Benthic cyanobacteria of the genus *Nodularia* are non-toxic, without gas vacuoles, able to glide and genetically more diverse than planktonic *Nodularia*

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Diversity and ecological features of cyanobacteria of the genus *Nodularia* from benthic, periphytic and soil habitats are less well known than those of *Nodularia* from planktonic habitats. Novel benthic *Nodularia* strains were isolated from the Baltic Sea and their morphology, the presence of gas vacuoles, nodularin production, gliding, 16S rRNA gene sequences, *rpoB*, *rbcLX* and *ndaF* genes, and *gvpA*-IGS regions were examined, as well as short tandemly repeated repetitive sequence fingerprints. Strains were identified as *Nodularia spumigena*, *Nodularia sphaerocarpa* or *Nodularia harveyana* on the basis of the size and shape of the different types of cells and the presence or absence of gas vacuoles. The planktonic strains of *N. spumigena* mostly had gas vacuoles and produced nodularin, whereas the benthic strains of *N. sphaerocarpa* and *N. harveyana* lacked gas vacuoles and did not produce nodularin (except for strain PCC 7804). The benthic strains were also able to glide on surfaces. In the genetic analyses, the planktonic *N. spumigena* and benthic *N. sphaerocarpa* formed monophyletic clusters, but the clusters were very closely related. Benthic strains determined as *N. harveyana* formed the most diverse and distant group of strains. In addition to phylogenetic analyses, the lack of the *gvpA*-IGS region and *ndaF* in *N. sphaerocarpa* and *N. harveyana* distinguished these species from the planktonic *N. spumigena*. Therefore, *ndaF* can be considered as a potential diagnostic tool for detecting and quantifying Baltic Sea bloom-forming, nodularin-producing *N. spumigena* strains. The data confirm that only one morphologically and genetically distinct planktonic species of *Nodularia*, *N. spumigena*, and at least two benthic species, *N. sphaerocarpa* and *N. harveyana*, exist in the Baltic Sea.

INTRODUCTION

The cyanobacterial genus *Nodularia* Mertens (Bornet & Flahault, 1886) comprises filamentous, uniseriate, heterocytous cyanobacteria, which occur in planktonic, benthic

and terrestrial habitats. Planktonic *Nodularia* strains produce nodularin hepatotoxin and form toxic blooms in brackish and saline water bodies around the world (Sivonen *et al.*, 1989; Harding *et al.*, 1995; Bolch *et al.*, 1999; del Carmen Pérez *et al.*, 1999).

Abbreviations: *gvpA*-IGS, intergenic spacer between two copies of gas vacuole protein A genes; ITS, internal transcribed spacer; *mcyE*, microcystin synthetase subunit E gene; ML, maximum-likelihood; MP, maximum-parsimony; *ndaF*, nodularin synthetase subunit F gene; NJ, neighbour-joining; PCA, principal component analysis; PC-IGS, phycocyanin operon intergenic spacer; PP1, protein phosphatase 1; *rbcLX*, ribulose-1,5-bisphosphate carboxylase/oxygenase subunit L and chaperonin-like protein X genes; *rpoB*, DNA-dependent RNA polymerase subunit B gene; STRR sequence, short tandemly repeated repetitive sequence.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AJ781131–AJ781151 (16S rRNA gene sequences), AJ781152–AJ781161 (*ndaF*), AJ781486–AJ781497 (*gvpA*-IGS), AJ783307–AJ783334 (*rpoB*) and AJ783675–AJ783703 (*rbcLX*).

To date, the diversity and toxic properties of bloom-forming planktonic *Nodularia* have been more widely studied than those of the benthic forms. A study of strains from the Baltic Sea showed that strains with gas vacuoles were phylogenetically distinct from, although closely related to, strains without gas vacuoles (Lehtimäki *et al.*, 2000). Genetic studies of three morphologically defined (Komárek *et al.*, 1993) planktonic species (*Nodularia baltica*, *Nodularia litorea* and *Nodularia spumigena*) and two benthic species (*Nodularia harveyana* and *Nodularia sphaerocarpa*) showed that only one genetically justified planktonic species, *N. spumigena* (Barker *et al.*, 1999; Laamanen *et al.*, 2001), and two benthic species,

N. harveyana and *N. sphaerocarpa*, exist in the Baltic Sea (Komárek *et al.*, 1993; Laamanen *et al.*, 2001). Furthermore, toxicity was confined to the planktonic *N. spumigena* (Laamanen *et al.*, 2001; Moffitt *et al.*, 2001). These studies indicate that considerable genetic variation exists in strains isolated from benthic habitats. Nevertheless, the lack of benthic isolates has restricted further phylogenetic analyses.

According to the taxonomic note of Stackebrandt *et al.* (2002), the phylogenetic position of a species should be determined by several methods, such as morphological, physiological, and 16S rRNA and protein-coding gene analysis, as well as genomic fingerprinting. Sequence analysis of housekeeping and other genes, such as DNA-dependent RNA polymerase subunit B (*rpoB*) (Palenik & Swift, 1996; Wilson *et al.*, 2000), ribulose-1,5-bisphosphate carboxylase/oxygenase subunit L (*rbcL*) and chaperonin-like protein X (*rbcX*) (Rudi *et al.*, 1998; Gugger *et al.*, 2002), have proved to be valuable targets of study for several cyanobacteria. However, thus far, they have not been used for analysis of *Nodularia* strains. Genomic fingerprinting, on the other hand, has been used to differentiate *N. spumigena* and *N. sphaerocarpa* (Lehtimäki *et al.*, 2000).

A species should form a phylogenetic cluster and comprise ecologically unique bacteria (Palys *et al.*, 1997; Cohan, 2002). Gas vacuole protein A gene (*gvpA*), encoding a basic structural protein of gas vacuoles, is often present as multiple copies in cyanobacteria (Barker *et al.*, 1999). As such, it determines whether the habitat of a cyanobacterium is in plankton or in the bottom of a water body. An analysis of *gvpA*-IGS (intergenic spacer between two copies of *gvpA*) revealed a genetically very homogeneous population of bloom-forming *N. spumigena* over a wide geographical area of the Baltic Sea (Barker *et al.*, 1999).

The ecological purpose of hepatotoxin production is not yet fully understood. Traditionally, cyanobacterial toxins have been regarded as repellents for zooplankton (e.g. Lampert, 1981). However, the effects of toxic planktonic *N. spumigena* on zooplankton are not only adverse, but may be neutral or even positive (Engström-Öst *et al.*, 2002; Koski *et al.*, 2002). Nodularin hepatotoxin is produced via a nodularin synthetase (*nda*; GenBank/EMBL/DDBJ accession no. AY210783) gene cluster. The *nda* genes seem to be recently derived from microcystin synthetase genes (Rantala *et al.*, 2004). These peptide synthetase gene clusters are ancient, sporadically distributed among cyanobacteria and have been lost from some derived lineages (Rantala *et al.*, 2004).

The genus *Nodularia* is special among the cyanobacteria as it is the only monophyletic heterocytous, filamentous cyanobacterial genus that contains two separate lineages that either have gas vacuoles (planktonic) or are without them (benthic). Our objective was to provide further information on the phylogenetic relationships of the two lineages by describing the phenotypic (morphology, gas vacuoles, nodularin production, gliding), genetic (16S

rRNA gene, *rpoB*, *rbcLX*, *ndaF* and *gvpA*-IGS) and genomic [short tandemly repeated repetitive (STRR) sequence fingerprinting] features of aged and freshly isolated planktonic and benthic *Nodularia* strains. In addition, the botanical taxonomy of the genus *Nodularia* (Komárek *et al.*, 1993), with an emphasis on benthic *Nodularia* species, is reviewed.

METHODS

Cyanobacterial strains. Novel benthic strains were isolated from littoral zones, growing on rock or plant surfaces under the water or in rock pools near the water level in the Gulf of Finland of the Baltic Sea. The strains were isolated by plating the samples onto agarose plates of Z8 (Rouhiainen *et al.*, 1995) and purified further into uniclonal cultures.

