rRNA sequences reflect the ecophysiology and define the toxic cyanobacteria of the genus *Nodularia*

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***Nodularia*, a member of the order *Nostocales*, is a bloom-forming filamentous cyanobacterium that possesses the ability to form toxic blooms. The toxin produced by *Nodularia*, nodularin, is a hepatotoxin, similar in structure to the heptapeptide toxin microcystin. Twenty-one strains of *Nodularia*, representing the species *Nodularia spumigena*, *Nodularia harveyana* and *Nodularia sphaerocarpa*, were analysed for toxin production by protein phosphatase inhibition assay and sequenced over the 16S rDNA region. Phylogenetic analysis of *Nodularia* 16S rDNA sequences found that *Nodularia* clustered into two main groups. An *N. spumigena* cluster was distinct from the benthic species *N. harveyana* and *N. sphaerocarpa*. There was no distinction between strains isolated from globally diverse locations. Nodularin-producing species were restricted to the single, evolutionally distinct cluster of *N. spumigena*. This observation has enabled the design of a specific 16S rRNA PCR for the rapid detection of nodularin-producing strains. Alignment of 16S rDNA sequences from toxic and non-toxic *Nodularia* with other members of the cyanobacteria allowed the design of both *Nodularia* generic and toxic *N. spumigena*-specific primers.**

Keywords: *Nodularia*, cyanobacteria, 16S rRNA, nodularin, evolution

Introduction

The cyanobacteria phylum is one of the 11 traditional phyla of the *Bacteria* (Schleifer & Ludwig, 1989; Woese, 1987). A number of cyanobacteria are of interest due to their bloom-forming potential and their ability to produce a wide range of secondary metabolites (Carmichael *et al.*, 1988; Patterson *et al.*, 1994). Currently, the genus *Nodularia* is defined taxonomically by its morphological characters and ecological requirements (Komárek *et al.*, 1993). *Nodularia* is a bloom-forming filamentous cyanobacterium, placed within the order *Nostocales* and family *Nostocaceae* (Komárek *et al.*, 1993; Nordin & Stein, 1980; Rippka *et al.*, 1979).

Species within the genus *Nodularia* are classified by a combination of morphological features and the ecological niche from which they are isolated (Komárek *et al.*, 1993). Historically, the classification of species

within the genus *Nodularia* has been controversial. Recently, the genus was revised by Komárek *et al.* (1993) to yield seven taxa. Taxonomically, the production of gas vesicles divides the *Nodularia* genus into two main groups: the benthic or terrestrial species *Nodularia harveyana*, *Nodularia sphaerocarpa* and *Nodularia willeii*; and the planktonic species *Nodularia spumigena*, *Nodularia baltica*, *Nodularia litorea* and *Nodularia crassa* (Komárek *et al.*, 1993).

Traditionally, cyanobacteria are identified according to their morphology, but due to several and variable morphotypes and the problems associated with laboratory culturing, the use of molecular techniques for cyanobacterial identification is now in common use. In the past, phylogenetic studies of *Nodularia* using 16S rRNA sequence analysis has been limited (Lehtimäki *et al.*, 1994; Barker *et al.*, 1999). However, a study of the 16S rDNA sequences of three Baltic Sea *Nodularia* isolates found each to be identical (Barker *et al.*, 1999). Phylogenetic studies of *N. spumigena* strains have been performed using the DNA sequence or RFLPs of the phycocyanin intergenic spacer (IGS) region and

Abbreviations: IGS, intergenic spacer; PP-2A, protein phosphatase 2A; RAPD, randomly amplified polymorphic DNA.

