Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*

Muriel Gugger,^{1†} Christina Lyra,¹ Peter Henriksen,² Alain Couté,³ Jean-François Humbert⁴ and Kaarina Sivonen¹

¹ Department of Applied Chemistry and Microbiology, PO Box 56, Biocenter Viikki, 00014 Helsinki University, Finland

² Department of Marine Ecology, National Environmental Research Institute, PO Box 358, DK-4000 Roskilde, Denmark

³ Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, 12 rue Buffon, 75005 Paris, France

⁴ INRA, UMR CARTEL, BP 511, 74203 Thonon-les-Bains cedex, France

Author for correspondence: Kaarina Sivonen. Tel: +358 9 19159270. Fax: +358 9 19159322. e-mail: kaarina.sivonen@helsinki.fi

Morphological analysis and sequencing of the 16S rRNA gene, the spacer region of the ribosomal operon (ITS1) and the *rbcLX* (RubisCO) region was performed on 26 *Anabaena* strains and 14 *Aphanizomenon* strains isolated from several lakes in Denmark, Finland and France. Based on their morphology, *Anabaena* strains differed from strains of *Aphanizomenon*: the vegetative cells, heterocysts and akinetes were significantly wider in *Anabaena* than in *Aphanizomenon*. Phylogenetic trees based on the 16S rDNA, ITS1 and *rbcLX* regions showed that the planktic *Anabaena* strains were not distinguishable from *Aphanizomenon* strains. The results of the clustering of *Anabaena* and *Aphanizomenon* strains based on 16S rDNA sequences showed that these two genera are not monophyletic. Sequence analysis of the 16S rDNA, ITS1-S and *rbcLX* regions of the planktic *Anabaena* strains showed that this genus is heterogeneous. In all methods, *Anabaena* strains that produced different toxic compounds (e.g. anatoxin-a, microcystin and an unknown neurotoxin) were clustered separately from each other but were grouped either with non-toxic *Anabaena* and/or *Aphanizomenon* strains. Our data suggest that the planktic *Anabaena* and *Aphanizomenon* isolates belong to the same genus, regardless of their morphological differences. Thus, a taxonomic revision of the two genera is required.

Keywords: *Anabaena*, *Aphanizomenon*, 16S rDNA, 16S–23S rDNA, RubisCO spacer

INTRODUCTION

The genera *Anabaena* and *Aphanizomenon* have been classified into the filamentous heterocystous cyanobacteria (subsection IV, family I) (Rippka *et al.*, 2001). Under the Botanical Code, the cyanobacterial genera *Anabaena* Bory ex Born. et Flah. and *Aphanizomenon* Morren ex Born. et Flah. belong to the order Nostocales (Komárek & Anagnostidis, 1989). The genus *Anabaena* is distinguished from the genus *Aphanizomenon* based on the botanical type species *Anabaena* (*An.*) *oscillarioides* Bory and *Aphanizomenon* (*Ap.*) *flos-aquae* (L.) Ralfs. Komárek & Kováčik (1989)

evaluated the morphological features used for the differentiation of *Anabaena* and *Aphanizomenon*, such as the structure of the trichomes, the elongated and narrowed terminal cells, the fascicle-like colonies and the development of the heterocysts. Only the structure of the trichomes appeared to be genus-specific. In addition, the occurrence of strains with intermediate characters in field samples indicated the problem of generic delimitation between *Anabaena* and *Aphanizomenon* (Komárek & Anagnostidis, 1989).

Anabaena and *Aphanizomenon* are able to fix nitrogen and form mass occurrences (water blooms) in fresh water and in the Baltic Sea (Sivonen *et al.*, 1990; Barker *et al.*, 2000). Comparative physiological studies have shown differences between strains of these genera, such as the higher growth affinity for light of *Ap. flos-aquae* PCC 7905 and the enhanced growth of an *Anabaena* strain under phosphate-limited conditions (De Nobel *et al.*, 1997, 1998). *Anabaena* species may synthesize neurotoxic alkaloids such as anatoxin-a, anatoxin-a(S) or saxitoxins and *Aphanizomenon* strains

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† Present address: Centre de Recherche Public – Gabriel Lippmann, 162a avenue de la Faïencerie, L-1511 Luxembourg, Grand Duché de Luxembourg.

