

Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods

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An investigation was undertaken of the genetic diversity of *Nodularia* strains from the Baltic Sea and from Australian waters, together with the proposed type strain of *Nodularia spumigena*. The *Nodularia* strains were characterized by using a polyphasic approach, including RFLP of PCR-amplified 16S rRNA genes, 16S rRNA gene sequencing, Southern blotting of total DNA, repetitive extragenic palindromic- and enterobacterial repetitive intergenic consensus-PCR, ribotyping and phenotypic tests. With genotypic methods, the *Nodularia* strains were grouped into two clusters. The genetic groupings were supported by one phenotypic property: the ability to produce nodularin. In contrast, the cell sizes of the strains were not different in the two genetic clusters. 16S rRNA gene sequences indicated that all the *Nodularia* strains were closely related, despite their different origins. According to this study, two genotypes of *Nodularia* exist in the Baltic Sea. On the basis of the taxonomic definitions of Komárek *et al.* (*Algol Stud* 68, 1–25, 1993), the non-toxic type without gas vesicles fits the description of *Nodularia sphaerocarpa*, whereas the toxic type with gas vesicles resembles the species *N. spumigena* and *Nodularia baltica*.

Keywords: *Nodularia* cyanobacterium, RFLP, 16S rDNA sequencing, REP- and ERIC-PCR, ribotyping

INTRODUCTION

The cyanobacterial genus *Nodularia* commonly form blooms in brackish waters such as the Baltic Sea in Europe (Sivonen *et al.*, 1989a; Kononen *et al.*, 1993) and in coastal lagoons and brackish water lakes in Australia (Baker & Humpage, 1994; Jones *et al.*, 1994). These blooms have caused numerous cases of animal poisoning (Francis, 1878; Nehring, 1993) due to a hepatotoxin called nodularin (Rinehart *et al.*, 1988). It is a small, cyclic pentapeptide and has an LD₅₀ of 50–70 µg kg⁻¹ when administered intra-

peritoneally in mouse (Rinehart *et al.*, 1988; Sivonen *et al.*, 1989a). The toxicity of nodularin results from its ability to inhibit the serine/threonine-specific protein phosphatases PP1 and PP2A (Ohta *et al.*, 1994). Therefore, nodularin is a potent tumour promoter and a possible carcinogen (Ohta *et al.*, 1994). Nodularin is produced continuously by some *Nodularia* strains (Lehtimäki *et al.*, 1994, 1997).

Cyanobacteria have traditionally been classified on the basis of morphology. The analysis of 16S rRNA genes has revealed that morphological characters do not necessarily result in a phylogenetically reliable taxonomy (Giovannoni *et al.*, 1988; Wilmotte, 1994). On the basis of morphological criteria, Walsby *et al.* (1995), Albertano *et al.* (1996), Hayes & Barker (1997) and Barker *et al.* (1999) found four different morphological types of *Nodularia* together in the Baltic Sea. One of them was not identified to the species level, whereas three were distinguished according to the criteria of Komárek *et al.* (1993) as *Nodularia litorea*,

Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; IGS, intergenic spacer; REP, repetitive extragenic palindromic; STRR, short, tandemly repeated sequence; UPGMA, unweighted pairs group method with averages.

The GenBank accession numbers for the *Nodularia* 16S rRNA gene sequences obtained in this study are AJ133155, AJ133158, AJ133171 and AJ133177–AJ133184.

Nodularia spumigena and *Nodularia baltica*. These planktonic species, as well as the benthic species *Nodularia harveyana* have also been recorded from the Baltic Sea by Komárek *et al.* (1993). The species were differentiated by ecology and morphology such as the presence of gas vesicles, the dimensions and shapes of vegetative cells, heterocytes and akinetes and the size and shape of trichomes (Komárek *et al.*, 1993). The properties of gas vesicles have also been used for taxonomic characterization of *Nodularia*, e.g. species-typical differences in the accumulation of gas vesicles have been recorded (Komárek *et al.*, 1993; Šmarda & Šmajs, 1996).

On the basis of the intergenic spacer region (IGS) of the phycocyanin coding sequence (PC-IGS), the genetic diversity of *Nodularia* populations in the southern Baltic Sea in 1996 was shown to be restricted to two distinct genotypes (Hayes & Barker, 1997). Later, the same authors assigned Baltic *Nodularia* isolates to three groups based on the PC-IGS, two groups based on the *gvpA*-IGS and three groups based on rDNA intergenic transcribed spacer (ITS) sequences (Barker *et al.*, 1999).

In this study, *Nodularia* isolates from the Baltic Sea and from Australian waters and reference strains were characterized by 16S rRNA gene analysis and by total-genome-based techniques. In addition, the ability to produce nodularin and the morphology of *Nodularia* strains were analysed.

METHODS

Cyanobacterial strains. The *Nodularia* strains were grown on Z8 medium with salt added and no nitrogen, as described in Lehtimäki *et al.* (1994). Other strains were grown on Z8 medium (Kotai, 1972) with or without nitrogen depending the nitrogen-fixation capability of the strains studied. The *Nodularia* strains were isolated from different geographical regions (Table 1). Most of them originated from the different parts of the Baltic Sea: the Arkona Sea (BY1, P38, GRD113), the Baltic proper (HKVV, F81, TEILI, 59/22, 55/15), the Gulf of Finland (AV1, AV3, AV33, HEM, GR8a, GR8b, TR183, UP16a, UP16f) and the Gulf of Bothnia (SR5b). The locations of isolation in the Baltic Sea for strains GR8, TR183 and UP16, which are not specified in the references, are respectively 59° 26' 82" N, 22° 54' 38" E; 59° 12' 85" N, 22° 02' 51" E; and 59° 41' 87" N, 23° 28' 22" E.

