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Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera

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Toxic and non-toxic cyanobacterial strains from *Anabaena*, *Aphanizomenon*, *Calothrix*, *Cylindrospermum*, *Nostoc*, *Microcystis*, *Planktothrix* (*Oscillatoria agardhii*), *Oscillatoria* and *Synechococcus* genera were examined by RFLP of PCR-amplified 16S rRNA genes and 16S rRNA gene sequencing. With both methods, high 16S rRNA gene similarity was found among planktic, anatoxin-a-producing *Anabaena* and non-toxic *Aphanizomenon*, microcystin-producing and non-toxic *Microcystis*, and microcystin-producing and non-toxic *Planktothrix* strains of different geographical origins. The respective sequence similarities were 99·9–100 %, 94·2–99·9 % and 99·3–100 %. Thus the morphological characteristics (e.g. *Anabaena* and *Aphanizomenon*), the physiological (toxicity) characteristics or the geographical origins did not reflect the level of 16S rRNA gene relatedness of the closely related strains studied. In addition, cyanobacterial strains were fingerprinted with repetitive extragenic palindromic (REP)- and enterobacterial repetitive intergenic consensus (ERIC)-PCR. All the strains except two identical pairs of *Microcystis* strains had different band profiles. The overall grouping of the trees from the 16S rRNA gene and the REP- and ERIC-PCR analyses was similar. Based on the 16S rRNA gene sequence analysis, four major clades were formed. (i) The clade containing filamentous heterocystous cyanobacteria was divided into three discrete groups of *Anabaena*/*Aphanizomenon*, *Anabaena*/*Cylindrospermum*/*Nodularia*/*Nostoc* and *Calothrix* strains. The three other clades contained (ii) filamentous non-heterocystous *Planktothrix*, (iii) unicellular non-heterocystous *Microcystis* and (iv) *Synechococcus* strains.

Keywords: cyanobacteria, 16S rRNA gene, RFLP, sequencing, REP, ERIC

INTRODUCTION

The chlorophyll-*a*-containing photosynthetic cyanobacteria occur in a wide range of habitats. In eutrophic fresh and brackish waters cyanobacteria form toxic water blooms which have caused human and animal poisonings (Ressom *et al.*, 1994; Kuiper-Goodman *et al.*, 1999). The most frequently found toxins in cyanobacterial blooms worldwide are hepatotoxic cyclic peptides, microcystins and nodularins (Sivonen & Jones, 1999). Mass occurrences of cyanobacteria that contain neurotoxins [anatoxin-a, anatoxin-a(S) and saxitoxins] have been found in Australia, Europe and North America (Sivonen & Jones, 1999).

Cyanobacteria are a morphologically diverse group of organisms ranging from unicellular to filamentous forms. Traditionally, the classification of cyanobacteria has been based on morphological characters,

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Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; LTRR, long tandemly repeated sequence; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; REP, repetitive extragenic palindromic; STRR, short tandemly repeated sequence; UPGMA, unweighted pairs group method with averages.

The GenBank/EMBL accession numbers for the cyanobacterial 16S rRNA gene sequences are AJ133151–AJ133154, AJ133156, AJ133157, AJ133159–AJ133170, AJ133172–AJ133176 and AJ133185

Table 1 Cyanobacterial strains used in this study

Toxin production is indicated as: N, neurotoxic (anatoxin-a); H, hepatotoxic (microcystins); NT, non-toxic; ND, toxicity not determined; NK; type of toxicity is not known. The strains for which toxicities are determined in this study are indicated in bold. The accession numbers of 16S rRNA genes indicated in the table are for sequences determined in this study. Culture collections: PCC, Pasteur Culture Collection, Paris, France; NIVA-CYA, Norwegian Institute for Water Research, Oslo, Norway; NIES, National Institute for Environmental Studies, Tsukuba, Japan. *Aphanizomenon gracile* PH-219 and *Aphanizomenon* sp. PH-271 were kindly provided by P. Henriksen (National Environmental Research Institute, Denmark), *Microcystis* sp. HUB5-3 by J. Fastner (Umwelt Bundes Amt, Germany) and *Nostoc* sp. 268 by B. Gromov (University of St Petersburg, Russia). References: 1, Asayama *et al.* (1996); 2, Giovannoni *et al.* (1988); 3, Herdman *et al.* (1979a); 4, Herdman *et al.* (1979b); 5, Kenyon *et al.* (1972); 6, Kondo *et al.* (2000); 7, Lachance (1981); 8, Leeuwangh *et al.* (1983); 9, Lehtimäki *et al.* (2000); 10, Lu *et al.* (1997); 11, Luukkainen *et al.* (1993); 12, Luukkainen *et al.* (1994); 13, Lyra *et al.* (1997); 14, Masephol *et al.* (1996); 15, Mazel *et al.* (1990); 16, Neilan *et al.* (1995); 17, Neilan *et al.* (1997a); 18, Neilan *et al.* (1997b); 19, Neilan *et al.* (1999); 20, Otsuka *et al.* (1999); 21, Rapala *et al.* (1993); 22, Rasmussen & Svenning (1998); 23, Rippka & Herdman (1992); 24, Rippka *et al.* (1979); 25, Rouhiainen *et al.* (1995); 26, Rudi & Jakobsen (1999); 27, Rudi *et al.* (1997); 28, Rudi *et al.*, 1998; 29, Sivonen *et al.* (1989); 30, Sivonen *et al.* (1990); 31, Sivonen *et al.* (1992); 32, Sivonen *et al.* (1995); 33, Turner (1997); 34, Turner *et al.* (1999); 35, Vezie *et al.* (1998); 36, Wilmotte (1994); 37, Wilmotte *et al.* (1992); 38, Yasuno *et al.* (1998).

Strain	Toxicity	Geographical origin	Accession no.	Reference
Anabaena sp.				
14	N*	Lake Sääksjärvi, Finland	AJ133152	13, 17, 21, 25, 29
37	N*	Lake Sääksjärvi, Finland	–	13, 25, 29
54	N*	Lake Sääksjärvi, Finland	–	13, 19, 25, 29
86	N*	Lake Villikkalanjärvi, Finland	AJ133151	13, 25, 29
123	N*	Lake Säyhteenjärvi, Finland	–	13, 19, 25, 29
130	N*	Lake Säyhteenjärvi, Finland	–	13, 21, 25
66A	H*	Lake Kiikkara, Finland	AJ133157	13, 19, 25, 31–32
66B	H*	Lake Kiikkara, Finland	–	13, 25, 31–32
90	H*	Lake Vesijärvi, Finland	AJ133156	13, 19, 25, 31–32
202A1	H*	Lake Vesijärvi, Finland	AJ133159	13, 19, 25, 31–32
202A1/35	H*	Lake Vesijärvi, Finland	–	13
202A2	H*	Lake Vesijärvi, Finland	–	13, 25, 31–32
277	NT†‡§¶	River Perniönjoki, Finland	AJ133160	13, 19, 25
PCC 6309	NT‡§¶	Freshwater, The Netherlands	–	3–5, 7, 23–24
PCC 7108	NT‡¶	Intertidal zone, USA	AJ133162	7, 16, 23–24
PCC 73105	NT‡§¶	Pond water, England	–	23, 16, 18
PCC 9208	NT‡¶	Soil, Spain	–	23
Anabaena circinalis				
NIES 41	ND	Lake Kasumigaura, Japan	–	16, 18
Anabaena cylindrica				
PCC 7122	NT‡§¶	Pond water, England	–	2–4, 7, 13, 23–24, 33–34, 36–37
Anabaena flos-aquae				
NIVA-CYA 83/1	H*	Lake Edlandsvatn, Norway	–	9, 13, 19, 25–28, 31–32
NIES 73	NT‡¶	Lake Kasumigaura, Japan	–	16, 18
Anabaena spiroides				
NIES 79	ND	Lake Kasumigaura, Japan	–	
Aphanizomenon sp.				
TR183	NT†‡§¶	The Baltic Sea	–	9, 13, 19
202	NT†‡§¶	Lake Vesijärvi, Finland	AJ133153	13, 19
PH-271	NT‡§¶	Lake Madesø, Denmark	–	
PCC 7905	NT‡§¶	Lake Brielse Meer, The Netherlands	AJ133154	9, 13, 16, 23
Aphanizomenon flos-aquae				
NIES 81	NT†‡§‖¶	Lake Kasumigaura, Japan		16, 18–19
Aphanizomenon gracile				
PH-219	NT‡§¶	Lake Nørre, Denmark	–	
Calothrix sp.				
PCC 7714	NT‡§¶	Pool, India	AJ133164	7, 23
Cylindrospermum stagnale				
PCC 7417	NT‡§¶	Soil, greenhouse, Sweden	AJ133163	3–4, 7, 23–24, 33–34, 36–37
Nodularia spumigena				
PCC 74301/1	NT‡§¶	Soil, Canada	–	9, 13, 23
Nostoc sp.				
152	H*	Lake Sääksjärvi, Finland	AJ133161	13, 19, 25, 30
159	NT‡§¶	Lake Haukkajärvi, Finland	–	28