The GenBank/EMBL accession numbers for the cyanobacterial sequences determined in this study are AJ293102–AJ293131 (16S rDNA), AJ293101, AJ293171–AJ293215 and AJ294540 (ITS1) and AJ293132–AJ293170 (*rbcLX*).

synthesize saxitoxins or anatoxin-a (Sivonen & Jones, 1999). Several *Anabaena* species also produce a wide variety of heptapeptide hepatotoxins, the microcystins (Sivonen *et al.*, 1992), while *Aphanizomenon ovalisporum* may synthesize alkaloid cytotoxins, the cylindropermopsins (Banker *et al.*, 1997, 2000).

Molecular studies have shown that *Anabaena* and *Aphanizomenon* strains are closely related. Strains of the two genera contained similar non-polar fatty acids, whole-cell fatty acids and whole-cell proteins (Lyra *et al.*, 1997; Li *et al.*, 1998; Gugger *et al.*, 2002). In addition, RFLP analysis and sequencing of the 16S rRNA gene have revealed a very close relationship between certain strains of *Anabaena* and *Aphanizomenon* (Neilan *et al.*, 1995; Rudi *et al.*, 1997; Lyra *et al.*, 1997, 2001; Itean *et al.*, 1999; Rudi & Jakobsen, 1999). However, the small number of *Aphanizomenon* strains investigated so far has hindered taxonomic conclusions at the generic level and has emphasized the need for further studies.

To study the relationship between the genera *Anabaena* and *Aphanizomenon*, we examined 26 strains of *Anabaena* and 14 strains of *Aphanizomenon* originating mainly from lakes in Denmark, Finland and France. The *Anabaena* strains belonged to several species based on classical morphological criteria and produced at least three types of toxic compounds, microcystins, anatoxin-a and an unknown neurotoxin. The *Aphanizomenon* strains were non-toxic and were classified as *Ap. flos-aquae* and *Aphanizomenon gracile*. The genotypic characteristics of the strains were investigated based on the 16S rDNA, the internal transcribed spacer between the 16S and 23S rDNA (ITS1) and *rbcLX* (RubisCO) with the intergenic spacer regions.

METHODS

Organisms and growth conditions. The strains used in this study are listed in Table 1. Morphological identification of the strains has been reported in previous studies (Sivonen *et al.*, 1989, 1992) or strains were identified in this study according to Geitler (1932). All strains were clonal isolates cultured in liquid Z8 medium (Kotai, 1972) with or without nitrogen at a constant temperature (20 °C) and continuous light (20 µmol quanta m⁻² s⁻¹). Microphotographs of the strains were taken using a Photometrics Sensys digital camera, PV Cam with Olympus (Provis) AX 70. The pictures were then processed with Image Pro Plus.

Toxicity. The toxicity of the strains has been described in previous studies (Sivonen *et al.*, 1989, 1992; Henriksen, 1996; Henriksen *et al.*, 1997; Lyra *et al.*, 2001) or was determined in this study by ELISA (enviroGard Microcystins Plate Kite, Strategic Diagnostics) or HPLC (Helwett Packard HP1090) as described in Lyra *et al.* (2001).

Morphology. The shapes and dimensions of the vegetative cells, heterocysts and akinetes from *Anabaena* and *Aphanizomenon* cultures were recorded during the exponential growth phase by light microscopy. Growth was measured as OD₇₅₀.

Oligonucleotide primers. Three sets of primers specific for cyanobacterial strains were used to amplify the 16S rDNA,

ITS1 and *rbcLX* (RubisCO) loci. The 16S rDNA was amplified and sequenced using the eubacterial primer 8F (Lane, 1991) and primer 1480 (5'-AGTCCTACCTTAGGC-ATCCCCCTCC-3'), specific for heterocystous cyanobacteria and designed in this study. To retrieve the complete sequence of both strands, the forward primers 400 (5'-AAGGCTCTTGGGTTGTAAACC-3') and 861 (5'-TAA-CGCGTTAAGTATCCC-3') and the reverse primers 521 (5'-ACCTGCGGACCCTTTACGC-3') and 1061 (5'-CTT-GGTAAGGTTCTTGCG-3') were used. ITS1 and the *rbcLX* loci were amplified and sequenced with the primers 16CITS and 23CITS (Neilan *et al.*, 1997b), CX, CW and DN (Rudi *et al.*, 1998) and Rubi (5'-AGGAGGATTTGTTTC-GCCTAGC-3'), designed in this study.