Morphology. Strains were grown for 2 weeks on Z8 medium without nitrogen and with salt in continuous light of 3–10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Lehtimäki *et al.*, 1994) prior to morphological analyses. Microscopic analysis and identification were carried out without knowing the strain identities. Strains were identified to species level following the taxonomy of Komárek *et al.* (1993). The sizes of vegetative cells, heterocytes and akinetes were determined under an inverted microscope (Leica DM IRB) with phase-contrast optics. From each strain and cell type, three replicate measurements were made and the mean value was used for identification. In addition, observations were made on the presence or absence of gas vacuoles, the shape of the terminal cells, and the colour and positioning of the akinetes. Micrographs of the strains were produced with a digital camera (Polaroid DMC 1).

Gliding experiments. *Nodularia* strains were plated onto Z8 agarose (Rouhiainen *et al.*, 1995) with salt and without nitrogen and grown for several weeks. Gliding capacity was detected by visual examination.

Nodularin analyses. Nodularin was extracted from approximately 5 mg lyophilized cells three times with 75 % aqueous methanol according to Fastner *et al.* (1998). Extracts were pooled, methanol was evaporated with a vacuum concentrator (Maxi Dry Plus) and water was added to obtain the original extraction volume. Prior to toxicity tests, extracts were filtered through 0.2 μm syringe filters (Acrodisc) and diluted to get toxin concentrations in the working range of toxicity assays. Samples were stored at -20°C until analysed by ELISA and protein phosphatase inhibition assay.

Samples were analysed according to the manufacturer's instructions with an Envirogard Microcystins Plate kit (Strategic Diagnostics), which is a direct competitive ELISA for quantitative detection of microcystins and nodularins. ELISA plates were read with a microplate photometer (iEMS Reader MF; Labsystems) at 450 nm.

In addition, protein phosphatase 1 (PP1) activity of samples was determined using a colorimetric protein phosphatase inhibition assay (An & Carmichael, 1994) with slight modifications. Samples were incubated with 5 U PP1 enzyme ml^{-1} (New England Biolabs) for 40 min at 37°C in the microplate photometer. Production of coloured *p*-nitrophenol from *p*-nitrophenol phosphate (Calbiochem) was determined at 405 nm. A standard curve was produced with known concentrations (0.5–5.0 $\mu\text{g l}^{-1}$) of microcystin-LR. The relative toxin concentration was determined from the linear region of the standard curve, i.e. the region between 20 and 80 % activity of PP1 enzyme.

Amplification of 16S rRNA, *rpoB*, *rbcLX* and *ndaF* genes and *gvpA*-IGS. Genomic DNA was isolated and purified with a DNeasy Plant Mini kit (Qiagen). The 16S rRNA gene with internal

transcribed spacer (ITS) sequence (approx. 1600 bp) was amplified with primers pA (Edwards *et al.*, 1989) and B23S with PCR cycling as described by Lepère *et al.* (2000). For amplification of over 500 bp of *rpoB*, primers (rpoBF, rpoBR, rpoBF2 and rpoBR2) and PCR cycling conditions described by Rajaniemi *et al.* (2005) were used. Region *rbcLX* (about 900 bp) was amplified with primers CW and DW and the PCR cycling conditions of Rudi *et al.* (1998). An approximately 800 bp region of *ndaF* of *Nodularia* was amplified with primers and PCR originally designed for *mcyE* (microcystin synthetase subunit E gene) from *Anabaena* (Vaitomaa *et al.*, 2003) with the exception of annealing at 52 °C. A fragment of about 252–350 bp of *gvpA*-IGS was amplified with primers PVAIF2 and PVAR2 (Barker *et al.*, 1999) in a total volume of 20 µl 1 × DyNAzyme II PCR buffer with 0.5 U DyNAzyme II DNA polymerase (Finnzymes), 0.5 µM each primer and 250 µM dNTP. The PCR cycling sequence was as follows: 94 °C, 4 min; 2 × (94 °C, 30 s; 40 °C, 30 s; 72 °C, 2 min); 38 × (94 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min); and 72 °C, 7 min.

STR fingerprinting. Fingerprinting was performed with primer set STRR1F/STRR3F or STRR1R/STRR3R (Wilson *et al.*, 2000), suitable for non-axenic cyanobacteria. The amplifications were performed in a total volume of 20 µl 1 × DyNAzyme II PCR buffer with 1 U DyNAzyme II DNA polymerase, each primer (0.5 µM) and dNTP (250 µM). The PCR cycling sequence was: 95 °C, 10 min; 30 × (94 °C, 1 min; 43 °C, 1 min; 65 °C, 5 min); and 65 °C, 16 min.

Sequencing. Cycle sequencing of 16S rRNA, *rpoB* and *rbcLX* genes was performed either in-house with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and Applied Biosystems PRISM 310 Genetic Analyser or by the Institute of Biotechnology (Helsinki, Finland). Amplification products were purified in-house for sequencing by Montage PCR Centrifugal Filter devices (Millipore) or Montage DNA Gel Extraction kits (Millipore). Fragments derived from *rpoB* of strains Bo53 and BECID35 were cloned with the pGEM Plant Mini kit (Promega) before sequencing. Sequencing primers for the 16S rRNA gene were forward and reverse primers pA and pH of Edwards *et al.* (1989). Primer sets used for amplification of *rpoB*, *rbcLX*, *gvpA*-IGS and *mcyE* were used for sequencing of the corresponding genes. Additional primers to sequence the *rbcLX* region were designed by Gugger *et al.* (2002) and by us (rbcLF, 5'-CAAACATGAATCTTAAGCAA-3'; rbcLR, 5'-GGCAATTTGCTTAAGATTTCAT-3'; rbcXF, 5'-CAACGTCGCCAG-CATCTAGAA-3'; rbcXR, 5'-GCGTTCTAGATGCTGGCGACG-3'). The synonymous and non-synonymous substitution rates of *rpoB*, *rbcL* and *rbcX* were calculated with the Synonymous/Non-synonymous Analysis Program (<http://www.hiv.lanl.gov>; Korber, 2001). The ambiguous nucleotides in the 16S rRNA gene observed in the present study were compared with corresponding positions in 16S rRNA secondary structure of *Escherichia coli* with ARB (Ludwig *et al.*, 2004).

Clustering of genetic and genomic data. The contigs were assembled with Trev (Bonfield *et al.*, 2002) and edited with Gap4 (Staden *et al.*, 2003) of the Staden Package and aligned with BIOEDIT (Hall, 1999). Phylogenetic nucleotide sequence trees were inferred with neighbour-joining (NJ) based on F84 distances and maximum-parsimony (MP) criteria of PAUP* (Swofford, 2001). The phylogenetic nucleotide sequence trees were also inferred with maximum-likelihood (ML) criteria of PHYLIP (Felsenstein, 1993). Phylogenetic amino acid sequence trees were inferred with NJ based on F84 distances and MP and ML criteria of PHYLIP. Bootstrap resamplings (1000) were performed for each consensus tree. The ML nucleotide sequence consensus trees of the 16S rRNA gene were an exception with 24 resamplings, as were *rpoB* and *rbcLX*, both with 50 resamplings. A UPGMA (unweighted pair-group method with arithmetic means) phenogram representing STRR fingerprints was constructed based on Dice similarities (based upon the presence or absence of bands) with the Bionumerics program (Applied Maths). In addition

to tree construction analyses, the clustering of genetic and genomic data was examined by principal component analysis (PCA) of CAP (PISCLES) of F84 distances based on the 16S rRNA gene, *rpoB* and *rbcLX*, as well as Dice distances of STRR fingerprints.