Information about the strains other than *Nodularia* used in this study is available from Rippka & Herdman (1992) (Pasteur Culture Collection strains, designated PCC), Skulberg (1990) (*Anabaena* sp. strain CYA83/1) and Lehtimäki *et al.* (1997) (*Aphanizomenon* sp. strain TR183). The amounts of nodularin produced by *Nodularia* strains were determined by HPLC (Lehtimäki *et al.*, 1994) and/or by ELISA with the EnviroGard Microcystins PlateKit (Strategic Diagnostics) according to the manufacturer's instructions. Axenicity was tested on TGY (tryptone/glucose/yeast extract) agar plates (Atlas, 1993) and by microscopy after Gram staining.

Morphology. The dimensions and the range of variation of vegetative cells ($n = 50$) and intercalary heterocytes ($n = 50$) were measured from axenic strains of *Nodularia* by light microscopy. The sizes of the akinetes were not measured, because they were not found in all strains and because incubations at high and low temperature, under low phosphorus or high salinity conditions did not induce them. Axenic cultures of 10–12 days of age were photographed.

Isolation and purification of DNA. Genomic DNA was isolated from the cultures by a procedure modified from Golden *et al.* (1988). Sucrose (20% w/v), lysozyme (10 mg ml⁻¹), sodium sarcosyl (1% w/v) and proteinase K (200 µg ml⁻¹) were used to lyse the cells. The Nucleon PhytoPure system (Amersham Life Science) was used to eliminate polysaccharides.

Southern blotting. For short, tandemly repeated sequences (STRR), a heptamer repeat oligonucleotide probe of *Nostoc* sp. PCC 7120 (5'-GGGGACTGGGGACTGGGGACTGGG-3', Bauer *et al.*, 1993) was used to study non-axenic *Nodularia* strains (Table 1). DNA was digested with *HincII*, separated by agarose gel electrophoresis, blotted onto membrane and probed as described by Rouhiainen *et al.* (1995). According to the Southern blotting results, ten *Nodularia* strains representing toxic and non-toxic isolates from different regions of the Baltic Sea and isolates from Australia (Table 1) were purified to axenic by the soft-agarose-plating method (Rouhiainen *et al.*, 1995).

RFLP of PCR-amplified 16S rRNA genes. 16S rRNA genes were amplified by PCR with primers fD1 and rD1 (Weisburg *et al.*, 1991) as described by Lyra *et al.* (1997). The amplified genes were digested with *AluI*, *DdeI*, *HaeIII*, *HhaI*, *MboI*, *MspI* and *RsaI*. The restriction products were electrophoresed and recorded with a video camera (model WW-BP500/G; Panasonic CCTV). The GELCOMP software (version 4, Applied Maths BVBA) and manual editing were used to verify that all bands were found and that no artifacts were included (Germer-Smidt *et al.*, 1998). The matrix of similarities was calculated on the basis of the Dice band-matching coefficient (Dice, 1945) and the dendrogram was constructed by using the unweighted-pairs-group-method-with-averages (UPGMA) clustering algorithm (Sneath & Sokal, 1973).

16S rDNA sequence. 16S rRNA genes were amplified in two parts, with primers pA/pF' and pD/pH' (Edwards *et al.*, 1989). The sequencing was done either by the solid-phase method (Hultman *et al.*, 1991) and an automated ALF sequencer (Pharmacia) or by the DyeTerminator sequencing kit and the ABI PRISM 377XL DNA sequencer (Perkin Elmer). The sequences were assembled by the XGAP program of the Staden package (Bonfield *et al.*, 1995) and aligned by the PILEUP program of the GCG package (Devereux *et al.*, 1984). Phylogenetic trees were constructed by the neighbour-joining method and DNA parsimony method of PHYLIP (Felsenstein, 1993). In this program, bootstrap analysis was used to evaluate the tree topologies by performing 1000 resamplings.

Nucleotide sequence accession numbers. Strain designations and 16S rRNA gene sequence accession numbers of cyanobacterial strains used in this study are as follows: *Nodularia* sp. BY1, AJ133177; *Nodularia* sp. GR8b, AJ133178; *Nodularia* sp. NSPI-05, AJ133179; *Nodularia* sp. NSOR-12, AJ133180; *Nodularia* sp. PCC 7804, AJ133181; *Nodularia* sp. UP16f, AJ133182; *Nodularia* sp. HKVV, AJ133183; *Nodularia* sp. PCC 73104/1, AJ133184; *Aphanizomenon* sp.

Table 1. *Nodularia* strains used in this study

Culture collections: PCC, Institut Pasteur Culture Collection, Paris, France; NS, S. Blackburn, CSIRO, Division of Marine Research, Tasmania, Australia. The remaining strains are authors' isolates from the Baltic Sea. References: 1, Bolch *et al.* (1996); 2, Bolch *et al.* (1999); 3, Camm & Stein (1974); 4, Castenholz (1989); 5, Fujii *et al.* (1997a); 6, Fujii *et al.* (1997b); 7, Giovannoni *et al.* (1988); 8, Herdman *et al.* (1979a); 9, Herdman *et al.* (1979b); 10, Komárek *et al.* (1993); 11, Kononen *et al.* (1993); 12, Lachance (1981); 13, Lehtimäki *et al.* (1994); 14, Lehtimäki *et al.* (1997); 15, Lyra *et al.* (1997); 16, Moisander *et al.* (1996); 17, Neilan (1996); 18, Neilan *et al.* (1995); 19, Neilan *et al.* (1997b); 20, Neilan *et al.* (1999); 21, Nelissen *et al.* (1994); 22, Nordin & Stein (1980); 23, Rinne & Tarkiainen (1978); 24, Rippka *et al.* (1979); 25, Sivonen *et al.* (1989a); 26, Sivonen *et al.* (1989b); 27, Šmarda *et al.* (1988); 28, Wilmotte (1994); 29, Wilmotte *et al.* (1994).