PCR amplification. PCR amplification of 16S rDNA and ITS1 was performed in a volume of 50 µl containing 2 µl of clonal culture, 200 µM dNTPs, 20 µM of each primer and 5 µl 10× PCR buffer. The template was boiled for 10 min prior to the addition of 1 U DynaZyme thermostable DNA polymerase (Finnzymes). The thermal-cycling conditions were 5 min denaturation at 97 °C, 30 cycles of 15 s denaturation at 94 °C, 30 s annealing at 48 °C and 1 min extension at 72 °C, followed by final 5 min elongation at 72 °C. The *rbcLX* locus was amplified as described by Rudi *et al.* (1998). The concentration of the amplified products was checked on 1% agarose gels and the products were purified with the Wizard PCR Preps DNA purification system (Promega).

Separation of the ITS1 PCR products by capillary electrophoresis. To determine the length variation of the ITS1, primers 16CITS and 23CITS (Neilan *et al.*, 1997b) were labelled at the 5'-end with 5'-6-carboxyfluorescein and 5'-4, 7,2',4',5',7'-hexachloro-6-carboxyfluorescein, respectively. PCR amplifications were performed as described above except that one-third of the primers were labelled. The PCR products were harvested according to the manufacturer's instructions and separated at 60 °C for 40 min with internal size standard GENESCAN 2500 using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer) with Performance Optimized Polymer 4 and the GENESCAN mode.

Sequencing. PCR products of the 16S rDNA and *rbcLX* were sequenced directly. Prior to sequencing, the ITS1 PCR products were either excised from the agarose gel or cloned using the pGEM-T Easy vector system (Promega) when several PCR products of the same size were detected by capillary electrophoresis. DNA sequencing was performed with the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer) according to the manufacturer's instructions. The sequenced fragments were assembled into contigs using GeneDoc version 2.6 (Nicholas & Nicholas, 1997).

Phylogenetic trees. The sequences were aligned using the program PILEUP of the GCG package version 10.1 (Genetics Computer Group, Madison, WI, USA) and corrected manually using GeneDoc version 2.6. Phylogenetic trees were constructed using the neighbour-joining method on Jukes and Cantor distances and the Wagner parsimony method of PHYLIP version 3.5c (Felsenstein, 1993). Bootstrap analysis of 500 resamplings was performed for each consensus tree. Only bootstrap values above 80% are indicated at the nodes of the trees. The trees were edited using TreeView version 1.6.1 (Page, 1996). The 16S rDNA and *rbcLX* sequences of *Microcystis* and *Planktothrix* and the ITS1-S sequence of *Nostoc* sp. PCC 7120 were chosen as outgroups for the respective trees.

Table 1. Cyanobacterial strains used in this study

Culture collections: PH, P. Henriksen (Denmark); PMC, National Museum of Natural History (France); NC, Niva-Cya, Norwegian Institute for Water Research (Norway); NIES, National Institute for Environmental Studies (Japan); PCC, Institut Pasteur (France). Strain IC-1 was provided by W. W. Carmichael (Wright State University, Dayton, OH, USA).