Table 1 (*cont.*)

Strain	Toxicity	Geographical origin	Accession no.	Reference
268	NT†‡§¶	Unknown, Russia	–	13, 19, 22, 25
PCC 6719	NT‡§¶	Soil water culture, USA	–	3–5, 7, 13, 15, 23–24
PCC 7120	NT†‡§‖¶	Unknown, USA	–	1, 3–4, 7, 9–10, 13–15, 19, 22–25, 27–28, 33–34, 36
Nostoc punctiforme				
PCC 73102	NT†‡§‖¶	*Macrozamia* sp., Australia	–	2–4, 7, 13, 15–16, 18–19, 22–24, 33–34, 36
Microcystis sp.				
130	NT†‡§¶	Lake Säyhteenjärvi, Finland	AJ133170	19, 25
199	H*	Lake Rusutjärvi, Finland	AJ133172	12, 19, 25, 32
205	H*	Lake Mallusjärvi, Finland	–	12, 19, 25, 32
265	NT†‡§¶	Lake Tuusulanjärvi, Finland	–	25
269	NT†‡§¶	River Raisionjoki, Finland	AJ133175	19, 25
GL060916	NT‡§¶	Lake Grand-Lieu, France	–	35
GL260735	H‡§¶	Lake Grand-Lieu, France	–	35
GL280641	NT‡§¶	Lake Grand-Lieu, France	AJ133173	35
HUB5-3	NT†‡§‖¶	Lake Pehlitzee, Germany	–	19
PCC 7005	NT†‡§‖¶	Lake Mendota, USA	–	3–4, 7, 16–19, 23–24, 33
Microcystis aeruginosa				
PCC 7941	H‡§¶	Lake Little Rideau, Canada	–	9, 13, 16–18, 23, 28, 33–34
NIES 44	NT†‡¶	Lake Kasumigaura, Japan	–	16, 18, 20
NIES 89	H†‡§¶	Lake Kawaguchi, Japan	–	6, 16–18, 33, 38
NIES 98	NT†‡§¶	Lake Kasumigaura, Japan	–	6, 16–18, 33, 38
Microcystis viridis				
NIES 102	H†‡§‖¶	Lake Kasumigaura, Japan	–	6, 16–19, 33, 38
Microcystis wesenbergii				
NIES 104	NT†‡§¶	Freshwater, Japan	AJ133174	6, 20, 38
Planktothrix sp.				
1/1	NT†‡§	Lake Långsjön, Finland	–	13, 25
2	NT†‡§¶	Lake Markusbölefjärden, Finland	AJ133185	13, 19, 25
18	NT†‡§	Lake Långsjön, Finland	–	13, 25, 32
27	NT†‡§	Lake Långsjön, Finland	–	13, 25
28	NT†‡§	Lake Markusbölefjärden, Finland	AJ133165	13
45	NT†‡§	Lake Enäjärvi, Finland	–	13, 25
49	H*	Lake Valkjärvi, Finland	AJ133167	11, 13, 19, 25, 32
97	H*	Lake Maarianallas, Finland	–	11, 13, 19, 25, 32
NIVA-CYA 126	H*	Lake Långsjön, Finland	AJ133166	11, 13, 19, 25, 32
NIVA-CYA 127	H*	Lake Vesijärvi, Finland	AJ133168	11, 32
NIVA-CYA 128/R	H*	Lake Vesijärvi, Finland	AJ133169	11, 13, 19, 32
Oscillatoria agardhii				
PCC 7805	NK†‡§¶	Lake Veluwemeer, The Netherlands	–	3–4, 8–9, 13, 23
NIES 204	H†§‖‡¶	Lake Kasumigaura, Japan	–	16, 18–19, 33
Oscillatoria acuminata				
PCC 6304	NT‡§¶	Source unknown	–	2–5, 23–24, 33, 36–37
Oscillatoria sancta				
PCC 7515	NT‡§¶	Greenhouse water tank, Sweden	–	2–4, 23–24, 33–34, 36–37
Synechococcus sp.				
GL150636	NT‡§¶	Lake Grand-Lieu, France	AJ133176	35

* Anatoxin-a or microcystin(s) have been characterized.

† Toxicity of the strain has been determined by mouse bioassay or another bioassay.

‡ Anatoxin-a has been screened by HPLC.

§ Microcystins have been screened by HPLC.

‖ Toxic compounds have been screened by a protein phosphatase inhibition assay.

¶ Hepatotoxicity of the strain has been screened by ELISA.

which can vary in different environmental or growth conditions and even be lost during cultivation. Komárek & Anagnostidis (1989) have estimated that more than 50 % of the strains in the culture collections have taxonomic names which do not agree with the morphological description of the taxon.

The phenotypic and genotypic classification of cyanobacteria relies mainly on the studies of Stanier and collaborators (Kenyon *et al*., 1972; Herdman *et al*., 1979a, b; Rippka *et al*., 1979). Recent studies using 16S rRNA gene sequencing have extended the knowledge of the phylogenies of microcystin- and nodularin-producing, planktic *Microcystis* (Neilan *et al*., 1997a; Otsuka *et al*., 1998) and *Nodularia* (Lehtimäki *et al*., 2000) strains. The two genera, comprising both toxic and non-toxic strains, were found to be very homogeneous (Neilan *et al*., 1997a; Lehtimäki *et al*., 2000). In addition, partial sequencing of the variable region of the 16S rRNA gene has revealed planktic non-heterocystous *Planktothrix* (*Oscillatoria agardhii*) as well as *Microcystis* strains to be closely related (Rudi *et al*., 1997). Neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains have recently been found to form a tight group by an RFLP study of the total 16S rRNA gene (Lyra *et al*., 1997). More 16S rRNA gene variability has been observed among the planktic *Anabaena* and *Nostoc* strains (Lyra *et al*., 1997; Rudi *et al*., 1997). Furthermore, strains belonging to the genus *Synechococcus* are highly divergent and are widely scattered across the evolutionary tree of cyanobacteria (Turner, 1997; Honda *et al*., 1999; Turner *et al*., 1999).