Strain	Axenicity	Nodularin production	Geographical origin	Date of isolation	References
HKVV	+	–	Brackish water, Baltic Sea	Not known	2, 6, 13, 15, 16, 20, 23
BY1	+	+	Brackish water, Baltic Sea	13/8/86	1, 2, 13–16, 20, 25, 26
P38 (= EIB)	–	+	Brackish water, Baltic Sea.	13/8/86	25, 26
GDR113	–	+	Brackish water, Baltic Sea	14/8/86	25, 26
F81	+	+	Brackish water, Baltic Sea	31/7/87	25
TEILI	–	+	Brackish water, Baltic Sea	4/8/87	25
59/22	–	+	Brackish water, Baltic Sea	8/8/87	25
AV1	–	+	Brackish water, Baltic Sea	8/8/87	5, 25
AV3	+	+	Brackish water, Baltic Sea	10/8/87	25
AV33	–	+	Brackish water, Baltic Sea	10/8/87	25
55/15 (= EIA)	–	+	Brackish water, Baltic Sea	1/9/87	25
HEM	+	+	Brackish water, Baltic Sea	10/9/87	13, 15, 20
SR5b	–	+	Brackish water, Baltic Sea	14/8/91	11
GR8a	–	+	Brackish water, Baltic Sea	3/8/92	–
GR8b	+	+	Brackish water, Baltic Sea	4/8/92	–
TR183	–	+	Brackish water, Baltic Sea	18/7/93	–
UP16a	+	–	Brackish water, Baltic Sea	22/7/94	–
UP16f	+	–	Brackish water, Baltic Sea	22/7/94	–
PCC 73104/1	+	–	Alkaline soil, Spotted Lake, BC, Canada	1972	1–4, 7–9, 12, 15, 17–22, 24, 28, 29
PCC 7804	+	+	Thermal spring, Dax, France	1966	2, 10, 12, 27
NSPI-05 (= PI9211-11)	+	+	Coastal water, Peel Inlet, Australia	11/12/92	–
NSOR-12 (= OR9301-08)	+	+	Coastal water, Orielton Lagoon, Tasmania, Australia	1/10/93	20

TR183, AJ133155; *Anabaena* sp. CYA83/1, AJ133158; and *Microcystis* sp. PCC 7941, AJ133171. Three sequences were retrieved from GenBank: *Nodularia* sp. BCNOD9427, AJ22447; *Nostoc* sp. PCC 7120, X59559; and *Oscillatoria agardhii* CYA18, X84811.

Repetitive extragenic palindromic (REP)- and enterobacterial repetitive intergenic consensus (ERIC)-PCR. Central composite design (Montgomery, 1997) was used to optimize the temperature and the concentrations of MgCl₂, nucleotides, enzyme, template and primers for REP- and ERIC-PCR amplification. Central composite design was provided by DATA ANALYSIS TOOLBOX (ProfMath Oy) of the MATLAB statistical software for Windows (The MathWorks). Conditions where the largest numbers of PCR products were amplified from the genome of *Nodularia* (PCC 73104/1) were chosen for REP- and ERIC-PCR analysis.

To examine the distribution of repetitive sequences in *Nodularia* strains, the primers pairs REP (1R-I, 2) and ERIC (1R, 2) were used (Versalovic *et al.*, 1991). Each 25 µl reaction contained 50 ng template DNA, 50 pmol primers,

4 µg BSA µl⁻¹ and 2.8 U Dynazyme DNA polymerase in Red Hot buffer [750 mM Tris/HCl (pH 9), 200 mM (NH₄)₂SO₄, 0.1% Tween 20] with 10% (v/v) DMSO. Concentrations of MgCl₂ and nucleotides were 3.3 mM and 700 µM (ERIC-PCR) or 6 mM and 500 µM (REP-PCR). PCR mixtures were overlaid with 25 µl mineral oil. PCR amplification was performed with an initial denaturation at 95 °C for 7 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 39 or 47 °C for 1 min with REP and ERIC primers, respectively, and extension at 65 °C for 8 min, with a single final extension at 65 °C for 16 min. PCR products were separated on 1% agarose gels containing ethidium bromide and the patterns were recorded with a video camera. The REP- and ERIC-PCR profiles were combined with the GELCOMP software. The similarities between stored pairs of tracks were calculated by the Dice band-based coefficient and the groupings were visualized by the UPGMA clustering algorithm.

Automated ribotyping. DNA extracts were fingerprinted with the RiboPrinter microbial characterization system

(Qualicon) as described by Bruce (1996) with the following modification: DNA, approximately 500 ng per strain, was used for the analysis instead of cells. Digestions were sometimes partial, which was probably due to inhibitory substances present in the samples. The image files were analysed by the GELCOMP software. The Dice similarity coefficient was used and patterns were clustered by UPGMA (GELCOMP).

RESULTS

Phenotypes of *Nodularia*

By light-microscopic examination, no groups of strains could be recognized on the basis of cell size. The length and width of vegetative cells varied from 3.1 to 4.7 µm and from 4.6 to 8.3 µm, respectively (Table 2). Vegetative cells were shorter than they were wide (Table 2, Fig. 1a–l), thus the length:width (l:w) ratio of vegetative cells varied from 0.52 (strain NSPI-05) to 0.91 (strain F81). Heterocysts were 4.2–7.4 µm long and 5.5–9.7 µm wide (Table 2). They were usually shorter than wide, but in some strains (F81, AV3, HEM), they were round (Table 2, Fig. 1c–e), meaning that the l:w ratio was 1.0. Furthermore, harsh culture conditions, e.g. starvation for phosphate used to germinate akinetes, increased the length of heterocysts until the cells were roundish. The lowest l:w ratio (0.52) was recorded for NSPI-05 strain. The length of cells never exceeded their width. The form of trichomes was variable (Fig. 1). According to nodularin and gas vesicle production, two groups of *Nodularia* could be recognized (Tables 1 and 2). Gas vesicles were not observed in non-toxic (HKVV, UP16a, UP16f and

PCC 73104/1) isolates or in some toxic isolates (HEM, BY1 and PCC 7804) (Table 2). However, strains HEM and BY1 had gas vesicles but lost them during storage in our laboratory.

Morphological analysis based on the criteria of Komárek *et al.* (1993) (see also Table 3) placed the non-toxic *Nodularia* strains as *N. sphaerocarpa*, while the toxic strains could be identified as *N. spumigena* or *N. baltica*. Based on the cell size, none of the strains belonged to *N. litorea*.