RESULTS AND DISCUSSION

Identification of *Nodularia* strains based on morphology, nodularin production and gliding

Following the morphological taxonomy of the genus *Nodularia* of Komárek *et al.* (1993), three different species were identified among our strains. Twelve strains were identified as *N. spumigena*, 11 as *N. sphaerocarpa* and four as *N. harveyana*. Strain PCC 7804, which has been previously identified as *N. sphaerocarpa* or *N. harveyana*, could not be identified to species level in the present study. Strains that were identified as *N. spumigena* were always isolated from plankton (Table 1) and were characterized by the presence of gas vacuoles (Table 2, Fig. 1A, C), the inability to glide and nodularin production (Table 2). Strains that did not produce nodularin, lacked gas vacuoles and were mainly isolated from different types of benthic habitats were identified as either *N. sphaerocarpa* or *N. harveyana* species. The main distinguishing morphological features between the two benthic species were: the width of the vegetative cells, i.e. wider in *N. sphaerocarpa* (mean width of 7.0 µm; Table 2, Fig. 1D–F) than in *N. harveyana* (mean width of 5.1 µm; Table 2, Fig. 1G–I); the shape of the terminal cells, i.e. blunt in *N. sphaerocarpa* (Fig. 1D–F) and conical in *N. harveyana* (Fig. 1G–I); and features of the akinetes (Table 2), i.e. spherical, often yellowish–ochre in colour and in long chains in *N. sphaerocarpa* (Fig. 1E) and oval, smaller in size and in chains of two to four in *N. harveyana* (Fig. 1I). In addition, it was noted that filaments of *N. harveyana* strains commonly terminated with heterocytes and that intercalary heterocytes were quite often in pairs (Fig. 1G–I).

Identification based solely on cell sizes/shapes and the presence or absence of gas vacuoles was not straightforward. Identification was especially difficult when the gas vacuoles were absent in strains of *N. spumigena*. Strains BY1 and PCC 9350 lost their gas vacuoles during cultivation, which seems to be common in cultured strains [Fig. 1B; see the discussions of Lehtimäki *et al.* (2000) and Laamanen *et al.* (2002)]. Nevertheless, nodularin production and gliding (Table 2) were the other two important distinguishing characters between the planktonic and benthic strains. Gliding was observed only by the benthic strains, which seems reasonable since those strains were isolated from bottom habitats where they most likely grew attached onto or in close proximity to surfaces. On a surface, filaments move towards more optimal growth conditions by gliding. Cyanobacteria have different motility strategies such as moving by pili (*Synechococcus* PCC 6803), polysaccharides (*Phormidium*, *Anabaena variabilis*) or oscillin protein forming fibres on the cell surface (*Oscillatoria*) (McBride,

Table 1. *Nodularia* strains used in this study

Culture collections: PCC, Pasteur Culture Collection of Cyanobacteria; UTEX, the Culture Collection of Algae at the University of Texas.

All species identifications previously used for the strains	Geographical origin	Year of isolation	Isolation habitat	Latest taxonomic designation(s)*
<i>N. spumigena</i> AV1	Baltic Sea (Gulf of Finland)	1987	Plankton	1
<i>N. spumigena</i> AV63	Baltic Sea (Gulf of Finland)	1987	Plankton	2, 3
<i>N. baltica</i> (<i>spumigena</i>) BY1	Baltic Sea (Arkona Sea)	1986	Plankton	2–4
<i>N. harveyana</i> Bo53	Boiensdorf resp.	1992–1994	Shallow coastal water	1
<i>N. harveyana</i> Hübel 1983/300	Baltic Sea (Hidden Sea)	1983	Benthic microbial mat	3, 5
<i>N. baltica</i> Hübel 1988/306	Baltic Sea (Kleiner Jasmunder Bodden)	1988	Plankton	3, 5
<i>N. spumigena</i> Hübel 1987/310	Baltic Sea (Vitter Bodden)	1987	Plankton	3
<i>N. spumigena</i> Hübel 1987/311	Baltic Sea (Vitter Bodden)	1987	Plankton	3
<i>N. sphaerocarpa</i> Fä19	Fährdorf/Isle of Poel	1992–1994	Shallow coastal water	1
<i>Nodularia</i> sp. (<i>spumigena</i>) F81	Baltic Sea (Baltic Proper)	1987	Plankton	1, 3
<i>N. spumigena</i> GR8b	Baltic Sea (Gulf of Finland)	1992	Plankton	2, 3
<i>N. spumigena</i> HEM	Baltic Sea (Gulf of Finland)	1987	Plankton	2–4
<i>N. sphaerocarpa</i> HKVV	Baltic Sea (Stockholm Archipelago)	Not known	Water	2, 3
<i>N. harveyana</i> BECID27	Baltic Sea (Gulf of Finland)	2001	Littoral zone, plant surface	1
<i>N. harveyana</i> BECID29	Baltic Sea (Gulf of Finland)	2001	Littoral zone, rock surface	1
<i>N. spumigena</i> NSPI-05	Peel Inlet, Australia	1992	Plankton	2, 3
<i>N. spumigena</i> NSOR-12	Oregon lagoon, Australia	1993	Plankton	2–4
<i>N. sphaerocarpa</i> ; <i>N. sphaerocarpa/harveyana</i> ; <i>N. spumigena</i> ; PCC 73104	Spotted lake, BC, Canada	1972	Alkaline soil	2–8, 9
<i>N. sphaerocarpa</i> ; <i>N. spumigena</i> PCC 73104/1	Spotted lake, BC, Canada	1972	Alkaline soil	2, 3, 6
<i>N. sphaerocarpa</i> ; <i>Nodularia</i> sp.; <i>N. sphaerocarpa/harveyana</i> PCC 7804	Dax, France	1966	Thermal spring	2–4, 6, 9
<i>N. spumigena</i> PCC 9350	Baltic Sea (Gulf of Finland)	1987	Plankton	6
<i>N. sphaerocarpa</i> Up16a	Baltic Sea (Gulf of Finland)	1994	Plankton	2, 3
<i>N. sphaerocarpa</i> Up16f	Baltic Sea (Gulf of Finland)	1994	Plankton	2, 3
Unidentified (<i>N. sphaerocarpa</i>), subsection IV.I BECID34†	Baltic Sea (Gulf of Botnia, The Quark)	2002	Littoral zone, plankton	1
<i>N. sphaerocarpa</i> BECID35	Baltic Sea (Gulf of Botnia, The Quark)	2002	Littoral zone, mat-like colony	1
<i>N. sphaerocarpa</i> BECID36	Baltic Sea (Gulf of Finland)	2002	A littoral pool, rock surface	1
<i>N. sphaerocarpa</i> Hübel 296	Not known	Not known	Not known	5
<i>N. spumigena</i> UTEX B 2092; <i>N. sphaerocarpa/harveyana</i>	Osoyoos, BC, Canada	1974	Alkaline soil	UTEX, 4, 7, 8
<i>N. harveyana</i> UTEX B 2093; <i>N. sphaerocarpa/harveyana</i> ; <i>Nodularia</i> sp.	Pond, San de Fuca, Whidbey Island, WA, USA	1974	Not known	UTEX, 4, 7, 8, 9

*Numbers refer to the following references: 1, this study; 2, Lehtimäki *et al.* (2000); 3, Laamanen *et al.* (2001); 4, Moffitt *et al.* (2001); 5, Komárek *et al.* (1993); 6, Rippka *et al.* (2001); 7, Rippka *et al.* (1979); 8, Nordin & Stein (1980); 9, Bolch *et al.* (1999).

†Identified morphologically as *N. sphaerocarpa*.

Table 2. Species identification of *Nodularia* strains and phenotypic features of the strains

Table shows: the sizes (µm) of the different types of cells (vegetative cells, heterocytes and akinetes); the presence (+) or absence (–) of gas vacuoles and, in parentheses, the number of gas vacuole protein A intergenic spacer (*gvpA*-IGS) copies observed with a PCR test; and nodularin production detected (+) or not detected (–) and, in parentheses, the presence (+) or absence (–) of the nodularin synthetase gene *ndaF*. NA, Not available; ND, not determined; NG, not classified to the genus level. The key for the species identification is described by Komárek *et al.* (1993).