Although the 16S rRNA molecule contains variable regions (Woese, 1987), it is too well conserved for studying species identity (Fox *et al*., 1992) or intraspecies variation (Ward *et al*., 1992). Techniques that are based on highly repetitive sequences have been used for identification of toxic, planktic *Anabaena*, *Nostoc* (Rouhiainen *et al*., 1995) and *Cylindrospermopsis* (Wilson *et al*., 2000) strains. Furthermore, fingerprinting of the repetitive extragenic palindromic (REP) elements and/or enterobacterial repetitive intergenic consensus (ERIC) sequences has been used for identification of symbiotic (Rasmussen & Svenning, 1998) and free-living cyanobacteria (Rasmussen & Svenning, 1998; Lehtimäki *et al*., 2000).

We have isolated planktic anatoxin-a (Sivonen *et al*., 1989), microcystin-producing (Luukkainen *et al*., 1993, 1994; Sivonen *et al*., 1992, 1990, 1995; Vezie *et al*., 1998) and non-toxic cyanobacterial strains from fresh (Finnish and French) and brackish waters and purified them axenically (Rouhiainen *et al*., 1995). In this study, we characterized microcystin-producing *Anabaena*, *Nostoc*, *Planktothrix* (*Oscillatoria agardhii*) and *Microcystis* strains, that belong to six known microcystin producing genera (Sivonen & Jones, 1999), by RFLP and sequencing of the 16S rRNA gene, and by REP- and ERIC-PCR. In addition, anatoxin-a-producing *Anabaena* strains, as well as non-toxic *Anabaena*, *Aphanizomenon*, *Calothrix*, *Cylindrospermum*, *Nostoc*, *Planktothrix*, *Oscillatoria* and *Synechococcus* strains were investigated.

METHODS

Cyanobacterial strains. The strains investigated are listed in Table 1. Non-nitrogen-fixing strains were grown in Z8 medium containing nitrogen (Kotai, 1972) whereas nitrogen-fixing strains were grown without nitrogen. The nitrogen-fixing *Nodularia* strain was grown in Z8 medium with salt and without nitrogen (Lehtimäki *et al*., 1994). The laboratory-cultured strains were characterized by the morphological criteria of Starmach (1966) and Tikkanen (1986). *Nostoc* sp. strain 152 and *Microcystis* sp. strains were identified only to genus level (Sivonen *et al*., 1990; Luukkainen *et al*., 1994). Neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains were distinguished by the typical characteristics of the latter genus, e.g. the hyaline elongated cells at the extremities of the trichomes and the flake-like bundles of the parallel trichomes as listed in Castenholtz (1989). The subsequent morphological identification of the neurotoxic *Anabaena* and the non-toxic *Aphanizomenon* strains placed these strains into the *Anabaena flos-aquae/circinalis/mendotae* (Sivonen *et al*., 1989; Rapala *et al*., 1993) and the *Aphanizomenon flos-aquae/gracile* species groups, respectively. The hepatotoxic *Anabaena* strains belonged into the *Anabaena flos-aquae/lemmermannii/circinalis* species group (Sivonen *et al*., 1992). All *Planktothrix* strains have been classified as *Oscillatoria agardhii* (Luukkainen *et al*., 1993) and renamed recently as *Planktothrix*. Due to difficulties in identification (the same strains were given different names by two experts or the same expert identified certain strains differently when studied more than once) and the loss of the morphological characteristics of the cultures kept in laboratory, it was decided to use only the genus and specify the organisms by strain numbers.

Toxin analyses. The anatoxin-a content of a total number of 26 cyanobacterial strains (indicated in bold in Table 1) was determined by HPLC with a Hewlett Packard HP 1090. Anatoxin-a was extracted from 20 mg lyophilized cyanobacteria with water under sonication (Braun Labsonic-U) on ice (10 min). Samples were filtered through GF 52 filters (Schleicher & Schuell) and concentrated on Oasis C_{18} cartridges (Waters). Before concentration, the pH of the samples was adjusted to 10 with 0·1 M NaOH. The toxin was eluted with 100 % methanol containing 0·01 % trifluoroacetic acid. The samples were subsequently dried and dissolved in 1 ml 10 % methanol. Hepatotoxins were extracted from 10 mg lyophilized cells with water under sonication (10 min). Samples were filtered and concentrated as above, without adjusting the pH. Hepatotoxins were eluted with 100 % methanol, dried and dissolved in 1 ml 20 % methanol. The toxin samples were subsequently filtered through a Gelman Acrodisc (0·2 μm). Anatoxin-a and hepatotoxins were analysed by HPLC with a diode array detector at 230 nm and 238 nm, respectively. The column for the detection of anatoxin-a was a Devosil ODS-5 column (4·6 × 150 mm; Nomura Chemical). The mobile phase was methanol/0·01 mol ammonium chloride l^{-1}, pH 4 (1 : 9, v/v; Harada *et al*., 1989). The column for the detection of hepatotoxins was a Hewlett Packard ODS Hypersil column (4·6 × 100 mm). The mobile phase was acetonitrile/0·01 mol ammonium acetate l^{-1} (24 : 76, v/v; Krishnamurthy *et al*., 1986). The flow rates were 0·9 ml min^{-1} for anatoxin-a and 1 ml min^{-1} for hepatotoxins. The injection volumes were 25 μl. The toxins were identified by their retention times and UV spectra. Purified anatoxin-a and microcystins were used as standards. Hepatotoxins were analysed by using an ELISA EnviroGard Microcystins Plate Kit as described in

the manufacturer's instructions (Strategic Diagnostics). Water extracts from lyophilized cyanobacteria (0·5–0·8 mg ml^{-1}) were used for the assay.

Extraction of DNA. Genomic DNA was extracted as described by Golden *et al.* (1988). DNA from *Aphanizomenon*, *Nodularia* and *Planktothrix* strains was extracted by a slightly modified method, with the addition of sucrose (10 %, w/v) and lysozyme (10 mg ml^{-1}) as described previously (Lehtimäki *et al.*, 2000).

RFLP of the amplified 16S rRNA gene. The 1500 bp fragments of 16S rRNA genes were amplified with primers fD1 and rD1 (Weisburg *et al.*, 1991) by PCR as described by Lyra *et al.* (1997). Amplified products were digested with *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Mbo*I, *Msp*I and *Rsa*I, according to Lyra *et al.* (1997). The restriction fragments were separated by electrophoresis in a 3 % agarose gel. The patterns of the restriction fragments were documented with a video camera (Panasonic CCTV, model WW-BP500/G). The images of restriction fragments were analysed using GelCompar software (version 4; Applied Maths BVBA) with manual editing. The similarity matrix was calculated with the Dice (1945) coefficient. Validity of the tree based on pairwise genetic distance values of restriction fragment data (Nei & Li, 1979; Nei, 1987) was estimated with the PHYLIP software package (Felsenstein, 1993). Four clustering techniques were used: unweighted pair-group method using arithmetic averages (UPGMA; Sneath & Sokal, 1973), neighbour-joining (NJ; Saitou & Nei, 1987), and Fitch and Kitsch methods of PHYLIP (Felsenstein, 1993).

Sequencing of 16S rRNA gene. Amplification and sequencing of the 16S rRNA genes were performed in two parts, with primers pA-pF′ and pD-pH′ (Edwards *et al.*, 1989). Sequencing was performed by either the solid phase (Hultman *et al.*, 1991) automated ALF sequencer (Pharmacia) or the cyclic sequencing methods using the dye termination sequencing kit (Perkin Elmer) and an ABI PRISM 377XL DNA sequencer (Perkin Elmer).