randomly amplified polymorphic DNA (RAPD)-PCR (Bolch *et al.*, 1996, 1999; Hayes & Barker, 1997; Neilan *et al.*, 1995). DNA sequencing of the *cpc*-IGS and analysis of *cpc*-IGS RFLP fragments from Baltic, Canadian and Australian *Nodularia* isolates by Hayes & Barker (1997) suggested that *Nodularia* genotypes may have a global distribution. Consecutive studies by Bolch *et al.* (1996, 1999) reviewed the *Nodularia* taxonomy by *cpc*-IGS RFLPs, sequencing and RAPD-PCR and compared these results to toxicity and morphology (Bolch *et al.*, 1999). Results indicated that overseas strains were genetically distinct from Australian strains, refuting the argument by Hayes & Barker (1997) that *Nodularia* genotypes are globally distributed. These conflicting studies supported the need for further study into *Nodularia* phylogeny and evolution.

Blooms of *Nodularia* are common within the estuaries and coastal lagoons of Australia (Jones *et al.*, 1994; Heresztyn & Nicholson, 1997), occur annually during summer months in the Baltic Sea (Sivonen *et al.*, 1989) and have been reported in the German North Sea coast (Nehring, 1993), New Zealand (Carmichael *et al.*, 1988) and North America (Galat *et al.*, 1990). Many *Nodularia* blooms are highly toxic due to their production of the cyclic pentapeptide hepatotoxin nodularin. The consumption of water-containing toxic *Nodularia* blooms has led to the death of domestic and native animals by massive liver haemorrhage in Australia, the Baltic Sea and New Zealand (Carmichael *et al.*, 1988; Francis, 1878; Nehring, 1993; Carmichael, 1994). In subacute doses, nodularin may act as a liver tumour promoter and initiator (Ohta *et al.*, 1994). For these reasons, rapid and accurate identification of toxic *Nodularia* in cyanobacterial bloom events is important.

In the present study, several strains of *N. spumigena*, *N. harveyana* and *N. sphaerocarpa*, isolated from geographically diverse regions, were analysed for the production of toxin and sequenced over the 16S rRNA gene region to determine if the current *Nodularia* morphological taxonomy is supported by 16S rRNA phylogeny. It was also an aim of this work to confirm or refute the proposed globality of *Nodularia* species distribution based on the sequence of 16S rDNA, an evolutionally significant character. These sequence data were also used to design PCR primers for the specific detection of toxic-bloom-forming strains of the genus *Nodularia* and for the identification of morphologically ambiguous strains from culturable and non-culturable environmental isolates.

Methods

Bacterial strains. Cyanobacterial strains used in this study (Table 1) were provided by the CSIRO Marine Laboratories (Hobart, Tasmania), Department of Applied Chemistry and Microbiology (University of Helsinki, Finland) and the Department of Biological Sciences (Wright State University, Dayton, OH, USA). Other strains were also obtained from Pasteur Culture Collection (PCC), the Culture Collection of

Algae at the University of Texas (UTEX) and the Czechoslovak Database of Algae and Cyanobacteria (CDAC). Cultures were maintained in BG-11 media at 25 °C with an illuminance of 1500 lx. All cell cultures were uni-algal. Heterotrophic bacteria were removed by filtering of cyanobacterial cultures through a 3.0 µm SS type MF-Millipore filter and washing with sterile milli-Q water.

Measurement of toxicity. Bioassay for nodularin content was performed using a protein phosphatase 2A (PP-2A) inhibition assay as previously described by An & Carmichael (1994). Toxin was liberated from cells by three freeze-thaw cycles. The sample was then incubated with PP-2A enzyme at 37 °C for 80 min. The toxicity of the sample was determined by assaying the inability of the PP-2A to convert the substrate *p*-nitrophenol phosphate to nitrophenol phosphate. The relative concentration of nodularin was determined from a standard curve of inhibition of PP-2A by given concentrations of microcystin.