Strain and its original species identifications	Our species identification*	Vegetative cells		Heterocytes		Akinetes		Gas vacuoles (<i>gvpA</i> -IGS)	Nodularin production (<i>ndaF</i>)
		Width	Length	Width	Length	Width	Length		
AV1, <i>N. spumigena</i>	<i>N. spumigena</i>	6·6	3·3	7·7	5·2	8·3	7·6	+	(+)
AV63, <i>N. spumigena</i>	<i>N. spumigena</i>	8·2	3·3	8·8	4·8	10·6	5·8	+	(+)
BY1, <i>N. baltica</i> (<i>spumigena</i>)†	<i>N. spumigena</i>	7·4	3·5	7·5	4·7	7·8	5·7	–	(+)
F81, <i>Nodularia</i> sp. (<i>spumigena</i>)	<i>N. spumigena</i>	5·9	3·4	6·8	6·9	7·3	8·3	+	(+)
GR8b, <i>N. spumigena</i> †	<i>N. spumigena</i>	6·8	3·1	8·3	6·2	9·9	7·4	+	(+)
HEM, <i>N. spumigena</i>	<i>N. spumigena</i>	8·0	4·4	9·3	10·1	12·4	11·6	+	(+)
Hübel 1988/306, <i>N. spumigena</i>	<i>N. spumigena</i>	7·4	3·2	9·0	5·4	8·3	6·6	+	(+)
Hübel 1987/310, <i>N. spumigena</i>	<i>N. spumigena</i>	7·4	3·6	8·1	4·4	8·3	5·4	+	(+)
Hübel 1987/311, <i>N. spumigena</i>	<i>N. spumigena</i>	7·3	3·6	7·9	5·0	8·9	6·7	+	(+)
NSOR-12, <i>N. spumigena</i> †	<i>N. spumigena</i>	8·0	3·2	8·6	5·6	9·5	6·8	+	(+)
NSPI-05, <i>N. spumigena</i> †	<i>N. spumigena</i>	10·1	4·1	11·1	5·3	13·0	7·1	+	(+)
PCC 9350, <i>N. spumigena</i>	<i>N. spumigena</i>	6·9	4·3	8·7	6·4	9·7	7·7	–	(+)
Fä19, <i>N. sphaerocarpa</i>	<i>N. sphaerocarpa</i> *	6·1	3·1	6·2	5·2	7·8	6·7	–	(–)
BECID34, unidentified subsection IV.III	NG	8·3	3·7	9·1	5·3	8·3	8·3	–	(–)
BECID35, <i>N. sphaerocarpa</i>	<i>N. sphaerocarpa</i> *	7·9	3·3	8·0	4·7	8·9	6·5	–	(–)
BECID36, <i>N. sphaerocarpa</i>	<i>N. sphaerocarpa</i> *	5·7	3·6	6·0	5·0	7·6	9·6	–	(–)
HKVV, <i>N. sphaerocarpa</i> †	<i>N. sphaerocarpa</i> *	7·8	4·0	8·7	6·1	9·5	7·3	–	(–)
PCC 73104, <i>N. sphaerocarpa</i> ; <i>N. spumigena</i> ; <i>N. sphaerocarpa/harveyana</i>	<i>N. sphaerocarpa</i> *	7·3	4·3	7·8	7·9	9·5	8·9	–	(–)
PCC 73104/1, <i>N. sphaerocarpa</i> ; <i>N. spumigena</i> †	<i>N. sphaerocarpa</i> *	6·6	3·1	8·0	5·8	9·5	8·6	–	(–)
PCC 7804, <i>N. sphaerocarpa</i> ; <i>Nodularia</i> sp.; <i>N. sphaerocarpa/harveyana</i> †	<i>Nodularia</i> sp.*	5·2	5·1	6·2	6·6	8·7	9·3	–	(+)
Up16f, <i>N. sphaerocarpa</i> †	<i>N. sphaerocarpa</i> *	8·1	3·2	8·6	5·4	8·7	7·3	–	(–)
Up16a, <i>N. sphaerocarpa</i>	<i>N. sphaerocarpa</i> *	8·5	3·6	9·0	6·2	9·9	9·3	–	(–)
UTEX B 2092, <i>N. spumigena</i> ; <i>N. sphaerocarpa/harveyana</i>	<i>N. sphaerocarpa</i> *	8·6	4·0	9·3	5·9	10·4	6·8	–	(–)
UTEX B 2093, <i>N. spumigena</i> ; <i>N. sphaerocarpa/harveyana</i>	<i>N. sphaerocarpa</i> *	6·2	3·1	7·8	7·5	NA	NA	–	(–)
Hübel 296, <i>N. sphaerocarpa</i>	<i>N. sphaerocarpa</i> *	10	3·9	9	6·3	12·9	9·9	–	(–)
BECID27, <i>N. harveyana</i>	<i>N. harveyana</i> *	4·6	2·0	6·3	5·9	NA	NA	–	(–)
BECID29, <i>N. harveyana</i> ‡	<i>N. harveyana</i> *	5·1	3·3	5·8	3·6	6·6	6·5	–	(–)
Bo53, <i>N. harveyana</i>	<i>N. harveyana</i> *	4·6	2·3	5·3	5·6	NA	NA	–	(–)
Hübel 1983/300, <i>N. harveyana</i> ‡	<i>N. harveyana</i> *	5·7	3·2	6·3	5·6	7·7	8·9	–	(–)

*Strains with gliding capacity.

†16S rRNA gene not sequenced in the present study.

‡Ambiguous nucleotides not observed in 16S rRNA gene.

§Determined by ELISA and protein phosphatase inhibition assay.

||Identified morphologically as *N. sphaerocarpa*.

Fig. 1. Strains of *Nodularia*. (A, B, C) Strains determined as *N. spumigena* (strains NSOR-12, BY1 and GR8b in A, B and C, respectively). (D, E, F) Strains determined as *N. sphaerocarpa* (strains BECID34, BECID36 and UTEX B 2092 in D, E and F, respectively). (G, H, I) Strains of *N. harveyana* (strains BECID27, Bo53 and Hübel 1983/300 in G, H and I, respectively). Heterocytes are indicated with black arrows and akinetes with white ones. Bars, 30 μ m.

2001). The mechanism by which *N. sphaerocarpa* and *N. harveyana* glide remains to be studied.

Phylogenetic relationships within the genus *Nodularia*

The different hierarchical tree constructions, NJ, MP and ML methods, used for analysis of the 16S rRNA, *rpoB* and *rbclX* genes (Fig. 2, Fig. 3 and Fig. 4) gave a similar general clustering of the strains. Non-hierarchical PCA (data not shown) was performed to test the reliability of hierarchical groups, such as the *N. sphaerocarpa* group, with less than 50 % bootstrap support. The PCA gave similar grouping and thus confirmed the reliability of hierarchical clustering.

The general scheme of phylogenetic clustering of the strains was in line with our morphological species identifications as the strains identified as belonging to different species clustered separately. The planktonic strains, which were identified as *N. spumigena*, were closely related to the *N. sphaerocarpa* strains but they formed a separate group. In

terms of morphological features, the distinct homogeneous clustering of the species (Figs 2, 3 and 4) was supported by the larger mean cell widths of *N. spumigena* compared to those of *N. sphaerocarpa* (Table 2) and the almost round, ochre-coloured akinetes, which often formed long chains in *N. sphaerocarpa* (Fig. 1E) in comparison to the oval, less numerous akinetes in *N. spumigena*. This result is in agreement with a recent morphological and phylogenetic study of various heterocytous filamentous cyanobacteria, such as *Anabaena* and *Aphanizomenon*, showing that shape, width and length of the akinetes were coherent with phylogenetic clustering (Rajaniemi *et al.*, 2005). Strains of *N. harveyana* formed a separate group, which was more diverse (Figs 2, 3 and 4, Table 3).