The sequences were assembled and edited using the Xgap program of the Staden package (Bonfield *et al.*, 1995) and aligned by using CLUSTAL W (Higgins & Sharp, 1989). The sequences have been submitted to EMBL/GenBank and the accession numbers are listed in Table 1.

Phylogenetic trees were constructed using the maximum-parsimony (MP) and the distance version of maximum-likelihood (ML) methods of PHYLIP, 3.5c (Felsenstein, 1993). In addition, the NJ method of the TREECON software package (van de Peer & de Wachter, 1994) was used for tree construction. The statistical significance of the branches was estimated by bootstrap analysis of the tree programs, involving the generation of 1000 trees. Distances for the NJ tree were estimated by the algorithm of Kimura (1980). The same method was also used to determine the distances of the DNA parsimony consensus tree, because it was not possible to calculate distances by the DNA parsimony program of PHYLIP.

REP- and ERIC-PCR. PCR reactions were carried out as described by Lehtimäki *et al.* (2000) with primers 1R-I and 2 (REP), and 1R and 2 (ERIC) (Versalovic *et al.*, 1991). The REP- and ERIC-PCR profiles were analysed by GelCompar software with manual editing. The similarity matrix was calculated by the Dice (1945) coefficient. The tree was constructed by the UPGMA algorithm of the GelCompar program.

RESULTS

RFLP of amplified 16S rRNA genes

Forty-two cyanobacterial strains were analysed by RFLP of the amplified 16S rRNA gene. In addition, data of 26 strains from the previous publication (Lyra *et al.*, 1997) were included in the tree construction. From a total of 68 axenic cyanobacterial strains 29 different genotypes were detected (Fig. 1). The NJ, UPGMA, Fitch and Kitsch methods from PHYLIP gave a congruent major grouping and therefore only the UPGMA dendrogram is presented (Fig. 1). The cophenetic correlation value of the similarity matrix was 92·1 %, thus the matrix was reliably represented by the UPGMA dendrogram.

In the RFLP study, the cyanobacteria were divided into four main branches (Fig. 1). The first branch contained all of the heterocystous cyanobacteria with the exception of *Calothrix* sp. strain PCC 7714. This strain formed a second branch with *Oscillatoria sancta* PCC 7515 and highly similar neurotoxic, hepatotoxic or non-toxic *Planktothrix* strains. *Oscillatoria acuminata* PCC 6304 formed the third branch with highly similar hepatotoxic or non-toxic *Microcystis* strains. The fourth branch contained *Synechococcus* sp. strain GL150636 (Fig. 1).

The heterocystous cyanobacteria were divided into two distinct clusters. One contained all *Anabaena* and *Aphanizomenon* strains, with the exception of *Anabaena flos-aquae* NIES 73. *Anabaena flos-aquae* NIES 73 was located alternatively with other *Anabaena* NIES strains (UPGMA, Kitsch) or it remained outside both clusters containing heterocystous strains (NJ, Fitch). Neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains shared identical or very similar 16S rRNA genotypes and grouped together. In addition, *Anabaena cylindrica* PCC 7122 and *Anabaena* sp. strains PCC 6309 and PCC 73105 shared identical RFLP patterns. *Nostoc* sp. strain 152 grouped with non-toxic *Anabaena* sp. strain 277. *Anabaena flos-aquae* (*lemmermannii*) NIVA-CYA 83/1 formed a group with the planktic, hepatotoxic and neurotoxic *Anabaena* species and the non-toxic *Aphanizomenon* strains isolated from Finnish, Danish and Japanese freshwaters and the Baltic Sea (Table 1). Planktic *Anabaena circinalis* NIES 41 and *Anabaena spiroides* NIES 79 grouped more loosely with the same cluster. The other cluster containing heterocystous strains was composed of *Nostoc*, *Nodularia* and *Cylindrospermum* strains. *Nostoc* sp. strains 268 and 159 grouped with *Nostoc punctiforme* PCC 71302.

The non-heterocystous cyanobacteria were divided into groups of unicellular *Microcystis* and filamentous *Planktothrix* (*Oscillatoria agardhii*) strains. *Microcystis* and *Planktothrix* strains from NIVA-CYA, NIES and PCC collections were very closely related to *Microcystis* and *Planktothrix* strains from our own culture collection. *Microcystis* as well as *Planktothrix* strains formed homogeneous groups. Three clustering techniques (Fitch, NJ, UPGMA) loosely grouped

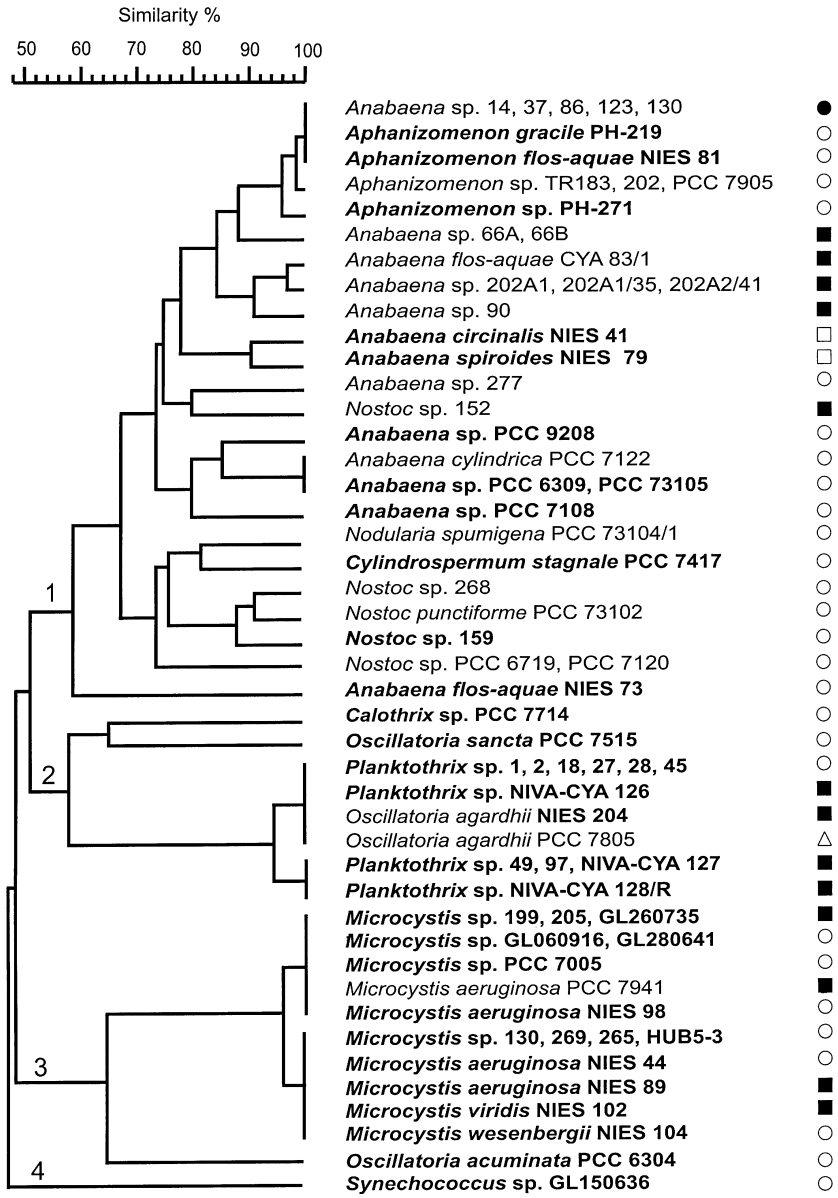


Fig. 1. UPGMA dendrogram based on RFLP of cyanobacterial 16S rRNA genes. The strains analysed in this study are indicated in bold and the strains analysed by Lyra *et al.* (1997) in plain type. The scale bar represents the similarity coefficient (Dice, 1945) of restriction patterns of 16S rRNA genes. Numbers 1–4 indicate the four main clades found by RFLP analysis of 16S rRNA gene. Symbols: ●, neurotoxic; ○, non-toxic; ■, hepatotoxic; □, toxicity not determined; △, type of toxicity not known.