DNA isolation, amplification and sequencing. Total genomic DNA was extracted using an SDS/lysozyme-based method as previously described by Neilan *et al.* (1995). Cells were incubated with 0.5 ml TNE buffer [50 mM Tris (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM NaCl] and lysozyme (Pharmacia) at a final concentration of 1 mg ml⁻¹ for 30 min at 55 °C. This was followed by the addition of 10 µl 20% SDS and 10 µl proteinase K (10 mg ml⁻¹) (Promega) and incubated for a further 10 min at 55 °C. DNA was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated from the supernatant by the addition of 1 vol. 4 M ammonium acetate and 1 vol. 2-propanol.

All PCR reactions were performed using 1 unit *Taq* polymerase (Fischer Biotech) in a 30 µl reaction mix containing 2.5 mM MgCl₂, 1 × *Taq* polymerase buffer (Fischer Biotech), 0.2 mM dNTPs (Boehringer Mannheim), 0.2 pM forward and reverse primer and 1 ng DNA template. The final volume was adjusted with sterile milli-Q water. Thermal cycling was performed in a PCR Sprint Temperature Cycling System machine (Hybaid) or a GeneAmp PCR System 2400 Thermocycler (Perkin Elmer). Amplification of the near-complete 16S rRNA gene was performed by PCR using oligonucleotide primers and conditions described by Neilan *et al.* (1997). The initial denaturation step at 94 °C for 5 min was followed by 30 cycles of DNA denaturation at 94 °C for 5 s, primer annealing at 55 °C for 10 s, strand extension at 72 °C for 45 s and a final extension step at 72 °C for 7 min. Amplification using the toxic *Nodularia*-specific primer, NTS [TGTGATGCAAATCTCA(C/A)A] and universal 16S rRNA reverse primer 1494R (Neilan *et al.*, 1997) was performed with thermal cycling conditions as above, with a primer annealing temperature of 63 °C. Amplification using the *Nodularia* generic primer, NS2 (GGCGAAGGCGCTCTACTA) and universal 16S rRNA reverse primer 1494R (Neilan *et al.*, 1997) was performed in a two-step thermal cycling procedure with an initial denaturation step of 94 °C for 5 min, 30 cycles of DNA denaturation at 94 °C for 5 s, primer annealing and strand extension at 69 °C for 45 s, and a final extension step at 72 °C for 7 min.

Automated DNA sequencing was performed using the PRISM Big Dye cycle sequencing system and a model 373 sequencer (Applied Biosystems). Oligonucleotide primers were synthesized using a model 8000 DNA synthesis system (Beckman).

All *Nodularia* 16S rDNA sequences were checked for ambiguities using the CHIMERA detection program in the

Table 1 Cyanobacterial strains used in this study and their ability to produce nodularin as determined by protein phosphatase inhibition assay