The 16S rRNA, *rpoB* and *rbclX* genes studied seem to have relatively similar evolutionary histories; therefore, the groupings obtained in this study can be considered to be reliable for phylogenetic delineation of *Nodularia* strains. Reliability of the tree topologies was also confirmed with larger taxon sampling of the 16S rRNA gene (data not

Strains of *N. spumigena* shared extremely high sequence similarity values for their 16S rRNA (98.5–100%), *rpoB* (97.6–100%) and *rbcLX* (99.1–100%) genes (Table 3). Nevertheless, STRR fingerprints (Fig. 5) showed more diversity (17.43–96.67%). The results of 16S rRNA gene analyses are coherent with the recent results on *N. spumigena*

In addition, hierarchical UPGMA (Fig. 5) and non-hierarchical PCA (data not shown) on STRR fingerprints gave similar groupings. The STRR fingerprints of *N. spumigena* showed that the Baltic Sea population is not clonal. The result is in agreement with previous studies on non-coding regions, such as the 16S–23S rRNA ITS, *gvpA*-IGS and phycocyanin operon intergenic spacer (PC-IGS) (Barker *et al.*, 1999, 2000; Laamanen *et al.*, 2001). These studies revealed variation among Baltic *N. spumigena* strains sharing identical 16S rRNA genes. In addition, fingerprinting methods such as RAPD-PCR on Baltic Sea and Australian strains (Bolch *et al.*, 1999), and repetitive extragenic palindromic sequence- and enterobacterial repetitive intergenic consensus-PCR on the Baltic Sea strains (Lehtimäki *et al.*, 2000) yielded a more diverse picture of the *N. spumigena* cluster than that shown by STRR in this study.

Fig. 3. Phylogenetic NJ tree based on *rpoB* (466 bp). Bootstrap values higher than 50 % for NJ, MP and ML, respectively, are indicated at the nodes in the trees. Accession numbers for the sequences obtained from the EMBL are indicated in parentheses. The *rpoB* sequences determined in the present study are indicated in bold. Bar, nucleotide substitution per site.

The benthic *N. sphaerocarpa* and *N. harveyana* were more diverse than the planktonic *N. spumigena*, as shown by all methods used. Among the *N. sphaerocarpa* strains, the similarity values of the 16S rRNA (98–100 %), *rpoB* (91.8–100 %) and *rbcLX* (95.5–100 %) gene sequences and the genomic ones of STRR (6.7–92.1 %) were lower than those observed in *N. spumigena* (Table 3). *N. harveyana* was the most diverse of the three species, as demonstrated by the relatively low similarity values of the 16S rRNA (97.5–99.6 %), *rpoB* (87.1–96.5 %) and *rbcLX* (89.4–99.3 %) gene sequences (Table 3). The STRR values (11.8–41.1 %) within *N. harveyana* were also low (Fig. 5).

N. harveyana strains formed one branch in the *rbcLX* tree (Fig. 4) and two branches in both the *rpoB* (Fig. 3) and the 16S rRNA gene (Fig. 2) trees. This implies that among the strains identified as *N. harveyana* either two species exist or the species is genetically diverse. The 16S rRNA gene similarity among *N. harveyana* strains is, however, greater than the 97 % threshold value for strains of the same species. Organisms with DNA–DNA similarity greater than 70 %, which is the boundary value for recognizing a

species (Wayne *et al.*, 1987), often share more than 97 % 16S rRNA gene similarity (Stackebrandt & Goebel, 1994). Therefore, 97 % similarity circumscribes the species, although bacterial species delineation should not be based only on rRNA gene similarities (Rosselló-Mora & Amann, 2001). Our study is the first one to include several *N. harveyana* strains. Strain Hübel 1983/300 (CDAC1983/300 in Moffitt *et al.*, 2001) is included in the taxonomic review of Komárek *et al.* (1993) and therefore it can be considered as a reference strain for the morphologically determined species. It was found to be highly divergent from the other strains of *Nodularia* by analysis of sequences of the 16S rRNA gene (Moffitt *et al.*, 2001) and the 16S rRNA ITS and PC-IGS regions (Laamanen *et al.*, 2001). Also in this study, this strain was the most divergent of all *N. harveyana* strains, as shown by all the molecular methods.

In this study, clear genetic discontinuities were not evident between the three *Nodularia* species, which was demonstrated by overlapping similarity values within and between the species (Table 3). If the boundary value of 97 % 16S rRNA gene similarity for identifying a species (Rosselló-Mora

Fig. 4. Phylogenetic NJ tree based on *rbcLX* (607 bp). Bootstrap values higher than 50 % for NJ, MP and ML, respectively, are indicated at the nodes. The *rbcLX* sequences determined in the present study are indicated in bold. Bar, nucleotide substitution per site.

& Amann, 2001) is applied, then *N. spumigena* and *N. sphaerocarpa* belong to a single species as demonstrated by our 16S rRNA data and the 16S rRNA similarities of 97.8–99.4 % (Moffitt *et al.*, 2001) and 98.6 % (Barker

et al., 1999), as well as greater than 99 % (Lehtimäki *et al.*, 2000) between planktonic *N. spumigena* and benthic *N. sphaerocarpa*. The greater than 97 % similarity value does not necessarily guarantee that strains share a high degree

Table 3. Similarity values of the 16S rRNA, *rpoB* and *rbcLX* gene sequences and identity values of RpoB and RbcLX amino acid sequences between *N. spumigena*, *N. sphaerocarpa* and *N. harveyana*

Species: 1, *N. spumigena*; 2, *N. sphaerocarpa*; 3, *N. harveyana*.

Nucleotide sequence	16S rRNA gene similarity (%)			<i>rpoB</i> similarity (%)			<i>rbcLX</i> similarity (%)		
	1	2	3	1	2	3	1	2	3
<i>N. spumigena</i>	98.5–100	97.9–99	96.6–100	97.6–98.3	91.2–93.5	87.3–92.7	99.1–100	95.5–98.0	88.3–90.1
<i>N. sphaerocarpa</i>		97.9–100	96.4–98.4		91.8–100	84.5–90.7		95.5–100	88.3–92.4
<i>N. harveyana</i>			97.5–99.6			87.1–96.5			89.4–99.3
Amino acid sequence				RpoB identity (%)			RbcLX identity (%)		
<i>N. spumigena</i>				99.3–100	96.1–98.7	96.7–98.7	100	96.7–98.1	90.1–90.5
<i>N. sphaerocarpa</i>					96.7–100	94.1–98.0		95.7–100	89.6–92.4
<i>N. harveyana</i>						97.4–100			92.4–99.5

Fig. 5. UPGMA phenogram based on cyanobacterial genomic fingerprints derived from STRR1F and STRR3F, as well as STRR1R and STRR3R PCR. Bar, similarity (%).

of DNA–DNA reassociation and thus are closely related (Stackebrandt & Goebel, 1994). A recent study on the 16S rRNA gene of planktonic and benthic *Anabaena* showed that the two forms were intermixed and shared similarities as high as 99.2% (Rajaniemi *et al.*, 2005). However, the strains formed several distinct clades, possibly representing different species. Evolution is a continuum and 16S rRNA and DNA–DNA similarities are arbitrary divisions for species. Sequence-based phylogeny, which uses evolutionary models with distinct types of nucleotide substitutions as well as sequence similarities, is a good approach to study speciation. The present phylogenetic study shows separate genetic grouping and distinct differences in phenotypic features between the groups despite the fact that the margins of the groups are not genetically clearly defined.

Specific features of genetic and genomic data

In this study, all 16S rRNA gene sequences of *N. spumigena*, *N. sphaerocarpa* and *N. harveyana* (Table 2), with the exception of *N. harveyana* BECID29 and Hübel 1983/300, had ambiguous nucleotides, which indicates that these strains carry at least two different 16S rRNA gene copies.