Strain	Geographical origin/year of isolation*	Toxicity/toxin†	Reference(s)‡
<i>Anabaena circinalis</i>			
86	Lake Villikalanjärvi, FI/1986	A	2, 8, 11, 22
123§	Lake Säyteenjärvi, FI/1986	A	2, 3, 8, 12, 18, 22, 23
90§	Lake Vesijärvi, FI/1986	M	3, 6, 8, 10, 12, 15, 18, 21–23
<i>Anabaena compacta</i>			
PH118	Lake Langesø, DK/1993	NT	
PH189	Lake Tuel, DK/1993	NT	
<i>Anabaena crassa</i> PH215	Lake Hjulby (Fyn), DK/1994	NT	
<i>Anabaena</i> cf. <i>cylindrica</i>			
PH133	Lake Arreso, DK/1993	N	
PMC9705	Dam of Champsanglard, FR/1997	NT	
<i>Anabaena flos-aquae</i>			
14	Lake Säaskjärvi, FI/1985	A	2, 7, 8, 12, 18, 22
37§	Lake Säaskjärvi, FI/1985	A	2, 8, 12, 21, 23
202A1§	Lake Vesijärvi, FI/1987	M	3, 5, 6, 8, 10, 12, 15, 18, 22, 23
<i>Anabaena lemmermannii</i>			
NC83/1§	Lake Edlandsvatnet, NW/1981	M	6, 8, 10, 12, 15, 17–20, 22, 23
66A§	Lake Säaskjärvi, FI/1986	M	3, 4, 6, 8, 10, 12, 18, 22, 23
202A2§	Lake Vesijärvi, FI/1987	M	3, 5, 6, 8, 10, 12, 22, 23
PH256	Lake Knud, DK/1994	M	13
<i>Anabaena</i> cf. <i>lemmermannii</i> PH262	Lake Knud, DK/1994	NT	13
<i>Anabaena macrospora</i> PMC9301	Lake Aydat, FR/1993	NT	
<i>Anabaena mendotae</i> PH57	Lake Velje Sø, DK/1993	NT	
<i>Anabaena planctonica</i> PH71	Lake Furesø, DK/1993	NT	
<i>Anabaena solitaria</i> 82	Lake Karpjärvi, FI/1986	NT	
<i>Anabaena spiroides</i>			
PMC9403	Lake Aydat, FR/1994	NT	
PMC9702	Dam of Champsanglard, FR/1997	NT	
<i>Anabaena</i> sp. PMC9701	Dam of Champsanglard, FR/1997	NT	
<i>Anabaena</i> sp. 277§	River Perniönjoki, FI/1991	NT	8, 12, 18, 22, 23
<i>Anabaena</i> sp. 299	Lake Vesijärvi, FI/1992	NT	18
<i>Anabaena</i> sp. IC-1	Cave Lake, ID, USA/1988	A	
<i>Aphanizomenon flos-aquae</i>			
NIES81§	Lake Kasumigama, JP/1978	NT	9, 14, 15, 22
202§	Lake Vesijärvi, FI/1987	NT	12, 18, 22, 23
TR183	Baltic Sea/1993	NT	10, 12, 18, 20, 22, 23
PMC9401	Lake Aydat, FR/1994	NT	
PMC9706	Dam of Champsanglard, FR/1997	NT	
PMC9707	Dam of Champsanglard, FR/1997	NT	
326	Lake Lohjanjärvi, FI/1998	NT	
PCC 7905§	Lake Brielse Meer, NL/?	NT	1, 9, 12, 20, 22, 23
<i>Aphanizomenon flos-aquae</i> var. <i>klebahnii</i>			
PH83	Lake Fure sø, DK/1993	NT	
PH218	Lake Birkerød, DK/1993	NT	
<i>Aphanizomenon</i> cf. <i>flos-aquae</i> PMC9501	Lake Chambon, FR/1995	NT	
<i>Aphanizomenon</i> cf. <i>gracile</i> PH271§	Lake Nørre, DK/1994	NT	22, 23
<i>Aphanizomenon gracile</i>			
PH219§	Lake Madesø, DK/1994	NT	22, 23
PMC9402	Lake Aydat, FR/1994	NT	

* DK, Denmark; FI, Finland; FR, France; JP, Japan; NL, The Netherlands; NW, Norway.

† A, Anatoxin-a; M, microcystin; N, neurotoxic; NT, no production of anatoxin-a or microcystin.

‡ References are indicated as: 1, Zevenboom *et al.* (1981); 2, Sivonen *et al.* (1989); 3, Sivonen *et al.* (1990); 4, Namikoshi *et al.* (1992a); 5, Namikoshi *et al.* (1992b); 6, Sivonen *et al.* (1992); 7, Rapala *et al.* (1993); 8, Rouhiainen *et al.* (1995); 9, Neilan *et al.* (1995); 10, Sivonen *et al.* (1995); 11, Lehtimäki *et al.* (1997); 12, Lyra *et al.* (1997); 13, Henriksen *et al.* (1997); 14, Neilan *et al.* (1997b); 15, Rapala *et al.* (1997); 16, Rudi *et al.* (1997); 17, Rudi *et al.* (1998); 18, Neilan *et al.* (1999); 19, Rudi & Jakobsen (1999); 20, Lehtimäki *et al.* (2000); 21, Rouhiainen *et al.* (2000); 22, Lyra *et al.* (2001); 23, Gugger *et al.* (2002).

§ Axenic culture.