Genetic diversity of non-axenic *Nodularia* strains

To screen the genetic heterogeneity within non-axenic *Nodularia* isolates, the presence of STRR sequences was studied for 17 strains. Most *Nodularia* strains had very simple hybridization patterns compared with the pattern of *Nostoc* sp. strain PCC 7120 (Fig. 2). The toxic Baltic strains were different from the non-toxic strain HKVV. The toxic Australian strains were dissimilar to the Baltic isolates and to each other.

Genetic characters of axenic *Nodularia* strains

From the 12 axenic *Nodularia* strains (Table 1), three closely related RFLP genotypes were found on the basis of restriction fragment patterns of 16S rRNA genes (Fig. 3). With this technique, the toxic Baltic strains were separated from the non-Baltic strains and from non-toxic Baltic strains. Of the seven restriction enzymes used, only two (*DdeI* and *MspI*) yielded different profiles, while the others (*AluI*, *HaeIII*, *HhaI*,

Table 2. Morphological characters of the axenic *Nodularia* isolates used in this study

Cell dimensions given are means of 50 measurements. The numbers in parentheses are the SD and the minimum and maximum values. The presence or absence of gas vesicles is indicated.

Strain	Size by light microscopy (µm)				Gas vesicles
	Vegetative cells		Heterocysts		
	Length	Width	Length	Width	
HEM	3.1 (0.68, 2.5, 5.0)	4.6 (0.64, 3.8, 6.3)	6.2 (0.39, 5.0, 7.5)	6.2 (0.39, 5.0, 7.5)	—*
BY1	4.4 (0.63, 3.8, 5.0)	5.1 (0.30, 5.0, 6.3)	5.1 (0.38, 5.0, 6.3)	6.1 (0.38, 5.0, 6.3)	—*
AV3	4.4 (0.68, 3.8, 6.3)	5.9 (0.90, 5.0, 7.5)	7.4 (0.34, 6.3, 7.5)	7.4 (0.34, 6.3, 7.5)	+
GR8b	4.4 (0.63, 3.8, 5.0)	6.8 (0.62, 6.3, 7.5)	5.1 (0.49, 3.8, 6.3)	7.6 (0.30, 7.5, 8.8)	+
F81	4.5 (0.62, 3.8, 5.0)	4.9 (0.69, 3.8, 6.3)	5.5 (0.62, 5.0, 6.3)	5.5 (0.62, 5.0, 6.3)	+
NSPI-05	4.3 (1.2, 2.5, 6.3)	8.3 (1.1, 7.5, 10.0)	5.1 (0.30, 5.0, 6.3)	9.7 (0.55, 8.8, 10.0)	+
NSOR-12	3.3 (1.0, 2.5, 5.0)	5.1 (0.25, 5.0, 6.3)	4.8 (0.44, 3.8, 5.0)	7.2 (0.57, 6.3, 7.5)	+
PCC 7804	3.2 (0.63, 2.5, 3.8)	5.2 (0.49, 5.0, 6.3)	4.2 (0.59, 3.8, 5.0)	6.7 (0.61, 6.3, 7.5)	—
HKVV	4.2 (0.60, 3.8, 5.0)	7.2 (0.69, 6.3, 8.8)	5.2 (0.49, 5.0, 6.3)	7.6 (0.38, 7.5, 8.8)	—
UP16a	4.1 (0.58, 3.8, 5.0)	5.4 (0.60, 5.0, 6.3)	5.5 (0.61, 5.0, 6.3)	7.6 (0.25, 7.5, 8.8)	—
UP16f	4.7 (0.79, 3.8, 6.3)	6.9 (0.72, 5.0, 7.5)	4.7 (0.79, 3.8, 6.3)	7.0 (0.63, 6.3, 7.5)	—
PCC 73104/1	4.3 (0.62, 3.8, 5.0)	6.6 (0.58, 6.3, 7.5)	5.0 (0, 5.0, 5.0)	7.5 (0, 7.5, 7.5)	—

* Gas vesicles have been observed previously.