Species*	Strain	Co-identity	Site of isolation	Isolation	Nodularin prodn†	Reference	Accession no.
<i>N. spumigena</i>	NSBL05		Lake Bullenmeri, VIC, Australia	S. Blackburn, 1993	—		AF268012
<i>N. spumigena</i>	NSBR01		Blackwood River, WA, Australia	T. Pitman, 1994	+		AF268016
<i>N. spumigena</i>	NSGL02A10		Gippsland Lakes, VIC, Australia	C. Bolch, 1993	+	Bolch <i>et al.</i> (1999)	AF268015
<i>N. spumigena</i>	NSKR07		Kaljon River, WA, Australia	T. Pitman, 1995	+		AF268013
<i>N. spumigena</i>	NSLA01		Lake Alexandrina, SA, Australia	P. Baker, 1990	+	Bolch <i>et al.</i> (1999)	AF268009
<i>N. spumigena</i>	NSLA02A4		Lake Alexandrina, SA, Australia	T. Pitman, 1994	+		AF268008
<i>N. spumigena</i>	NSOR04		Orielton Lagoon, TAS, Australia	C. Bolch, 1993	+	Bolch <i>et al.</i> (1999)	AF268010
<i>N. spumigena</i>	NSOR10		Orielton Lagoon, TAS, Australia	S. Blackburn, 1993	+		AF268014
<i>N. spumigena</i>	NSOR12		Orielton Lagoon, TAS, Australia	S. Blackburn	+		AF268011
<i>N. spumigena</i>	NSPH02		Peel Inlet, WA, Australia	S. Blackburn, 1992	+		AF268007
<i>N. spumigena</i>	NSPH05A14		Peel Inlet, WA, Australia	T. Pitman, 1994	+		AF268017
<i>N. spumigena</i>	L575		Lake Ellesmere, New Zealand	W. Carmichael, 1980	+‡	Carmichael <i>et al.</i> (1988)	AF268006
<i>N. spumigena</i>	BY1		Baltic Sea	K. Sivonen	+	Lehtimäki <i>et al.</i> (1994)	AF268004
<i>N. spumigena</i>	HEM		Baltic Sea	K. Sivonen	+	Lehtimäki <i>et al.</i> (1994)	AF268005
<i>N. spumigena</i> / <i>N. sphaerocarpa</i>	HKVV		Baltic Sea	K. Sivonen	—	Lehtimäki <i>et al.</i> (1994)	AF268024
<i>N. spumigena</i>	PCC 73104 ^T	UTEX-B2091 ^T , ATCC 29167 ^T , CCAP-1452/4 ^T	Alkaline soil, Spotted Lake, BC, Canada	R. Rippka, 1979	—	Rippka <i>et al.</i> (1992)	AF268023
<i>N. harveyana</i>	PCC 7804		Thermal Spring, Dax, France	R. Pourriot, 1966	+	Rippka <i>et al.</i> (1992)	AF268019
<i>N. spumigena</i>	UTEX-B2092		Alkaline soil, Osoyoos, BC, Canada	J. Stein, 1974	—		AF268022
<i>N. harveyana</i>	UTEX-B2093	PCC 9336	Pond, San de Fuca, Whidbey Island, WA, USA		—	Nordin & Stein (1980); Rippka <i>et al.</i> (1992)	AF268021
<i>N. harveyana</i>	CDAC1983/300		Island Hiddensee, Lagoon-Gellen, Germany	Habel, 1983	—	Komárek <i>et al.</i> (1993)	AF268020
<i>N. sphaerocarpa</i>	CDAC1966/93-1		Thermal Spring, Dax, France	R. Pourriot, 1966	—	Komárek <i>et al.</i> (1993)	AF268018
<i>Anabaena</i> sp.	PCC 7210		Unknown	R. Haselkorn	ND	Ligon <i>et al.</i> (1991)	X59559
<i>Anabaena</i> <i>cylindrica</i>	NIES19		England		ND		
<i>Cylindrospermopsis</i>	AWT205		Ornamental Pond, NSW, Australia		ND	Hawkins <i>et al.</i> (1997)	
<i>Lyngbya</i>	AWT211		Unknown		ND		
<i>Aphanizomenon</i>	NIES81		Lake Kasumigaura, Japan		ND		
<i>Synechocystis</i>	PCC 6803		Fresh water, CA, USA	R. Kunisawa, 1968	ND	Rippka <i>et al.</i> (1979)	
<i>Microcystis</i> <i>aeruginosa</i>	PCC 7806		Braakman Reservoir, The Netherlands	R. Rippka, 1972	ND	Rippka <i>et al.</i> (1992)	

* *Nodularia* strains listed were sequenced over the 16S rDNA region. The *Anabaena* strain was used in the phylogenetic analysis. All other cyanobacterial genera listed were used to determine the specificity of *Nodularia* 16S rRNA oligonucleotide primers.

†+ or — indicates the presence or absence of toxin as determined by PP-2A inhibition assay. ND, Not determined.

‡ L575 culture was unavailable for toxicity assay; however, it has been shown to produce nodularin (Carmichael *et al.*, 1988).

Ribosomal Database Project II (<http://www.cme.msu.edu/RDP/cgis/chimera.cgi>).