RESULTS

Morphological and morphometric characteristics of *Anabaena* and *Aphanizomenon* strains

The trichomes were coiled (Fig. 1a) to straight (Fig. 1b) in *Anabaena* strains, whereas they appeared

straight and flexuous in *Aphanizomenon* strains (Fig. 1c, d). Some strains lost the diacritical features on which the original identification of the strains at the species level was made: *Ap. flos-aquae* lost the fascicle-like colonies (see Fig. 1d), *Anabaena spiroides* lost the regularity of the coiled trichomes and the akinetes

Fig. 1. Morphology of the trichomes in *Anabaena* and *Aphanizomenon* isolates. (a) *Anabaena spiroides* PMC9702. (b) *Anabaena lemmermannii* 202A2. (c) *Aphanizomenon flos-aquae* PMC9707. (d) *Aphanizomenon flos-aquae* TR183. Heterocysts are indicated by short arrows and akinetes are indicated by long arrows. Bars, 10 µm.

were not aggregated in the centre of the colonies of *Anabaena lemmermannii*. The size range of the cells did not correspond to the ones described for the species designation in Geitler's monograph. The vegetative cells of *Anabaena* strains were round or barrel-shaped (Fig. 1a, b), whereas they were cylindrical in *Aphanizomenon* (Fig. 1c, d). Most of the vegetative cells were 5–7 µm long in both genera (Fig. 2). The mean width of these cells was < 4 µm for *Aphanizomenon* strains and > 4 µm for *Anabaena* strains (Fig. 2). The heterocysts were round to oval in *Anabaena* and cylindrical in *Aphanizomenon* strains. The heterocysts were significantly ($P < 0.05$; Mann–Whitney U test) wider in *Anabaena* than in *Aphanizomenon* strains (Fig. 2), they were 5–7 µm long and at the intercalary position in

both genera. *Anabaena* sp. 277 and *Ap. flos-aquae* NIES 81 and PMC 9401 rarely produced heterocysts and lost their capacity to grow in medium without nitrogen. On the other hand, strains such as *An. flos-aquae* 14, *Anabaena* sp. IC-1, *An. lemmermannii* 66A, *An. cf. lemmermannii* PH262, *Anabaena solitaria* 82 and *Ap. flos-aquae* PCC 7905 grew very well in Z8 medium without nitrogen but seldom showed differentiation of the cells to heterocysts and akinetes. The shapes of akinetes in the different *Anabaena* strains varied, whereas *Aphanizomenon* strains had only cylindrical akinetes. The akinetes were significantly ($P < 0.05$; Mann–Whitney U test) wider in *Anabaena* strains than in *Aphanizomenon* strains (Fig. 2). The terminal and vegetative cells were similar in *Anabaena*

Fig. 2. Morphometric comparison of the lengths and widths of the different cells of *Anabaena* and *Aphanizomenon* trichomes. Frequencies of various size classes of vegetative cells, heterocysts and akinetes are indicated. Filled bars indicate *Aphanizomenon* strains and unfilled bars indicate *Anabaena* strains; n , number of cells measured.

strains, with the exception of the conical terminal cells of *Anabaena* cf. *cylindrica* PH133. The terminal cells of several *Aphanizomenon* strains were hyaline and often elongated. Gas vesicles were present in all strains except *An.* cf. *cylindrica* PH133.

Genetic diversity of *Anabaena* and *Aphanizomenon* strains according to 16S rRNA gene sequences

Thirty almost-complete (1465 bp) 16S rDNAs sequenced in this study and 10 previously sequenced 16S rDNAs (Lyra *et al.*, 2001) of *Anabaena* and *Aphanizomenon* strains were used to construct the phylogenetic trees. The neighbour-joining and parsimony 16S rDNA trees were similar. Thus, only the parsimony tree for the phylogenetic relationship between *Anabaena* and *Aphanizomenon* strains is presented (Fig. 3).

The 16S rDNA tree revealed that strains of the genera *Anabaena* and *Aphanizomenon* were intermixed and thus not monophyletic (Fig. 3). The main clusters, 1, 2 and 3, were supported by high bootstrap values. Cluster 1 contained all anatoxin-a-producing *Anabaena* strains, 14, 37, 86, 123 and IC-1, non-toxic *Anabaena* strains PH57 and PH262 and non-toxic *Ap. flos-aquae*/*Ap. gracile* strains 202, PH219, PMC9706, TR183, PCC 7905, PMC9501 and NIES81. The *Anabaena* and *Aphanizomenon* strains of cluster 1 shared high similarity values of at least 99.5%. Cluster