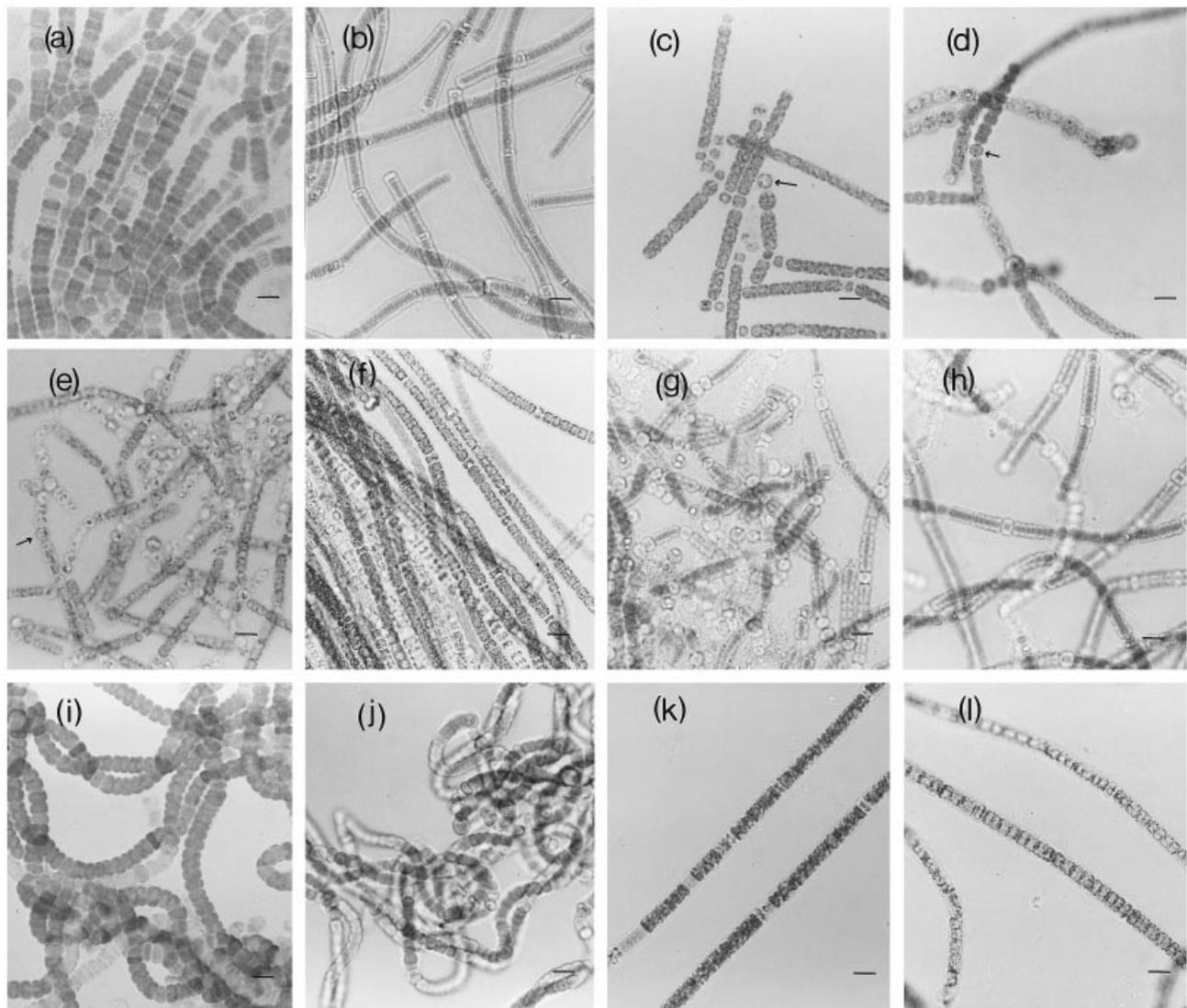


Fig. 1. Photomicrographs of *Nodularia* isolates: (a) HKVW, (b) BY1, (c) F81, (d) AV3, (e) HEM, (f) GR8b, (g) UP16a, (h) UP16f, (i) PCC 73104/1, (j) PCC 7804, (k) NSPI-05 and (l) NSOR-12. Bars, 10 μ m. Some of the heterocytes are marked with arrows.

*Mbo*I and *Rsa*I) produced similar patterns (data not shown) for the genus *Nodularia*. For cyanobacteria other than *Nodularia*, these five enzymes yielded different patterns.

The almost complete 16S rRNA gene sequences (1444–1445 bp) were determined for eight *Nodularia* strains, which were chosen on the basis of RFLP genotypes. A total of 1395 nucleotide sites were used to generate phylogenetic trees on the basis of the neighbour-joining method and the DNA parsimony method (Fig. 4). The topologies of these trees were highly similar. The minor differences between the two trees were for the nodes that were not supported by the bootstrap analysis (Fig. 4a, b). The internal branch

lengths estimated by the distance method (Fig. 4a) were shorter than those estimated by the parsimony method (Fig. 4b).

The distance and parsimony methods gave two statistically supported clusters of *Nodularia* (Fig. 4), which were in agreement with the results from RFLP of PCR-amplified 16S rRNA genes (Fig. 3). One cluster comprised toxic strains and the other non-toxic strains (Fig. 4). However, the differences between strains in these two clusters were low, e.g. it was only 1.3% between PCC 7804 and the non-toxic strains. The most dissimilar toxic strains, BY1 and PCC 7804, were 99.0% similar in 16S rRNA gene sequence, meaning that similarity within *Nodularia* strains originating

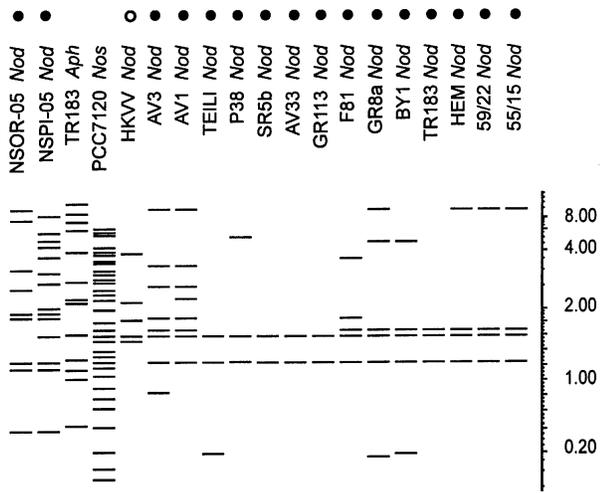


Fig. 2. Southern hybridization analysis of *HincII*-digested total DNAs from several non-axenic *Nodularia* sp. strains (*Nod*), one *Aphanizomenon* sp. strain (*Aph*) and one *Nostoc* sp. strain (*Nos*). The size marker (kb) on the right was generated by digestion of lambda DNA with *Bst*EII. Symbols: ●, toxic; ○, non-toxic.

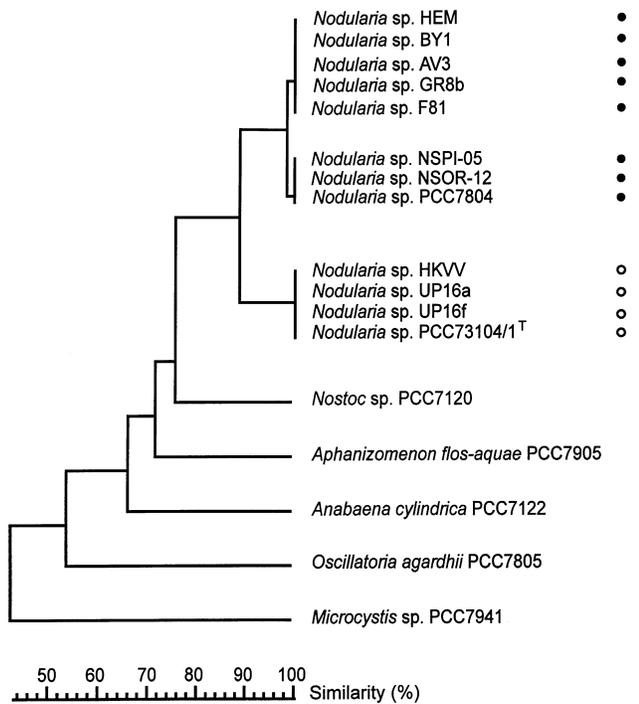


Fig. 3. UPGMA dendrogram (GELCOMP) of RFLP analysis of 16S rRNA genes of several *Nodularia* strains and other cyanobacterial genera. The restriction enzymes used were: *AluI*, *DdeI*, *HaeIII*, *HhaI*, *MboI*, *MspI* and *RsaI*. Symbols: ●, toxic; ○, non-toxic; T, proposed type strain of *N. spumigena*.