Phylogenetic analyses. All 16S rRNA gene sequences used in this study were aligned using the programs PILEUP from GCG and the multiple-sequence alignment tool from CLUSTAL W (Thompson *et al.*, 1994). Manual alignment of the output was also performed with consideration for the secondary structure of the 16S rRNA molecule. The input order of taxa was randomized for all sequence alignments and phylogenetic inference programs. Genetic distances (D) between strains were calculated using the formula described by Jukes & Cantor (1969), $D = -\frac{3}{4} \ln(1 - \frac{4}{3}d)$, where d is the level of sequence dissimilarity. The phylogenetic inference protocols DNADIST, NEIGHBOR, SEQBOOT and CONSENSE were supplied by the PHYLIP package (version 3.57c) (Felsenstein, 1985). Sequence manipulation and phylogeny programs were accessed through the Australian National Genome Information Service (Sydney, Australia).

RESULTS AND DISCUSSION

Phylogenetic analysis of *Nodularia* 16S rDNA sequences

Near-complete sequences from position 27F to 1494R of the 16S rRNA gene were generated for 21 strains of *Nodularia* during this study (Table 1). All *Nodularia* strains sequenced were most similar, by BLAST analysis, to the *Nodularia* strain BCNOD9427 16S rRNA gene sequence (accession no. AJ224447) in the EMBL database (similarity 96–99%).

The current morphological taxonomy of *Nodularia* describes the genus as being composed of seven species (Komárek *et al.*, 1993). This phylogenetic study concentrated on three main species, i.e. those which were available for molecular investigations. A phylogenetic distance tree (Fig. 1) was obtained from the alignment of *Nodularia* sequences with *Nodularia* sp. BCNOD9427 and *Anabaena* PCC 7120^T, the type strain from a closely related genus of the order *Nostocales* used as an outgroup. Evolutionary relationships (Fig. 1) and genetic distances were determined by the alignment of 1322 bp from within the 16S rRNA gene sequence. Parsimony and maximum-likelihood analysis confirmed the tree topology presented is the most probable.

Strains of *N. spumigena* isolated from blooms in Australia, New Zealand and the Baltic Sea clustered together as one group. This *N. spumigena* group could further be separated into two subgroups: one containing Australian strains only; and the other group containing strains from the Baltic Sea, New Zealand and from Lake Alexandrina in Australia. *N. harveyana* and *N. sphaerocarpa* are morphologically distinct from *N. spumigena* due to their inability to produce gas vesicles. *N. harveyana* and *N. sphaerocarpa* strains were phylogenetically distinct from the *N. spumigena* clade (Fig. 1). This benthic group may be polyphyletic, although delineation of the benthic strains into the species *N. harveyana* and *N. sphaerocarpa* was not possible due to the lack of strains representative of these species available for this study. Future analysis

Fig. 1. Phylogenetic representation of the genus *Nodularia* based on the alignment of 1322 bases within the 16S rRNA gene sequences used. Genetic distances (D) between strains were calculated using the formula described by Jukes & Cantor (1969), $D = -\frac{3}{4} \ln(1 - \frac{4}{3}d)$, where d is the level of sequence dissimilarity. The phylogenetic inference protocols DNADIST, NEIGHBOR, SEQBOOT and CONSENSE were supplied by the PHYLIP package (version 3.57c) (Felsenstein, 1985). Scale bar, 5 base substitution per 1000 nucleotides. Significant bootstrap values (Felsenstein, 1985) calculated from 1000 bootstrap trees are indicated at the nodes. ¹*N. spumigena* NSBL05 appeared non-toxic by the PP-2A assay. ²*N. harveyana* PCC 7804 appeared toxic by the PP-2A assay.

of these groups should be performed to determine the correct speciation of the benthic strains. The line of descent separating the planktonic cluster from the benthic group gave a bootstrap value of 38.5% (Fig. 1).