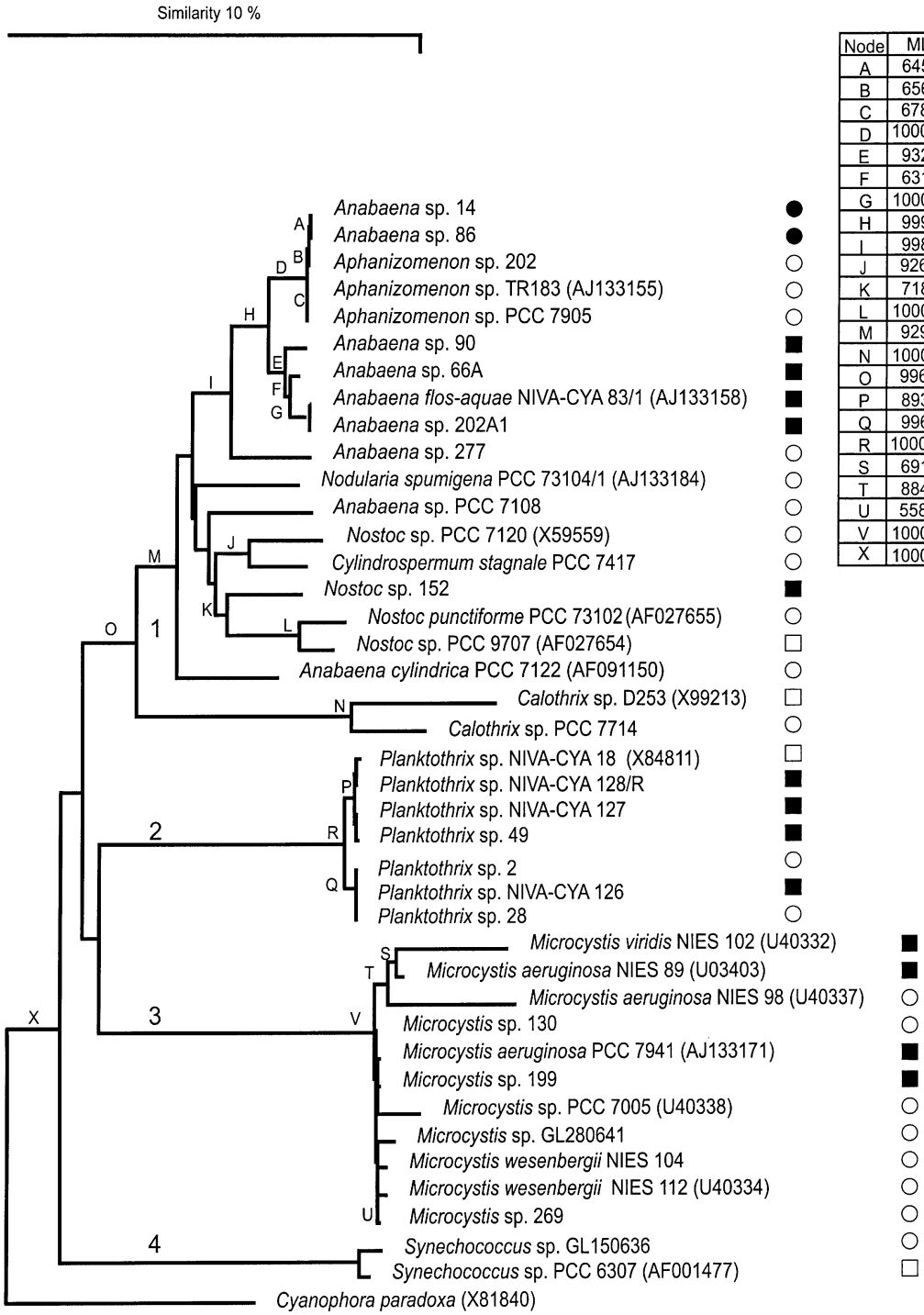
Oscillatoria acuminata PCC 6304 with *Microcystis* strains, while four algorithms (Fitch, Kitsch, NJ, UPGMA) loosely grouped the filamentous heterocystous *Calothrix* sp. strain PCC 7714 with the *Planktothrix* strains and *Oscillatoria sancta* PCC 7515 (data not shown).

All four tree construction methods supported the inner branching of the groups containing strains of (i) neurotoxic *Anabaena* and non-toxic *Aphanizomenon*, (ii) hepatotoxic *Anabaena* and *Anabaena* NIVA-CYA 83/1, (iii) *Anabaena* from PCC, (iv) *Nostoc* PCC 73102, *Nostoc* 268 and 159, (v) *Planktothrix* and (vi) *Microcystis* strains (Fig. 1).

Sequences of the 16S rRNA genes

Nineteen representatives of the RFLP genotypes from Finnish lakes and the French Lake Grand-Lieu, and five strains from PCC and NIES were sequenced (Table 1). MP, ML and NJ methods were used to analyse 1425 (of 1442–1482) positions. The phylogenetic trees constructed by these methods were largely congruent and therefore only the NJ tree is presented (Fig. 2). However, bootstrap values for all methods are presented. The overall bootstrap values of the MP tree were lower than the values for ML and NJ trees. Most branches of the MP, ML and NJ trees were supported by high bootstrap values.

The cyanobacteria studied here were divided into four main branches (Fig. 2). The first branch constituted the filamentous cyanobacteria, *Anabaena*, *Aphanizomenon*, *Nostoc*, *Nodularia*, *Cylindrospermum* and *Calothrix*, capable of forming heterocysts and akinetes. The second branch contained non-heterocystous, filamentous *Planktothrix* strains. The third and fourth branches contained non-heterocystous



Node	ML	MP	NJ
A	645	895	903
B	656	694	617
C	678	600	978
D	1000	1000	1000
E	932	691	929
F	631	630	630
G	1000	997	998
H	999	936	998
I	998	986	998
J	926	626	927
K	718	711	727
L	1000	1000	1000
M	929	663	972
N	1000	1000	1000
O	996	782	1000
P	893	725	957
Q	996	1000	1000
R	1000	1000	1000
S	691	860	769
T	884	559	872
U	558	530	530
V	1000	1000	1000
X	1000	1000	1000

Fig. 2. Phylogenetic tree for cyanobacterial 16S rRNA gene sequences. Bootstrap values higher than 500 are indicated in the table for ML, MP and NJ trees. The latter tree is presented. Scale bar represents 10 % similarity of 1425 nt. EMBL accession numbers for the sequences are indicated in parentheses. Numbers 1–4 indicate the four main clades found by 16S rRNA gene sequence analysis. Symbols: ●, neurotoxic; ○, non-toxic; ■, hepatotoxic; □, toxicity not determined.

unicellular *Microcystis* and *Synechococcus* strains. The plastid *Cyanophora paradoxa* was chosen as an outgroup.