from different regions (Table 1) was remarkably high. Grouping within the cluster containing the toxic strains was not supported statistically. Furthermore,

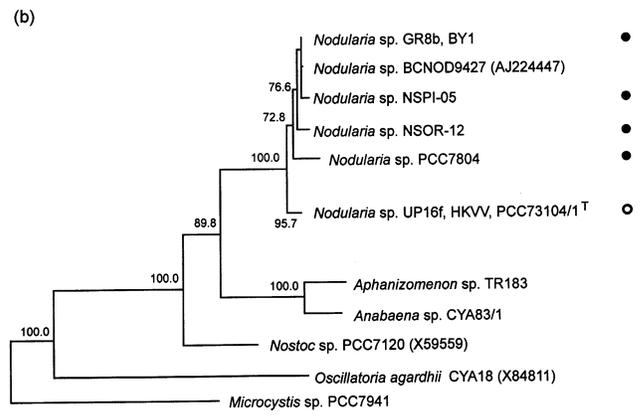
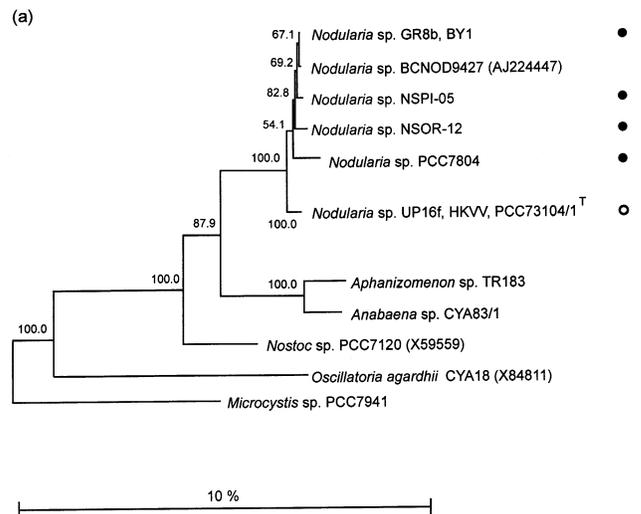


Fig. 4. Phylogenetic dendrograms based on cyanobacterial 16S rRNA gene sequences and constructed by (a) the neighbour-joining method and (b) the parsimony method (PHYMLIP). Scale bar represents 10% similarity of 1395 nucleotides. Percentages of 1000 bootstrap replicates are indicated near the nodes (only those values > 50% are shown). Sequences from GenBank are marked with accession numbers. Symbols: ●, toxic; ○, non-toxic; T, proposed type strain of *N. spumigena*.

the 16S rDNA sequences of three non-toxic strains (PCC 73104/1, HKVV and UP16f) and of two toxic strains (BY1 and GR8b) were identical (Fig. 4).

REP- and ERIC-PCR patterns grouped the *Nodularia* strains in two groups (Fig. 5). The toxic strains formed one group. In this group, the strains from Australia and France were separated from the Baltic strains. With these techniques and the ribotyping analysis, the non-toxic strains (PCC 73104/1, HKVV and UP16f) were separated from the toxic strains (Figs 5 and 6). Strains PCC 73104/1 and HKVV were more similar than strain UP16f according to these techniques (Figs 5 and 6). In the RiboPrinter system, the DNA sample of strain PCC 7804 was only partially digested, even after an extra purification step with the Nucleon

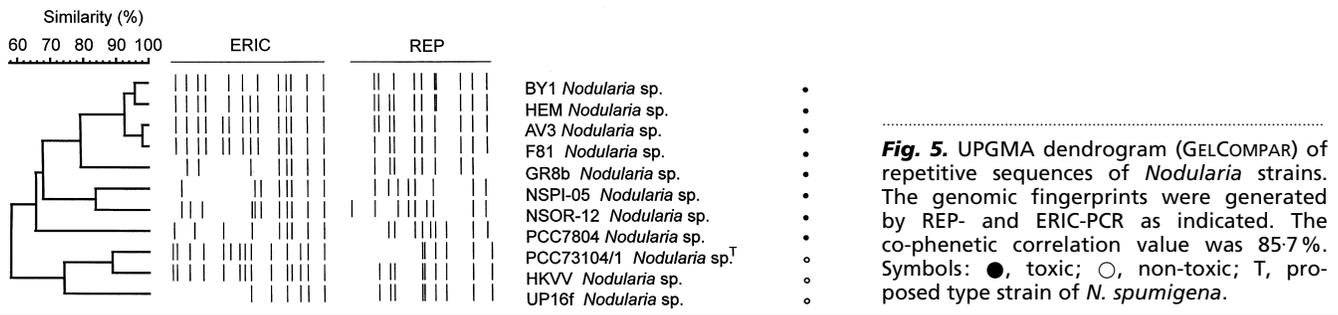


Fig. 5. UPGMA dendrogram (GELCOMPAR) of repetitive sequences of *Nodularia* strains. The genomic fingerprints were generated by REP- and ERIC-PCR as indicated. The co-phenetic correlation value was 85.7%. Symbols: ●, toxic; ○, non-toxic; T, proposed type strain of *N. spumigena*.