Percentage similarities and the pairwise genetic distance matrix was produced by excluding gaps within the sequence alignment. Analysis of similarities produced when gaps were excluded from the alignment showed that the *Nodularia* genus was supported by similarities of greater than 96%, whereas similarities to the outgroup *Anabaena* were less than 94% (data not shown). The *N. spumigena* cluster was supported by percentage similarities of greater than 98.7% and were up to 100% similar. Similarities within the *N. harveyana*/*N. sphaerocarpa* group were greater than 94.7%. The percentage similarities and genetic distances support the *Nodularia* strains analysed in this study as a distinct genera from the *Anabaena* used as an outgroup in this study.

Evolutionary distribution of nodularin production throughout the genus *Nodularia*

Production of nodularin was determined for all strains used in this study (Table 1). Since nodularin inhibits PP-2A at approximately the same potency as microcystin (Honkanen *et al.*, 1990, 1991), the apparent concentration of nodularin was determined using a standard curve of PP-2A inhibition by standard concentrations of microcystin-LR (data not shown). All *N. spumigena* strains isolated from Australia and the Baltic Sea, excluding *N. spumigena* NSBL05, exhibited high levels of toxicity. All toxic *Nodularia* strains produced nodularin at levels of greater than 8000 pmol (mg protein)⁻¹ and were as high as 62000 pmol (mg protein)⁻¹ for *N. spumigena* NSKR07 in comparison to *Microcystis aeruginosa* PCC 7806, which contained levels of microcystin at 3000 pmol (mg protein)⁻¹. The detection limit of the standard curve was 33 pmol, and all strains below this limit were deemed non-toxic. The taxonomically controversial strain *N. spumigena*/*N. sphaerocarpa* HKVV, *N. spumigena* strain PCC 73104^T and *N. spumigena* strain UTEX-B2092 appeared not to produce toxin. All strains of *N. harveyana* and *N. sphaerocarpa* analysed showed no toxic effect, with the exception of *N. harveyana* PCC 7804, which appeared to be toxic by the protein phosphatase assay. Toxin data appeared to be consistent with previous toxin data (Bolch *et al.*, 1999; Neilan *et al.*, 1999) with the exception of PCC 73104^T, which was previously described as toxic (Neilan *et al.*, 1999) but was found to be non-toxic in this study.

Toxic strains of *N. spumigena* that were examined by 16S rDNA sequencing were all members of a phylogenetically distinct group (Fig. 1). Biosynthesis of the hepatotoxin nodularin appears to be restricted to a single *N. spumigena* cluster. The non-toxic *N. spumigena* NSBL05 did not appear, however, to be phylogenetically different from other toxic bloom isolates (Fig. 1). This isolate is thought to contain a natural mutation or repressed nodularin biosynthesis (Bolch *et al.*, 1999; M. C. Moffitt, unpublished data). Non-toxic strains *N. spumigena* PCC 73104^T and *N. spumigena* UTEX-B2092 were in a separate group from the toxic cluster and appeared in the non-toxic *N. harveyana*/*N. sphaerocarpa* polyphyletic group. All non-toxic strains defined taxonomically as *N. harveyana* and *N. sphaerocarpa* by classical methods (Komárek *et al.*, 1993) appeared to be delineated from the major toxic *N. spumigena* group in this study. Toxic strain *N. harveyana* PCC 7804 did, however, group with these non-toxic strains (Fig. 1). Toxin from this strain is thought to be a nodularin derivative, having a different HPLC retention time from that of nodularin found in toxic *N. spumigena* (Bolch *et al.*, 1999).