The ambiguous nucleotides occurred frequently at both complementary nucleotides in paired regions corresponding to *Escherichia coli* 16S rRNA secondary structure loop 6 (*N. spumigena* Hübel 1987/311 and AV1, as well as *N. sphaerocarpa* Up16a, PCC 73104, BECID36, Hübel 296, UTEX B 2092 and UTEX B 2093), loop 9 (*N. spumigena* PCC 9350, Hübel 1988/306, Hübel 1987/310, Hübel 1987/311, HEM, AV1, AV63 and F81, as well as *N. sphaerocarpa* Up16a, PCC 73104 and Hübel 296), loop 22 (the eight

N. spumigena strains, *N. sphaerocarpa* Fä19 and UTEX B 2093, as well as *N. harveyana* BECID27), loop 28 (the eight *N. spumigena* strains) and loop 37 (*N. sphaerocarpa* Fä19, BECID36 and UTEX B 2093). Mutations seemed to accumulate in these regions because the correction mechanism for the initial mutation caused mutation at the complementary site instead of reversing the initial mutation. Ambiguous nucleotides were also sometimes found at only one complementary nucleotide position and in non-paired regions of the loops. Mutations do not seem to be beneficial since they have not spread to all other 16S rRNA gene repeats. This present study showed that planktonic and benthic strains of *Nodularia* carry more than one different copy of the 16S rRNA gene and thus purifying selection (concerted evolution) does not play a major role in the evolution of the 16S rRNA gene of these heterocytous cyanobacteria. Genetic studies have shown that a wide range of planktonic cyanobacteria carry several 16S rRNA gene copies in a single strain, which are devoid of purifying selection (Iteman *et al.*, 2002), and thus also several rRNA operons (Iteman *et al.*, 2002; Gugger *et al.*, 2002; Laamanen *et al.*, 2002).

Synonymous substitutions exceeded non-synonymous substitutions in *rpoB* and *rbclX* regions of the *Nodularia* strains studied here, indicating that negative selection (purifying selection) plays an important role in the evolution of these protein regions. Therefore, these gene regions may be utilized as phylogenetic tools for strains of the genus *Nodularia*. The amino acid sequence of the C-terminal end of *rbclX* was very heterogeneous and, for example, *Nodularia* strain PCC 7804 contained a frame-shift mutation in this

region. Therefore, the C-terminal end of *rbcX* of the studied strains was not included in the alignment and the possible neutral or positive selection evolving the C-terminal end of *rbcX* was not tested.

The main *Nodularia* groups in the *rbcLX* tree were the same as those in the 16S rRNA gene and *rpoB* trees. Therefore, the *rbcLX* region of *Nodularia* strains did not seem to be laterally transferred, in contrast to the *rbcLX* of other planktonic cyanobacteria strains, such as those from the genera *Anabaena* and *Aphanizomenon* (Rudi *et al.*, 1998; Gugger *et al.*, 2002). The slight differences found between 16S rRNA gene, *rpoB* and *rbcLX* trees are potentially caused by the low number of nucleotide and amino acid differences in the sequences of the studied *Nodularia* strains.

The *Nodularia* strains also shared a similar intergenic sequence between the *rbcL* and *rbcX* genes, which separated *Nodularia* from the other heterocytous filamentous strains, such as those from the genera *Anabaena* and *Nostoc*. The intergenic sequence between *rbcX* and the 1,5-bisphosphate carboxylase/oxygenase subunit S gene (*rbcS*) was more variable and grouped the *Nodularia* in a similar way to the 16S rRNA, *rpoB* and *rbcLX* genetic clustering. In addition, *rpoB* of the *Nodularia* strains carried an indel (insertion or deletion) of the same length, with the exception of *N. harveyana* strains BECID27, BECID29 and Bo53. The indels of strains BECID27 and Bo53, which were grouped together with all genetic and genomic methods used (Figs 2, 3, 4 and 5), as well as the indel of BECID29, were 23 bp longer than the indels of other *Nodularia* strains. An indel of 95 bp was also observed in the *gvpA*-IGS of PCC 9350, HEM, F81 and AV63, sharing identical 16S rRNA gene, *rpoB* and *rbcLX* sequences and highly similar STRR fingerprints (80.10–96.67%). Thus, it seems that the fairly conserved indels are useful for phylogenetic classification, since they confirm the groupings based on nucleotide substitutions. These tools have been recommended as sole (Gupta, 2000a, b) or additional tools in phylogenetic studies of organisms (Woese, 2000).

Genomic STRR fingerprinting revealed high variability within the benthic *Nodularia* strains, especially among *N. harveyana*, *N. sphaerocarpa* BECID36, Få19, UTEX B 2092 and UTEX B 2093, and *Nodularia* sp. PCC 7804. This tool identifies and confirms the very close genomic similarity of *Nodularia* strains sharing identical or almost identical genetic similarity values. However, strains such as *N. harveyana* BECID29 and 12 *Nodularia* strains sharing more than 97% 16S rRNA gene similarity shared no bands in their STRR profiles. Therefore, the different STRR patterns do not necessarily indicate distant relatedness of the strains. The low numerical value of STRR fingerprints can cause bias in determining the grouping of more diverse *Nodularia* strains. However, the method has been successfully used for differentiation and identification of symbiotic cyanobacteria such as *Nostoc* (Rasmussen & Svenning, 1998).

***gvpA*-IGS and *ndaF* and their value as indicators of the presence of gas vacuoles and nodularin production**

PCR amplification resulted in one *gvpA*-IGS product (located between two copies of *gvpA* genes) from *N. spumigena* and thus indicated the presence of at least two *gvpA* genes, *gvpA1* and *gvpA2*, in the *N. spumigena* strains studied. Also, *N. spumigena* strains without visible gas vacuoles (BY1 and PCC 9350) gave a positive result (Table 2) showing that the genes in these strains are possibly silent. PCR did not amplify any *gvpA*-IGS product from the benthic *N. sphaerocarpa* and *N. harveyana* strains (Table 2) implying that the benthic strains carry only one copy of the *gvpA* or no copy at all. Results indicate that the planktonic strains, which have gas vacuoles, generally have a higher number of gas vacuole genes than the benthic *Nodularia* strains, which lack gas vacuoles. Nevertheless, the presence of *gvpA* in the benthic strains may enable production of gas vacuoles under certain circumstances, such as in hormocytes/hormogonia. Hormogonia of cyanobacteria usually contain gas vacuoles (Damerval *et al.*, 1989).

ndaF was present in all *N. spumigena* strains and each one of them produced nodularin (Table 2). In addition, *ndaF* and nodularin production were detected in *Nodularia* sp. strain PCC 7804, which was isolated from a thermal spring and produces a nodularin variant (Beattie *et al.*, 2000). These results show that the presence of *ndaF* is a good indicator of nodularin production. In the future, *ndaF* from *Nodularia*, similarly to *mcyA* and *mcyE* from *Anabaena* and *Microcystis* strains (Vaitomaa *et al.*, 2003; Tillet *et al.*, 2001), may prove to be a valuable tool for detection and early warning tests of nodularin-producers in water environments. The sequence divergence of *ndaF* among *N. spumigena* and *Nodularia* sp. PCC 7804 considered phylogenetically indicates that the *ndaF* (*mcyE*), 16S rRNA and *rpoB* genes have co-evolved, as suggested previously by Rantala *et al.* (2004).

The data on nodularin production and the presence of *ndaF* provide further support to the idea that nodularin production is generally inherent in planktonic *N. spumigena*, in contrast to the benthic *Nodularia* strains (Lehtimäki *et al.*, 2000; Laamanen *et al.*, 2001; Moffitt *et al.*, 2001; Moffitt & Neilan, 2001).

The relationship of phylogenetic and genetic data to the ecological features of *Nodularia* species

Genes encoding microcystin synthetases have been repeatedly lost during the evolution of microcystin- and nodularin-producing cyanobacteria (Rantala *et al.*, 2004). Benthic *Nodularia* strains do not produce nodularin or microcystin and are always without gas vesicles. Therefore, it is tempting to hypothesize that the benthic *Nodularia* species have also lost their gas vacuoles and nodularin synthetases once they adopted the benthic habitat. The

reason would have been that the nodularin synthetases were no longer needed in the benthic habitat and their synthesis required an extra expenditure of energy. Nevertheless, the absence of nodularin synthetase in benthic *Nodularia* does not support the hypothesis that microcystins and nodularins are targeted against grazers. In the benthic littoral habitats of the Baltic Sea, a number of grazers exist and thus it might be advantageous to produce nodularin. Benthic cyanobacteria such as *N. harveyana* can, however, produce other unknown compound(s) that are toxic to the grazers, other cyanobacteria, bacteria and fungi (Pushparaj *et al.*, 1999) and have a similar role to nodularin.