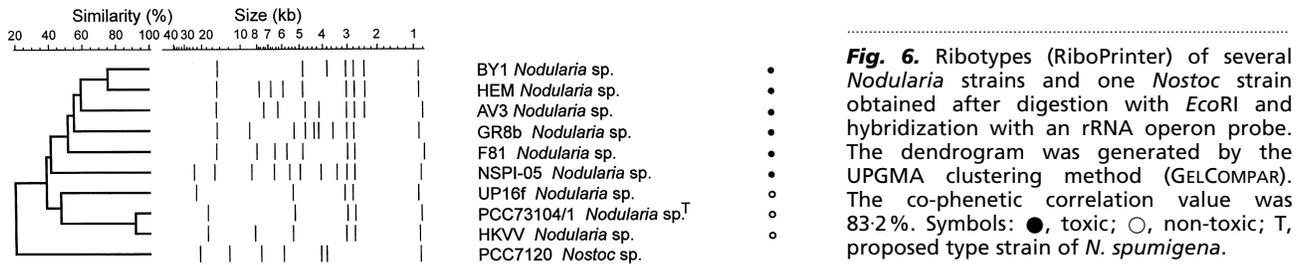


Fig. 6. Ribotypes (RiboPrinter) of several *Nodularia* strains and one *Nostoc* strain obtained after digestion with *EcoRI* and hybridization with an rRNA operon probe. The dendrogram was generated by the UPGMA clustering method (GELCOMPAR). The co-phenetic correlation value was 83.2%. Symbols: ●, toxic; ○, non-toxic; T, proposed type strain of *N. spumigena*.

PhytoPure system and, therefore, this sample was not included in the results.

DISCUSSION

Phenotypic characteristics such as the ability to form gas vesicles, which regulate the buoyancy of planktonic cyanobacteria, have been used to differentiate between the species of *Nodularia* (Smarda *et al.*, 1988; Komárek *et al.*, 1993). In the laboratory, cyanobacterial isolates may lose their gas vesicles. Therefore, the ability to produce gas vesicles may not be a useful character to identify cultured strains, as confirmed in this study (see Table 2, BY1 and HEM strains). However, all of the toxic *Nodularia* strains examined were able to produce gas vesicles and it remains to be proven whether the non-toxic strains are unable to make these structures. Using cell size as a taxonomic criterion for cultured isolates of cyanobacteria also proved to be problematic, since this character may vary according to the growth conditions. The cell size of cultured *Nodularia* strains was not reflected in the 16S rRNA gene similarity. Morphological characterization of unicellular *Merismopedia* and *Microcystis* strains has also been unsuccessful in distinguishing genetic subclusters created by 16S rDNA sequencing (Palinska *et al.*, 1996; Otsuka *et al.*, 1998). In this study, the phenotypic character of *Nodularia* strains that was consistent with the genotypic analysis was nodularin production. By all of the genotypic methods, the non-toxic *Nodularia* strains were differentiated from the toxic ones. Previously, using RAPD-PCR and *cpcBA* IGS sequence data, Bolch *et al.* (1999) found genotypic distinctions between most toxic and non-toxic *Nodularia* isolates. Thus, nodularin production might be used as a marker in the taxonomy of *Nodularia*. In contrast to our study,

no correlation between 16S rRNA gene evolution and toxicity of *Microcystis* strains was observed (Neilan *et al.*, 1997a), although the *Microcystis* strains were clustered similarly by RFLP of ITS and toxin production (Neilan, 1996).

The occurrence of phylogenetic types of cyanobacteria and their distribution in marine ecosystems have been surveyed by sequencing 16S rRNA genes obtained from DNA isolated directly from the environment. The discovery of cyanobacterial clusters SAR6/SAR7 in both the Pacific and Atlantic Oceans (Giovannoni *et al.*, 1990; Schmidt *et al.*, 1991; Fuhrman *et al.*, 1993; Mullins *et al.*, 1995) revealed that this cyanobacterial cluster is distributed globally in the seas. Our study and the study of Bolch *et al.* (1999) of cyanobacterial sequences retrieved from laboratory cultures showed geographically diverse strains of *Nodularia* to be very close relatives. Similar findings were reported for *Microcystis* (Neilan *et al.*, 1997a).

16S rRNA gene-based analysis and fingerprinting techniques revealed similar genotypic diversity for *Nodularia* strains. Previously, Vinuesa *et al.* (1998) had shown these techniques to be consistent when characterizing *Bradyrhizobium* strains.

Our results indicated that two closely related *Nodularia* genotypes are found in the Baltic Sea. One genotype consists only of non-toxic strains (UP16a, UP16f and HKVV). With 16S rRNA gene sequencing, these strains were identical to the proposed type strain of *N. spumigena*, PCC 73104/1 (Rippka *et al.*, 1979; Nordin & Stein, 1980), which is a soil isolate. All genetic markers separate it and other non-toxic strains from the toxic strains. The toxic strains form another genotype, which most closely fits the descriptions of *N.*

Table 3. Morphological and ecological properties of planktonic and benthic *Nodularia* species

Data on morphological and ecological properties were taken from Komárek *et al.* (1993) and data on gas vesicles were derived from Šmarda & Šmajs (1996). Phytoplankton includes microscopic aquatic organisms that float freely or have weak swimming abilities. The organisms found aggregated in the littoral zone are the metaphyton, which is synonymous with the term tychoplankton used previously. The euplankton refers to organisms living in the open waters. The term periphyton refers to microscopic organisms attached to objects projecting above the bottom sediment, whereas organisms living on or in it are benthic. NA, Not applicable.