The 16S rRNA gene sequences of neurotoxic *Anabaena* sp. strains (14 and 86) were identical. The neurotoxic *Anabaena* and the non-toxic *Aphanizomenon* strains

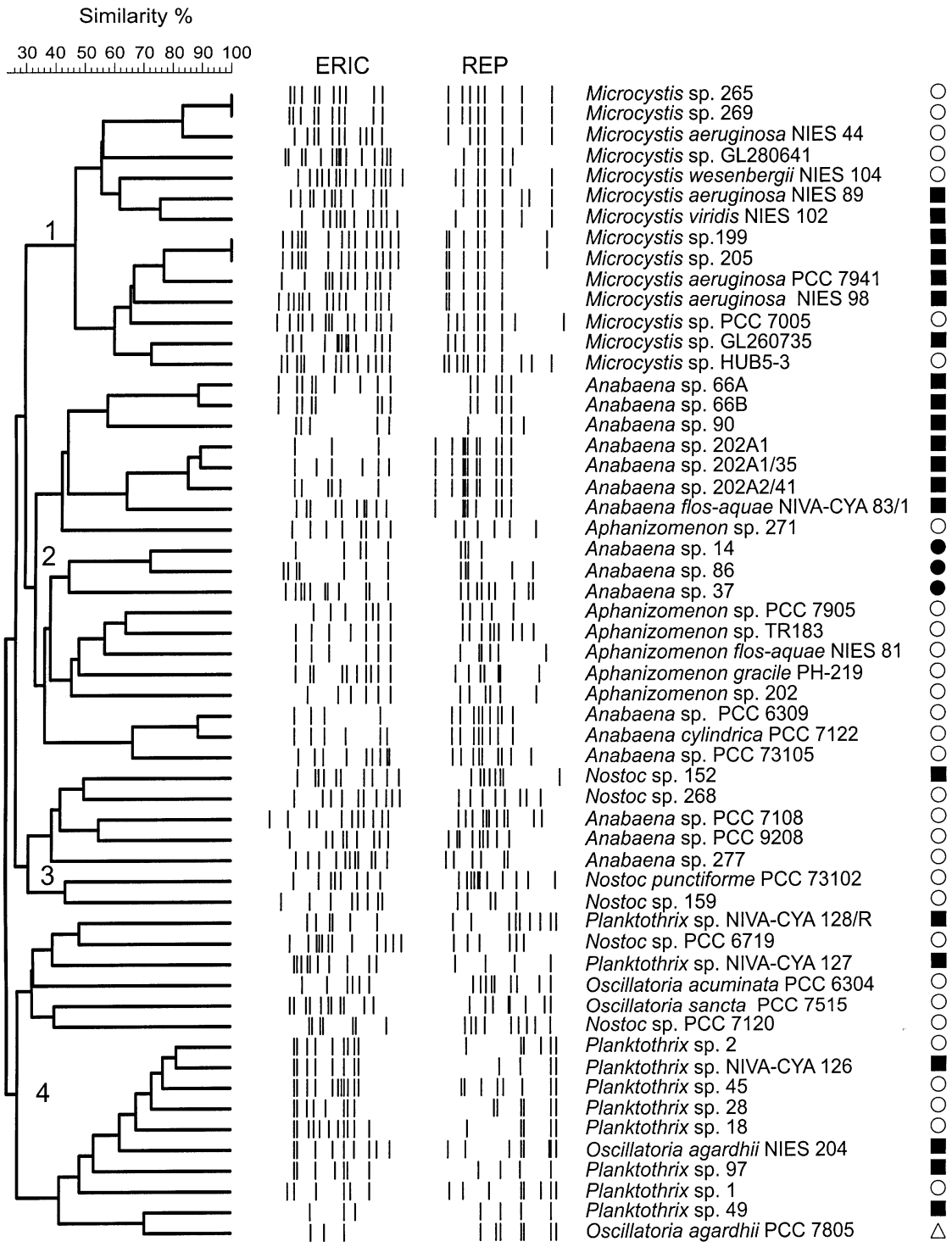


Fig. 3. UPGMA dendrogram based on cyanobacterial genomic fingerprints generated by REP- and ERIC-PCR. Scale bar represents the similarity coefficient (Dice, 1945) of the fingerprints of cyanobacterial strains. Numbers 1–4 indicate the four groups of cyanobacteria found by genomic fingerprinting. Symbols: ●, neurotoxic; ○, non-toxic; ■, hepatotoxic; □, toxicity not determined; △, type of toxicity not known.

shared remarkably high similarity values ranging from 99·9 to 100% and formed a tight group, which was supported by high bootstrap values in the MP, ML and NJ trees (Fig. 2). The similarity values within the *Anabaena*/*Aphanizomenon* cluster that also contained hepatotoxic and non-toxic *Anabaena* strains varied

from 95·7 to 100 %. The non-toxic *Anabaena* 277 and the hepatotoxic *Anabaena* NIVA-CYA 83/1 shared the lowest similarity value within the *Anabaena*/*Aphanizomenon* cluster.

Within the group comprising *Nostoc*, *Cylindrospermum* and *Anabaena* strains the 16S rRNA genes were more variable, sharing similarity values from 93·5 to 98 %. *Nostoc* sp. strain PCC 7120 and *Cylindrospermum stagnale* PCC 7417 grouped together, sharing 96·9 % 16S rRNA gene similarity. Interestingly, *Anabaena cylindrica* PCC 7122 was outside the branches that contained other *Anabaena*, *Aphanizomenon*, *Nostoc*, *Cylindrospermum* and *Nodularia* strains in the trees constructed using ML and NJ methods, while this strain was loosely grouped with the *Anabaena* and *Aphanizomenon* strains in the MP tree. Similarly, *Anabaena* sp. strain PCC 7108 was found with the *Anabaena*/*Aphanizomenon* (ML, NJ) or with the cluster that contained strains belonging to several heterocystous genera (MP). *Anabaena cylindrica* PCC 7122 and the two branches that contained heterocystous strains shared similarity values from 94 to 95 % and from 93·4 to 94·8 %, respectively. *Anabaena* PCC 7108 and these two clusters shared similarity values from 94·1 to 94·5 % and from 93·8 to 95·8 %, respectively.

Calothrix strains were outside the other branches of heterocystous, filamentous cyanobacteria with similarity values less than 90·2 %. The two *Calothrix* strains were moderately close relatives, sharing 95·1 % sequence similarity.

Three branches of non-heterocystous *Planktothrix*, *Microcystis* and *Synechococcus* were found. *Planktothrix* and *Microcystis* strains formed two very homogeneous clusters. Within these clusters, the 16S rRNA gene sequence similarities varied from 99·3 to 100 % and from 94·2 to 99·9 %, respectively. The two clusters of *Microcystis* and *Planktothrix* strains were as far apart from each other as from the clusters containing heterocystous strains (similarity values less than 89·7 %). *Synechococcus* sp. strain GL150636 formed a distinct branch, sharing less than 86·7 % sequence similarity with the strains sequenced. *Synechococcus* sp. strain GL150636 and *Synechococcus* sp. strain PCC 6307 shared 99·2 % similarity, indicating very close relatedness.