Low numbers of nucleotide substitutions may also indicate that *N. spumigena* diverged from the other *Nodularia* strains a relatively short time ago. *N. spumigena* is the only *Nodularia* species that inhabits a narrow planktonic ecological niche. It is possible that the more heterogeneous and more nutrient-rich benthic habitat, with its different types of substrates, has allowed higher, possibly also faster, diversification of *Nodularia* than the planktonic habitat. Salinity variations also account for the higher observed heterogeneity of the benthic habitat. The occurrence of benthic *Nodularia* species in water pools above the water layer (Table 1; Nordin & Stein, 1980) results in exposure to high variations in salinity.

The differences in STRR profiles found in the present study may indicate genomic rearrangements such as changes in genome size and content, which has been linked to the adaptation of an organism to an ecological niche in many publications (Thomson *et al.*, 2003; Rocap *et al.*, 2003). For example, marine cyanobacteria have smaller genomes than their freshwater relatives, possibly reflecting the homogeneous and nutrient-poor marine environment (Thomson *et al.*, 2003). STRR fingerprinting cannot reveal directly differences in either the genome size or the content. However, it shows the potential recombination of genomes by which possible changes of genome size and content may occur.

Although *N. sphaerocarpa* and *N. harveyana* are more distantly related than *N. spumigena* and *N. sphaerocarpa*, clear phenotypic or ecological differences between the former two species have not yet been established. In this study, both *N. sphaerocarpa* and *N. harveyana* strains were devoid of gas vacuoles and were able to glide (Table 2). The strains did not produce nodularin, with the exception of strain PCC 7804. Komárek *et al.* (1993) considered *N. harveyana* to be halotolerant and *N. sphaerocarpa* to be a freshwater species. Seven of the *N. sphaerocarpa* strains used in this study were isolated from the brackish water of the Baltic Sea (Table 1) and one strain (UTEX B 2093) was from a pond with salinity of 18‰ (Nordin & Stein, 1980). The *N. harveyana* strains were isolated from highly similar habitats. These facts do not point to differences in halotolerance or salinity adaptation, but physiological experiments will help to unravel the possible differences.

Designations of benthic *Nodularia* strains

The delineation of *N. spumigena* type strains PCC 73104 and PCC 73104/1 here as *N. sphaerocarpa* is in agreement with their morphological and genetic classification into the *N. sphaerocarpa* cluster in other studies (Bolch *et al.*, 1999; Lehtimäki *et al.*, 2000; Laamanen *et al.*, 2001; Moffitt *et al.*, 2001). According to our results, and the fact that *N. spumigena* was already described in 1886 according to the botanical system, a new bacteriological type strain ought to be assigned to *N. spumigena*. Therefore, for example, strain AV1 or GR8b might be nominated as the new type strain of *N. spumigena*. Strain PCC 73104, in turn, could be appointed as the type strain of *N. sphaerocarpa*.

Furthermore, our morphological and genetic data identified strains PCC 7804, UTEX B 2092 and UTEX B 2093 as *N. sphaerocarpa*. Previous morphological and genetic data have designated PCC 7804 as *N. sphaerocarpa* (Lehtimäki *et al.*, 2000), *Nodularia* sp. (Laamanen *et al.*, 2001; Rippka *et al.*, 2001) or *N. harveyana*/*sphaerocarpa* (Moffitt *et al.*, 2001). The latest morphological and genetic study of Moffitt *et al.* (2001) classified both UTEX B 2092 and UTEX B 2093, originally classified as *N. spumigena* and *N. harveyana* (UTEX culture collection), as *N. harveyana*/*sphaerocarpa*. In the present study, PCC 7804, UTEX B 2092 and UTEX B 2093 formed a supported genetic group with *N. spumigena* that was clearly separate from the *N. harveyana* reference strain, Hübel 1983/300. The genetic analyses distinguished PCC 7804 from PCC 73104, since the strains were subdivided into distinct lineages, indicating that these strains might belong to two distinct species. Strain PCC 7804 showed only 65% DNA–DNA hybridization value with PCC 73104 (Lachance, 1981) and thus was considered to belong to a different species than PCC 73104 (Rippka *et al.*, 2001). The current species concept, however, allows more flexible DNA–DNA similarity frontiers of a single species when each strain from more than one genomic group share the same phenotypic property (Rosselló-Mora & Amann, 2001). PCC 7804 and PCC 73104 lacked gas vacuoles and thus putatively inhabit the same ecological niche, the benthic habitat. Therefore, in the absence of a clear phenotypic difference, which separates PCC 7804 from PCC 73104, it may be justified to assign PCC 7804 and PCC 73104 to a single species.

Conclusions

In this study, it was shown that within the genus *Nodularia* at least three different species, *N. spumigena*, *N. sphaerocarpa* and *N. harveyana*, exist. The morphologically defined species are closely related, but clearly distinguishable phylogenetically and, in the case of *N. spumigena*, phenotypically. *N. spumigena* is characterized by nodularin production, the presence of *ndaF*, gas vacuoles and possibly one copy of *gvpA*-IGS and thus possibly two *gvpA* genes. The benthic *Nodularia* are, in turn, able to glide, non-nodularin-producing, without *ndaF*, gas vacuoles and *gvpA*-IGS

fragment, and thus with one *gvpA* gene or no *gvpA* gene at all. *N. harveyana* is diverse and may in fact include two species. DNA–DNA hybridization will give more firm taxonomic conclusions of the three species determined in the present study. Future studies will also reveal if there are ecological differences between *N. sphaerocarpa* and *N. harveyana*.