Character	<i>N. baltica</i>	<i>N. harveyana</i>	<i>N. litorea</i>	<i>N. sphaerocarpa</i>	<i>N. spumigena</i>
Trichome:					
Width (µm)	4·8–6	4–5	10–15	5–7	6·8–12
Cell length (µm)	1·8–4·8	1·5–2·5	2–4	3·4–6	2–4
Heterocysts:					
Length × width (µm)	2–5 × 5–7·5	3–5 × 4–6	4·4–6·8 × 10–15	4–6 × 5–7	3·5–5 × 9–13·7
Akinetes:					
Length × width (µm)	4–8 × 5·5–9	4–8 × 6–7	6–10 × 14–15	7–12·7 × 7–11·9	6–10·8 × 10–12
Observations	Sometimes solitary or in rows	In series of two to sixteen	Solitary or in short series	In series	In series or discontinuous rows, rarely solitary or in twos
Gas vesicles:					
Present?	Yes	No	Yes	No	Variable
Density (µm ⁻²)	140/160–180	NA	215/230–240	NA	139/160–180
Ecology	Metaphyton or phytoplankton; marine, brackish and saline waters	Benthic or periphyton; saline pools and lakes, thermal springs and marshes with high salinity	Phytoplankton; marine, brackish and saline waters	Periphyton; streams and freshwater lakes. Probably also subaerophytic on flooded alkaline soils	Metaphyton and euplankton; marine, brackish and saline waters

baltica and *N. spumigena* (Table 3). According to the nomenclature of Komárek *et al.* (1993), the proposed type strain PCC 73104/1 is not typical of *N. spumigena*.

The phenotypic characters of the non-toxic *Nodularia* strains fit most closely to the description of *N. sphaerocarpa*, even without the recorded data of akinetes (Table 3). When akinetes were present, like in the HKVV strain, they occurred in series and were more or less spherical. The inability to produce gas vesicles is common in all typical strains of this species (Komárek *et al.*, 1993). Although strains UP16a and UP16f have been isolated from the planktonic community, it is possible that these non-toxic strains without gas vesicles belong to benthic species. This suggestion is based on the small number (three) of non-toxic strains among approximately 100 toxic *Nodularia* strains we have isolated from the Baltic Sea. On the other hand, it might be that these non-toxic strains are rare in the planktonic community of the Baltic Sea. This is supported by the analysis of field samples; blooms samples containing *Nodularia* were frequently toxic (Sivonen *et al.*, 1989b; Kononen *et al.*, 1993). Strain HKVV has previously been classified as *N.*

sphaerocarpa by using morphological criteria (Lehtimäki *et al.*, 1994; Bolch *et al.*, 1999). The morphological dimensions of this strain and some other strains were found to be variable in different studies (this study; Bolch *et al.*, 1999). The difference in these features is probably due to the different growth conditions. Previously, different growth conditions such as pH, temperature and salinity were reported to have no effect on dimensions of vegetative cells and heterocytes of *Nodularia* strains (Nordin & Stein, 1980). In contrast, all of the morphological traits of unicellular halotolerant cyanobacteria were reported to be dependent on growth conditions, especially salinity (Garcia-Pichel *et al.*, 1998).

On the basis of cell size, it was not possible to identify strain BY1 as *N. baltica* or *N. spumigena* in this study. Bolch *et al.* (1999) also failed to identify it to the species level. By *cpcBA* sequence analysis, this strain has been placed with isolates BCNOD9401 and BCNOD9427 (Bolch *et al.*, 1999), which were isolated from the Baltic Sea and which have previously been designated as *N. litorea* or *N. spumigena* according to trichome widths (Hayes & Barker, 1997). By 16S

rDNA sequence analysis, strain BY1 grouped with isolate BCNOD9427 (this study; Hayes & Barker, 1997).

In this study, all of the toxic *Nodularia* strains, regardless of origin, belonged to the second genotype, which usually had the ability to form gas vesicles and which fits to the description of *N. spumigena* and *N. baltica* (Komárek *et al.*, 1993). Even though the *Nodularia* isolates were closely related by 16S rDNA sequence, the fingerprinting methods showed that there were differences among the isolates. However, the profiles of repetitive sequences, which have previously been shown to exist in cyanobacterial genomes (Versalovic *et al.*, 1991; Rasmussen & Svenning, 1998), indicated high genetic homogeneity among the toxic Baltic strains of *Nodularia*. The toxic strains NSPI-05 and NSOR-12 from Australia and PCC 7804 from France were found to be different from the Baltic Sea strains by all genotypic methods. Similarly, Bolch *et al.* (1996, 1999) have shown the Australian strains to be different from the Baltic strains. We also screened STRR sequences of non-axenic *Nodularia* strains with this method, which was applied previously to *Anabaena* in our laboratory (Rouhiainen *et al.*, 1995). Although most of the *Nodularia* strains had only a few locations with STRR sequences, this method gave the same information as the other fingerprinting methods.

DNA–DNA homology studies have been used to measure the degree of relatedness between organisms with high 16S rRNA sequence similarity (Stackebrandt & Goebel, 1994). According to these authors, less than 70% DNA homology is expected for species having less than 97% sequence similarity. Here, *Nodularia* strains PCC 73104/1 and PCC 7804 had 98.7% sequence similarity. According to the study of Lachance (1981), these strains had 65% relative binding. Therefore, these strains may be from the same genospecies, because the DNA relatedness of 65% is not much below the lower boundary level suggested by Wayne *et al.* (1987). Whether the *Nodularia* strains in the present study belong to one genospecies needs to be determined by DNA–DNA hybridization studies, since identity of 16S rRNA sequences does not guarantee species identity (Fox *et al.*, 1992).

Nodularia strains that belong to the same genospecies may exhibit a cluster of morphotypes. Previously, *Microcystis* strains that share high sequence similarities of the 16S rDNA (Otsuka *et al.*, 1998) and of the 16S–23S ITS (Otsuka *et al.*, 1999) have been suspected to be members of the same genospecies showing various morphological expressions. Furthermore, similar results were found with the genus *Merismopedia* (Palinska *et al.*, 1996).

Nodularia frequently form massive water-blooms in many brackish-water bodies. This genus can also produce hepatotoxin, which causes health hazards for animals and human beings. In this study, we have clarified the taxonomy of this genus by using different genotypic and phenotypic markers. We have shown a

clear genotypic distinction between toxic and non-toxic *Nodularia* strains. Whether this separation is valid in natural *Nodularia* populations remains to be investigated.

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