REP and ERIC fingerprinting

The strains belonging to the genera known to produce anatoxin-a or microcystins were chosen for REP- and ERIC-PCR analysis. *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* (*Oscillatoria agardhii*) genera, containing identical or highly similar strains, when analysed by RFLP or by sequencing of the 16S rRNA genes were differentiated by REP- and ERIC-PCR. These methods gave highly reproducible fingerprints (data not shown). Tree construction by a UPGMA algorithm was possible, but the reliability of the cluster analysis was not high as shown by a low (76·2 %) cophenetic correlation value. Fingerprinting differentiated all studied strains except two identical pairs of non-toxic *Microcystis* sp. strains 265 and 269 and toxic *Microcystis* sp. strains 199 and 205 (Fig. 3). Thus this method is suitable when high resolution is needed. Four clear clusters were formed: (i) *Microcystis*, (ii) *Anabaena*/*Aphanizomenon*, (iii) *Nostoc*/*Anabaena* and (iv) *Nostoc*/*Oscillatoria*/*Planktothrix* group (Fig. 3). The *Anabaena*/*Aphanizomenon* group was divided into two subgroups.

DISCUSSION

Four major clades of cyanobacteria were found in the 16S rRNA gene analysis. Morphologically different neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains formed an extremely tight cluster, which was highly supported by bootstraps values of MP, ML and NJ trees. The grouping was also supported by RFLP analysis of the 16S rRNA gene and by REP and ERIC fingerprinting. Previously, molecular taxonomic studies have indicated the grouping of *Anabaena* together with *Aphanizomenon* strains (e.g. Neilan *et al*., 1995; Rudi *et al*., 1997; Lu *et al*., 1997; Lyra *et al*., 1997; Rudi & Jakobsen, 1999), but in this study high similarity (more than 99·9 %) at the 16S rRNA gene level was revealed.

Similarly, morphological differences between *Oscillatoria* and *Microcoleus* genera (Wilmotte *et al*., 1992) and within the *Merismopedia* genus (Palinska *et al*., 1996) are not reflected at the 16S rRNA gene level. On the other hand, the unicellular cyanobacteria that are morphologically similar and simple can be phylogenetically different (Ward *et al*., 1992).

Generally, the 16S rRNA gene sequence and RFLP analysis gave similar results. In the RFLP study, *Anabaena* sp. strain PCC 7108 and *Anabaena cylindrica* PCC 7122 grouped with the cluster containing *Anabaena* and *Aphanizomenon* strains. In the sequence analysis, these strains grouped similarly (MP) or *Anabaena* PCC 7108 grouped with the *Cylindrospermum*, *Nodularia* and *Nostoc* strains while *Anabaena cylindrica* PCC 7122 reminded outside both of these clusters (ML, MP). The low bootstrap values of the branches containing *Anabaena* PCC 7108 and PCC 7122 strains as well as the shared similarity values between these strains and other nitrogen-fixing cyanobacteria indicate that these strains are as closely related to the *Anabaena* and *Aphanizomenon* as to the *Cylindrospermum*, *Nodularia* and *Nostoc* strains. In addition, *Nostoc* 152 can belong to both clusters, as shown by the different groupings of the strain in the 16S rRNA gene analyses and the shared similarity values of *Nostoc* 152 and the other nitrogen-fixing strains. The most distant heterocystous filamentous *Calothrix* species, strain PCC 7714, grouped with *Oscillatoria sancta* PCC 7515 when studied by RFLP. In sequence analysis, the *Calothrix* strains formed a distinct cluster. The disparity could be due to errors in the RFLP method caused by undetectable restriction

fragments (small fragments) or fragment length differences.

The distances in the trees created by 16S rRNA gene RFLP and sequencing revealed that the evolutionary relationships of the *Cylindrospermum*, *Nostoc*, *Anabaena* PCC 7108 and PCC 7122 strains are not clear. The low bootstrap values of the nodes within the cluster analysed by 16S rRNA gene sequencing are consistent with this result. Wilmotte (1994) and Turner (1997) have also reported the uncertain grouping of the strains belonging to these three genera by 16S rRNA gene sequence analysis. Furthermore, an RFLP study of the 16S–23S rDNA spacer region has revealed that a subset of *Nostoc* strains grouped with a cluster containing *Anabaena* and *Aphanizomenon*, while the rest of the *Nostoc* strains formed a distinct subgroup with *Anabaena* strains PCC 7120 and ATCC 27892 (Lu *et al*., 1997). The latter strains have been proposed to belong to *Nostoc* genera by Rippka & Herdman (1992).

Planktothrix strains isolated from the Finnish lakes shared very high 16S rRNA gene sequence similarities (99·3–100 %). The high similarity of the *Planktothrix* NIVA-CYA strains has been determined previously by Rudi *et al*. (1997). Our strains were highly similar to the *Planktothrix* sp. strain NIVA-CYA 18 and *Oscillatoria agardhii* NIES 204 strains, which have been placed into one of the five subgroups containing *Oscillatoria* strains (Turner, 1997). These two strains have been proposed to belong to the *Planktothrix* genus by Anagnostidis & Komárek (1988). The red-pigmented *Planktothrix* sp. strain NIVA-CYA 128/R was grouped tightly with green-pigmented *Planktothrix* strains. Previously, the red-pigmented NIVA-CYA 128/R strain was separated from green-pigmented *Planktothrix* strains by a phenotypic whole-cell protein analysis (Lyra *et al*., 1997). Wilmotte *et al*. (1992) found that different pigment content and the presence and absence of chromatic adaptation did not correlate with significant divergence at the 16S rRNA gene level.

The remarkable 16S rRNA gene sequence similarity of 99·9–100 % of neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains indicate that these strains could be different phenotypes of the same species. However, the studies of Lachance (1981) revealed that two strains sharing as high as 99·8 % rRNA gene similarity can share as little as 25 % DNA–DNA reassociation and thus not belong to the same species. A species is generally defined as a group of strains if they share approximately 70 % or greater DNA–DNA relatedness and 5 °C or less ΔT_m, and if their phenotypic characteristics agree with this definition (Wayne *et al*., 1987).

Planktic *Anabaena* sp. strain 277 and the other planktic *Anabaena* and *Aphanizomenon* strains shared sequence homology of less than 96·6 %. It is known that strains with 16S rRNA gene sequence similarity values below 97·5 % are unlikely to have more than 60–70 % DNA similarity and that they are unlikely to belong to the same species (Stackebrandt & Goebel, 1994). 16S rRNA gene sequence analysis is a superior method when the similarity values of strains are below 97 %. Thus *Anabaena* 277 may belong to a different species than the other planktic *Anabaena* and *Aphanizomenon* strains. Furthermore, planktic *Anabaena* and *Aphanizomenon* strains seem to be well diverged from *Anabaena* PCC 7108 and *Anabaena cylindrica* PCC 7122. The two latter strains are not closely related to each other, because the 16S rRNA gene sequence similarity value between them was only 93·5 %. *Anabaena* PCC 7108 and PCC 7122 have been found to share a DNA–DNA reassociation value of 32 % (Lachance, 1981). This value was 94·6 % between *Anabaena* strains PCC 7122 and PCC 6309 (Lachance, 1981). Their relatedness (as well as *Anabaena* sp. strain PCC 73105) at the species level was also shown here by the shared RFLP profile of the 16S rRNA gene. Thus, PCC 7122 and PCC 6309 may belong to the same species and PCC 7108 may belong to a different species. The 100 % RFLP homology of the 16S rRNA gene between *Anabaena* sp. strains PCC 6309, PCC 7122 and PCC 73105 indicates that these strains may well belong to the *Anabaena cylindrica* species.