According to the present 16S rDNA sequence analysis, *N. spumigena* PCC 73104^T, the type strain for *N. spumigena*, and *N. spumigena* UTEX-B2092 did not

phylogenetically cluster within the *N. spumigena* group. The ecological niche from which both strains were isolated was alkaline soil (Table 1), whereas strains within the *N. spumigena* phylogenetic cluster were isolated from planktonic algal bloom events. This clustering of *N. spumigena* PCC 73104^T is consistent with results of *cpc*-IGS sequencing and RAPD-PCR performed by Bolch *et al.* (1999). The speciation of the non-toxic *Nodularia* strain HKVV is taxonomically incomplete and, as a result, has been confused in the past (Fujii *et al.*, 1997; Lehtimäki *et al.*, 1994). Analysis of 16S rDNA in this study indicated that the non-toxic Baltic strain HKVV should be considered to be in the non-toxic group of *N. sphaerocarpa* and that this may be the correct speciation for this strain. Benthic strains of *N. spumigena* may also be reclassified within the group of benthic *Nodularia*. Since the type strain of *N. spumigena* is PCC 73104^T, reclassification of the toxic-bloom-forming clade may be required.

Amongst the toxic cluster, there appears to be no delineation between strains isolated from geographically distinct regions and there appears to be a continuous cline of globality amongst toxic-bloom-forming *N. spumigena*. Similar results were obtained in a previous study which analysed *cpc*-IGS sequences of *Nodularia* (Hayes & Barker, 1997). Hayes & Barker (1997) detected two distinct genotypes, one genotype which included Baltic strains BCNOD9427 and BY1 and the Canadian strain PCC 73104^T, and a second genotype containing Baltic and Australian isolates. Contrary to results of *cpc*-IGS RFLP analysis (Bolch *et al.*, 1999; Hayes & Barker, 1997), this study did not show that PCC 73104^T was in the same cluster as BY1 and BCNOD9427. However, PCC 73104^T did cluster with the Baltic Sea strain HKVV. Recent findings resulting from *cpc*-IGS sequencing and RAPD-PCR were able to divide Australian strains into two main groups and show that they were distinct from overseas strains with the exception of strain BY1 (Bolch *et al.*, 1999). The 16S rRNA phylogenetic tree created in this study was similar to the phylogenetic tree resulting from RAPD-PCR created by Bolch *et al.* (1999) with the toxic Baltic strain BY1 clustering with the toxic *N. spumigena* group and the overseas benthic *N. harveyana* and *N. sphaerocarpa* strains clustering distinct from planktonic and toxic *N. spumigena* Baltic Sea strains BCNOD9427 and HEM. New Zealand strain L575, which was not included in the Bolch *et al.* (1999) study, also clustered within the group of toxic *N. spumigena*. The combination of these data indicate that the phylogenetics of the small subunit RNA is reflected by the genomic evolution for this group of cyanobacteria. Alternatively, the structure of phycocyanin genes in this group may have experienced adaptive evolution with respect to the strain's ecological niche.

The clustering of toxic cyanobacterial strains within a single phylogenetically defined species as occurs in *Nodularia* has also been shown to occur within the genera *Cylindrospermopsis* and *Anabaena* (Beltran &

Fig. 2. Analysis of 16S rRNA gene regions using cyanobacterial (Neilan *et al.*, 1997) and *Nodularia*-specific oligonucleotide primers. Lanes 1–18 correspond to PCR fragments from strains *N. spumigena* L575, HEM, BY1, NSOR10, NSBL05, NSPH05A14, *N. spumigena*/*N. sphaerocarpa* HKVV, *N. sphaerocarpa* CDAC 1966/93-1, *N. harveyana* PCC 7804, *N. harveyana* CDAC 1983/300, *Anabaena cylindrica* NIES19, *Cylindrospermopsis raciborskii* AWT205, *Lyngbya* AWT211, *Aphanizomenon* NIES81, *Synechocystis* PCC 6803, *Microcystis aeruginosa* PCC 7806, *Anabaena* PCC 7210 and no DNA (control), respectively. The marker lanes (M) contain Φ X174/HaeIII. The cyanobacteria-specific 16S rDNA PCR amplified a 400 bp fragment from all strains analysed (CYAN). The *Nodularia* genus-specific 16S rDNA PCR amplified a 780 bp fragment from all *Nodularia* strains analysed in this study (NOD). The toxic *N. spumigena*-specific 16S rDNA PCR amplified a 200 bp fragment from all toxic planktonic bloom-forming *Nodularia* (TOX).

