in strains of the genus Anabaena. *Applied and environmental microbiology*, **76**, 701-9.
16. Simola,O., Wiberg,M., Jokela,J., Wahlsten,M., Sivonen,K. and Syrjä,P. (2012) Pathologic findings and toxin identification in cyanobacterial (Nodularia spumigena) intoxication in a dog. *Veterinary pathology*, **49**, 755-9.
17. Vaitomaa,J., Rantala,A., Halinen,K., Rouhiainen,L., Tallberg,P., Mokelke,L. and Sivonen,K. (2003) Quantitative real-time PCR for determination of microcystin synthetase e copy numbers for microcystis and anabaena in lakes. *Applied and environmental microbiology*, **69**, 7289-97.
18. Koskenniemi,K., Lyra,C., Rajaniemi-Wacklin,P., Jokela,J. and Sivonen,K. (2007) Quantitative real-time PCR detection of toxic Nodularia cyanobacteria in the Baltic Sea. *Applied and environmental microbiology*, **73**, 2173-9.
19. Vestola J, Jokela J, Fewer DP, Shishido T, Wahlsten M, Wang H, Rouhiainen L & Sivonen K. Unpublished. The non-ribosomal biosynthesis of novel variants of the antifungal glycolipopeptide hassallidin, produced by several cyanobacterial genera.