It seems that almost all strains within the heterogeneous *Nostoc*, as well as *Calothrix*, group represent individual species, because the similarity values of 16S rRNA gene sequences within the group were below 97·5 %. In contrast, there seems to be no species variation inside the three homogeneous clusters containing *Anabaena* and *Aphanizomenon* strains, *Planktothrix* strains and *Microcystis* strains. The species classifications based on 16S rRNA genes are not supported on phenotypic grounds except in the case of *Planktothrix*. The *Microcystis* strains have been found to share high sequence similarities of the 16S rRNA gene (Otsuka *et al*., 1998) and of the 16S–23S internal transcribed spacer (Otsuka *et al*., 1999). Toxic and non-toxic *Microcystis* strains isolated from Finnish lakes were grouped tightly with *Microcystis* strains from PCC and NIES culture collections, which have been shown to belong to the major *Microcystis* cluster by 16S rRNA gene analysis (Neilan *et al*., 1997a; Turner, 1997). In addition, the different *Microcystis* species as classified by morphological features observed microscopically, such as cell size, cell arrangements in colonies and the existence of gas vesicles (Komárek, 1991), have been found to integrate into one species in DNA–DNA reassociation and DNA base composition analyses (Kondo *et al*., 2000). It remains to be seen if the *Anabaena* and *Aphanizomenon* genera as distinguished by characteristics of the latter genus (Castenholtz, 1989) also share high levels of DNA–DNA relatedness.

The unicellular *Synechococcus* strains were as distantly related to the unicellular *Microcystis* genus as to the filamentous genera. *Synechococcus* sp. strain GL150636 from the French Lake Grand-Lieu was

remarkably similar to the freshwater *Synechococcus* sp. strain PCC 6307, which has been found to belong to one of the polyphyletic branches that also contains marine *Synechococcus* and *Prochlorococcus* strains (Turner, 1997; Urbach *et al*., 1998; Honda *et al*., 1999). These branches also contain other unicellular or filamentous non-heterocystous strains (Turner, 1997; Honda *et al*., 1999).

In this study we have shown that REP and ERIC genomic fingerprinting allows quick determination and grouping of closely related strains. Furthermore, the number of fragments produced by REP and ERIC fingerprinting was adequate and it varied only moderately among studied strains, which made numerical analysis and tree construction possible. Closely related neurotoxic *Anabaena* and non-toxic *Aphanizomenon* strains, *Microcystis* strains and *Planktothrix* strains were differentiated to a high degree by REP and ERIC fingerprinting. The method even differentiated very similar strains of *Anabaena* (14 and 86), *Aphanizomenon* (TR183 and PCC 7905) and *Planktothrix* (49 and 127). The most striking feature was the grouping of the *Nostoc* sp. strains PCC 6719 and PCC 7120 with the *Planktothrix* strains. The DNA–DNA reassociation and RFLP studies of the 16S rRNA gene have shown that *Nostoc* PCC 6719 and PCC 7120 probably belong to the same species, since the strains share 96% DNA–DNA homology (Lachance, 1981) and 100% 16S rRNA gene RFLP homology (Lyra *et al*., 1997). In addition, these strains were found to be identical in the hybridization study with a short terminal repeated repetitive (STRR) sequence probe (Mazel *et al*., 1990). Good resolution of strains belonging to the symbiotic *Nostoc* (Rasmussen & Svenning, 1998) and free-living *Nodularia* and *Nostoc* (Rasmussen & Svenning, 1998; Lehtimäki *et al*., 2000) genera has also been obtained with REP and/or ERIC fingerprinting. REP and ERIC (Versalovic *et al*., 1991) fingerprinting is an excellent method when closely related, axenic strains are studied.

Previous Southern blot analysis and PCR fingerprinting revealed the existence of STRR sequences among genera that contain filamentous heterocystous strains (Mazel *et al*., 1990; Rouhiainen *et al*., 1995; Rasmussen & Svenning, 1998; Wilson *et al*., 2000; Lehtimäki *et al*., 2000) or unicellular *Microcystis* (Asayama *et al*., 1996) strains. In addition, long tandemly repeated repetitive (LTRR) sequences have been used for characterization of mainly *Nostoc* strains (Masephol *et al*., 1996; Rasmussen & Svenning, 1998). STRR and LTRR sequences appear to be superior when studying non-axenic cyanobacteria (Rasmussen & Svennig, 1998; Lehtimäki *et al*., 2000).

In this study toxicity was not consistent with the genetic analysis of a wide range of cyanobacterial genera. Previously, Neilan *et al*. (1997a) found no correlation between 16S rRNA genes and the toxicity of *Microcystis* strains. This genotypic distinction between toxic and non-toxic cyanobacteria has been indicated by several genotypic analyses of *Nodularia* strains (Lehtimäki *et al*., 2000). The best way to differentiate toxic and non-toxic *Microcystis*/*Planktothrix*/*Anabaena* strains might be to sequence the domains of peptide synthetases that are multienzyme complexes responsible for microcystin production (Neilan *et al*., 1999).

There was no geographical distribution pattern of specific genotypes among the freshwater cyanobacteria in this study. For example the *Microcystis* strains forming two identical pairs by REP- and ERIC-PCR were isolated from four different Finnish lakes. Cyanobacterial 16S rRNA gene sequences from the Pacific Ocean and the Atlantic Ocean isolates have revealed that some cyanobacterial strains are globally distributed (Giovannoni *et al*., 1990; Schmidt *et al*., 1991; Fuhrman *et al*., 1993; Mullins *et al*., 1995). In addition, Bolch *et al*. (1999) and Lehtimäki *et al*. (2000) have found very closely related cultured *Nodularia* strains from geographically separated areas.

The different topologies between trees based on cyanobacterial 16S rRNA genes and $tRNA^{Leu}$ introns suggest that lateral gene transfer of these introns has occurred in cyanobacterial radiation (Rudi & Jakobsen, 1999). In addition, comparisons of 16S rRNA and *rbcLX* (encoding D-ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit and chaperonin-like protein) phylogenies indicate lateral DNA transfer among cyanobacteria (Rudi *et al*., 1998). Recently, it was stated that lateral gene transfer cannot be limited to special categories of genes (Doolittle, 1999). Even the most used and trusted chronometers, rRNA genes, can be transferred as shown by complete exchange of rRNA genes between bacteria (Asai *et al*., 1999). In this study, the overall agreement of the trees based on the 16S rRNA gene and REP and ERIC total genome fingerprinting showed that the 16S rRNA gene represents the total genome. In addition, the studies of Vinuesa *et al*. (1998) and Lehtimäki *et al*. (2000) revealed similar genotypic diversity of *Bradyrhizobium* and *Nodularia* strains by these two methods. The studies of whole-cell protein (the gene products representing the total genome) and RFLP of the 16S rRNA gene gave fairly congruent results (Lyra *et al*., 1997). Thus, lateral transfer of 16S rRNA genes might not play a major role among the studied strains.

In summary, the trees based on the numerous full length 16S rRNA gene sequences have revealed four homogeneous groups of planktic strains: (i) non-toxic *Aphanizomenon*, anatoxin-a-producing *Anabaena* and hepatotoxin-producing *Anabaena* (this study); (ii) non-toxic and microcystin-producing *Microcystis* (Neilan *et al*., 1997a; Otsuka *et al*., 1998; this study); (iii) non-toxic and nodularin-producing *Nodularia* (Lehtimäki *et al*., 2000); and (iv) non-toxic and microcystin-producing *Planktothrix* (this study). Non-toxic strains belonging to *Anabaena*, *Cylindrospermum* and *Calothrix*, and non-toxic and hepatotoxin-producing strains belonging to *Nostoc* were more loosely related.

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