Neilan, 2000; L. Hardman, personal communication). In contrast, microcystin production within various genera of cyanobacteria does not appear to correlate with phylogeny according to 16S rRNA gene sequence analysis (Neilan *et al.*, 1997). This suggests, in evolutionary terms, that the ability to produce nodularin has occurred recently and is confined to globally distributed strains of *N. spumigena*. It would also indicate that the prolific gene transfer observed in microcystin-producing cyanobacteria is not occurring between species of *Nodularia* (Neilan *et al.*, 1999).

Specific 16S rDNA probes for the detection of *Nodularia*

Specific primers were designed from alignment of the 16S rDNA sequences from all *Nodularia* analysed in this study with other members of order *Nostocales* (data not shown). A specific forward PCR primer (NS2) for the identification of members of the *Nodularia* genus was designed at position 705 of the *Nodularia* 16S rDNA sequence. The *Nodularia*-specific primer amplified members of the genus *Nodularia* only and was unable to amplify other members of the cyanobacteria (*Cylindrospermopsis*, *Aphanizomenon* and *Anabaena*) (Table 1), including members of the *Nostocales*, as well as strains from the genera *Microcystis*, *Lyngbya* and *Synechocystis* (Fig. 2). Within the group of *Nodularia* strains studied, the identification of toxic *N. spumigena* strains was possible with a specific primer (NTS) designed at position 1250. The corresponding PCR using this toxic *Nodularia*-specific primer identified all nodularin-producing *N. spumigena* strains within the toxic-bloom-forming cluster and was unable to amplify non-toxic *Nodularia* strains and toxic strains from other genera (Fig. 2). In addition, these primers were able to amplify the

Nodularia-specific regions of the 16S rRNA gene from DNA extracted from a bloom sample predominantly containing toxic *N. spumigena* (M. C. Moffitt & B. A. Neilan, unpublished data). This primer, however, was unable to amplify DNA from the toxic strain *N. harveyana* PCC 7804.

This study was unable to analyse the phylogeny of the entire *Nodularia* morpho-genus, since the other four species are uncommon and not preserved or available as cultures. To accurately define the speciation of *Nodularia* as determined by the phylogenetic analysis of the 16S rDNA sequence, further analysis of representatives from all species of *Nodularia* is required. The *Nodularia* genus-specific primer presented here was useful for the amplification of a 780 bp region of the 16S rRNA gene from members of the *Nodularia* genus as defined by the species sequenced in this study. There was no amplification of this region of the 16S rRNA gene from other cyanobacteria tested. The toxic *N. spumigena*-specific primer was useful for the amplification of a 200 bp region from the 16S rRNA gene of toxic strains analysed in this study. However, this primer did not amplify this region in the toxic strain *N. harveyana* PCC 7804, which is not a bloom-forming strain of *N. spumigena*. The toxic *N. spumigena*-specific primer was useful for the detection of cultured and uncultured toxic and planktonic strains. Currently, identification of a toxic *Nodularia* bloom event requires the microscopic analysis of cellular material and analysis of toxicity by expensive techniques such as HPLC, requiring high-density bloom material. This study reports the creation of *Nodularia* genus-specific and toxic-bloom-forming *N. spumigena*-specific 16S rDNA oligonucleotide primers. These primers have the ability to detect low levels of *Nodularia* DNA in environmental samples, providing a quick and cheap

method for determining the presence of potential bloom-forming *Nodularia* in predisposed waterways. Hence, this primer may become useful in the rapid detection of toxin-producing strains in environmental bloom events.

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