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REFERENCES

- An, J. & Carmichael, W. W. (1994). Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. *Toxicon* **32**, 1495–1507.
- Barker, G. L. A., Hayes, P. K., O'Mahony, S. L., Vacharapiyasophon, P. & Walsby, A. E. (1999). A molecular and phenotypic analysis of *Nodularia* (Cyanobacteria) from the Baltic Sea. *J Phycol* **35**, 931–937.
- Barker, G. L., Handley, B. A., Vacharapiyasophon, P., Stevens, J. R. & Hayes, P. K. (2000). Allele-specific PCR shows that genetic exchange occurs among genetically diverse *Nodularia* (Cyanobacteria) filaments in the Baltic Sea. *Microbiology* **146**, 2865–2875.
- Beattie, K. A., Kaya, K. & Codd, G. A. (2000). The cyanobacterium *Nodularia* PCC 7804, of freshwater origin, produces [L-Har²]nodularin. *Phytochemistry* **54**, 57–61.
- Bolch, C. J. S., Orr, P. T., Jones, G. J. & Blackburn, S. I. (1999). Genetic, morphological, and toxicological variation among globally distributed strains of *Nodularia* (Cyanobacteria). *J Phycol* **35**, 339–355.
- Bonfield, J. K., Beal, K. F., Betts, M. J. & Staden, R. (2002). Trev: a DNA trace editor and viewer. *Bioinformatics* **18**, 194–195.
- Bornet, E. & Flahault, C. (1886). Revision des Nostocacées hétérocystées. *Ann Sci Nat Bot* **7**, 177–262.
- Cohan, F. M. (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457–487.
- Damerval, T., Castets, A. M., Guglielmi, G., Houmard, J. & Tandeau de Marsac, N. (1989). Occurrence and distribution of gas vesicle genes among cyanobacteria. *J Bacteriol* **171**, 1445–1452.
- del Carmen Pérez, M., Bonilla, S., de León, L., Šmarda, J. & Komárek, J. (1999). A bloom of *Nodularia baltica-spumigena* group (Cyanobacteria) in a shallow coastal lagoon of Uruguay, South America. *Algol Stud* **93**, 91–101.
- Edwards, U., Rogall, T., Blocker, H., Emde, M. & Bottger, E. G. (1989). Isolation and direct complete determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.
- Engström-Öst, J., Koski, M., Schmidt, K., Viitasalo, M., Jónasdóttir, S. H., Kokkonen, M., Repka, S. & Sivonen, K. (2002). Effects of toxic cyanobacteria on a plankton assemblage: community development during decay of *Nodularia spumigena*. *Mar Ecol Prog Ser* **232**, 1–14.
- Fastner, J., Flieger, I. & Neumann, U. (1998). Optimised extraction of microcystins from field samples – a comparison of different solvents and procedures. *Water Res* **32**, 3177–3181.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, WA, USA.
- Gugger, M., Lyra, C., Henriksen, P., Coute, A., Humbert, J. F. & Sivonen, K. (2002). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Int J Syst Evol Microbiol* **52**, 1867–1880.
- Gupta, R. S. (2000a). The natural evolutionary relationships among prokaryotes. *Crit Rev Microbiol* **26**, 111–131.
- Gupta, R. S. (2000b). The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev* **24**, 367–402.
- Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Harding, W. R., Rowe, N., Wessels, J. C., Beattie, K. A. & Codd, G. A. (1995). Death of a dog attributed to the cyanobacterial (blue-green algal) hepatotoxin nodularin in South Africa. *J S Afr Vet Assoc* **66**, 256–259.
- Hindák, F., Šmarda, J. & Komárek, J. (2003). *Nodularia moravica* spec. nova, a new benthic freshwater nostocalean species (Cyanophyta/Cyanobacteria/Cyanoprokaryota). *Algol Stud* **109**, 241–253.
- Iteman, I., Rippka, R., Tandeau de Marsac, N. & Herdman, M. (2002). rDNA analyses of planktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. *Microbiology* **148**, 481–496.
- Komárek, J., Hübel, M., Hübel, H. & Šmarda, J. (1993). The *Nodularia* studies. 2. Taxonomy. *Algol Stud* **68**, 1–25.
- Korber, B. (2001). HIV signature and sequence variation analysis. In *Computational Analysis of HIV. Molecular Sequences*, pp. 55–72. Edited by A. G. Rodrigo & G. H. Learn. Dordrecht, the Netherlands: Kluwer Academic.
- Koski, M., Schmidt, K., Engström-Öst, J., Viitasalo, M., Jónasdóttir, S. H., Repka, S. & Sivonen, K. (2002). Calanoid copepods feed and produce eggs in the presence of toxic cyanobacteria *Nodularia spumigena*. *Limnol Oceanogr* **47**, 878–885.
- Laamanen, M. J., Gugger, M. F., Lehtimäki, J. M., Haukka, K. & Sivonen, K. (2001). Diversity of toxic and nontoxic *Nodularia* isolates (Cyanobacteria) and filaments from the Baltic Sea. *Appl Environ Microbiol* **67**, 4638–4647.
- Laamanen, M. J., Forsström, L. & Sivonen, K. (2002). Diversity of *Aphanizomenon flos-aquae* (cyanobacterium) populations along a Baltic Sea salinity gradient. *Appl Environ Microbiol* **68**, 5296–5303.
- Lachance, M. A. (1981). Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. *Int J Syst Bacteriol* **31**, 139–147.
- Lampert, W. (1981). Inhibitory and toxic effects of blue-green algae on *Daphnia*. *Int Rev Gesamt Hydrobiol* **66**, 285–298.
- Lehtimäki, J., Sivonen, K., Luukkainen, R. & Niemelä, S. I. (1994). The effects of incubation time, temperature, light, salinity, and phosphorous on growth and hepatotoxin production by *Nodularia* strains. *Arch Hydrobiol* **130**, 269–282.
- Lehtimäki, J., Lyra, C., Suomalainen, S., Sundman, P., Rouhiainen, L., Paulin, L., Salkinoja-Salonen, M. & Sivonen, K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *Int J Syst Evol Microbiol* **50**, 1043–1053.

- Lepère, C., Wilmotte, A. & Meyer, B. (2000).** Molecular diversity of *Microcystis* strains (Cyanophyceae, Chroococcales) based on 16S rDNA sequences. *Syst Geogr Plants* **70**, 275–283.
- Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004).** ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- McBride, M. J. (2001).** Bacterial gliding motility: multiple mechanisms for cell movement over surfaces. *Annu Rev Microbiol* **55**, 49–75.
- Moffitt, M. C. & Neilan, B. A. (2001).** On the presence of peptide synthetase and polyketide synthase genes in the cyanobacterial genus *Nodularia*. *FEMS Microbiol Lett* **196**, 207–214.
- Moffitt, M. C., Blackburn, S. I. & Neilan, B. A. (2001).** rRNA sequences reflect the ecophysiology and define the toxic cyanobacteria of the genus *Nodularia*. *Int J Syst Evol Microbiol* **51**, 505–512.
- Nordin, R. N. & Stein, J. R. (1980).** Taxonomic revision of *Nodularia* (Cyanophyceae/Cyanobacteria). *Can J Bot* **58**, 1211–1224.
- Palenik, B. & Swift, H. (1996).** Cyanobacterial evolution and prochlorophyte diversity as seen in DNA-dependent RNA polymerase gene sequences. *J Phycol* **32**, 638–646.
- Palys, T., Nakamura, L. K. & Cohan, F. M. (1997).** Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. *Int J Syst Bacteriol* **47**, 1145–1156.
- Pushparaj, B., Pelosi, E. & Jüttner, F. (1999).** Toxicological analysis of the marine cyanobacterium *Nodularia harveyana*. *J Appl Phycol* **10**, 527–530.
- Rajaniemi, P., Hrouzek, P., Kaštovská, K., Willame, R., Rantala, A., Hoffmann, L., Komárek, J. & Sivonen, K. (2005).** Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int J Syst Evol Microbiol* **55**, 11–26.
- Rantala, A., Fewer, D. P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T. & Sivonen, K. (2004).** Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc Natl Acad Sci U S A* **101**, 568–573.
- Rasmussen, U. & Svenning, M. M. (1998).** Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Appl Environ Microbiol* **64**, 265–272.
- Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979).** Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.
- Rippka, R., Castenholz, R. W., Itaman, I. & Herdman, M. (2001).** Form-genus VII. *Nodularia* Mertens 1822. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 574–575. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.
- Rocap, G., Larimer, F. W., Lamerdin, J. & 21 other authors (2003).** Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* **424**, 1042–1047.
- Rosselló-Mora, R. & Amann, R. (2001).** The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.
- Rouhiainen, L., Sivonen, K., Buikema, W. J. & Haselkorn, R. (1995).** Characterization of toxin-producing cyanobacteria by using an oligonucleotide probe containing a tandemly repeated heptamer. *J Bacteriol* **177**, 6021–6026.
- Rudi, K., Skulberg, O. M. & Jakobsen, K. S. (1998).** Evolution of cyanobacteria by exchange of genetic material among phylogenetically related strains. *J Bacteriol* **180**, 3453–3461.
- Sivonen, K., Kononen, K., Carmichael, W. W., Dahlem, A. M., Rinehart, K. L., Kiviranta, J. & Niemelä, S. I. (1989).** Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and structure of the toxin. *Appl Environ Microbiol* **55**, 1990–1995.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002).** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Staden, D., Judge, P. & Bonfield, J. K. (2003).** *Managing Sequencing Projects in the GAP4 Environment. Introduction to Bioinformatics. A Theoretical and Practical Approach*. Edited by S. A. Krawetz & D. D. Womble. Totowa, NJ: Human Press.
- Swofford, D. L. (2001).** PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0b8. Sunderland, MA: Sinauer Associates.
- Thomson, N., Bentley, S., Holden, M. & Parkhill, J. (2003).** Fitting the niche by genomic adaptation. *Nat Rev Microbiol* **1**, 92–93.
- Tillet, D., Parker, D. L. & Neilan, B. A. (2001).** Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcyA*) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl Environ Microbiol* **67**, 2810–2818.
- Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Møkelke, L. & Sivonen, K. (2003).** Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. *Appl Environ Microbiol* **69**, 7289–7297.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wilson, K. M., Schembri, M. A., Baker, P. D. & Saint, C. P. (2000).** Molecular characterization of the toxic cyanobacterium *Cylindrospermopsis raciborskii* and design of a species-specific PCR. *Appl Environ Microbiol* **66**, 332–338.
- Woese, C. R. (2000).** Interpreting the universal phylogenetic tree. *Proc Natl Acad Sci U S A* **97**, 8392–8396.