2 was divided into two subclusters, 2a and 2b, containing six non-toxic *Ap. flos-aquae*/*Ap. gracile* strains (PMC9401, PMC9402, PMC9707, 326, PH218 and PH83) and five non-toxic *Anabaena* strains (PH215, PMC9301, PMC9403, PMC9701 and PMC9702), with similarity values > 99.7% and 98.9%, respectively. The overall sequence similarity of cluster 2 (> 98%) supported by high bootstrap values (up to 83% in both phylogenetic reconstruction methods) revealed a tight grouping between the strains of the two genera. Cluster 3 contained all hepatotoxic *Anabaena* strains, 202A1, NC83/1, 202A2, 66A, PH256 and 90, and the non-toxic *Anabaena* sp. strain 299 and *An. solitaria* strain 82. The 16S rDNA sequences of *Anabaena* sp. strain 299 and *An. lemmermannii* strain PH256 were similar, while the sequences of *An. solitaria* 82 and the other *Anabaena* strains of cluster 3 shared similarity values < 97.9%. The four distinct branches contained non-toxic strains, *An.* cf. *cylindrica* PMC9705 and *Ap. gracile* PH271 (branch 4), the two non-toxic *Anabaena compacta* strains PH118 and PH189 (branch 5), the non-toxic *Anabaena* sp. strain 277 and *Anabaena planctonica* strain PH71 (branch 6) and neurotoxic *Anabaena* cf. *cylindrica* strain PH133 (branch 7). The two *Anabaena* strains 277 and PH71 and the other *Anabaena* strains in the tree shared similarity values < 97%. *An.* cf. *cylindrica* PH133 and the other *Anabaena* strains shared sequence similarity < 94.6%.

ITS1 length polymorphism. The lengths and numbers of ITS1 PCR products of *Anabaena* and *Aphanizomenon* strains are listed in Table 2. The amplified fragments contained the ITS1 region as well as 150 nucleotides of the 16S rRNA gene and 50 nucleotides of the 5' end of the 23S rRNA gene. Multiple bands were observed in agarose gel electrophoresis (data not shown). Capillary

ITS1 sequences. All ITS1 fragments of the strains *An* cf.

Table 2. Lengths of the long and short ITS1 amplified products and the three types of ITS1-S sequence in *Anabaena* and *Aphanizomenon* strains

Lengths are expressed as numbers of nucleotides. The sequences represented by underlined numbers have been obtained by cloning.

Strain	Length of ITS1 product		ITS1-S type		
	ITS1-L	ITS1-S	α	β	γ
<i>An. circinalis</i> 86	700	475	272		
<i>An. circinalis</i> 123*	700	475	272		
<i>An. circinalis</i> 90*	709	476, 478	<u>274</u>		
<i>An. compacta</i> PH118	700, 710	494	290		
<i>An. compacta</i> PH189	701	493	290		
<i>An. crassa</i> PH215	684, 689	463, 466	<u>262</u> , <u>265</u>		
<i>An. cf. cylindrica</i> PH133	705	498			295
<i>An. cf. cylindrica</i> PMC9705	701	474, 501		<u>298</u>	
<i>An. flos-aquae</i> 14	700	475	272		
<i>An. flos-aquae</i> 37*	700	475	272		
<i>An. flos-aquae</i> 202A1*	703	503	298		
<i>An. lemmermannii</i> NC83/1*	704	503	299		
<i>An. lemmermannii</i> 66A*	710	505	300		
<i>An. lemmermannii</i> 202A2/41*	704	503	298		
<i>An. lemmermannii</i> PH256	710	478, 501	276		
<i>An. cf. lemmermannii</i> PH262	701	473, 500	<u>272</u>	<u>298</u>	
<i>An. macrospora</i> PMC9301	689	467	265		
<i>An. mendotae</i> PH57	701	473, 500		<u>298</u>	
<i>An. planctonica</i> PH71	694	486	<u>282</u>		
<i>An. solitaria</i> 82	741	496	293		
<i>An. spiroides</i> PMC9403	687	466	265		
<i>An. spiroides</i> PMC9702	694	482	280		
<i>Anabaena</i> sp. PMC9701	704	482, 486	<u>279</u>		
<i>Anabaena</i> sp. 277*	662	448	249		
<i>Anabaena</i> sp. 299	710	478	276		
<i>Anabaena</i> sp. IC-1	700	475	272		
<i>Ap. flos-aquae</i> NIES81*	710, 733	475, 492	273		
<i>Ap. flos-aquae</i> 202*	700, 716	490, 499		288	
<i>Ap. flos-aquae</i> TR183	716	491		288	
<i>Ap. flos-aquae</i> PMC9401	689	467, 501	266		
<i>Ap. flos-aquae</i> PMC9706	713	467	266		
<i>Ap. flos-aquae</i> PMC9707	689	467, 502	266		
<i>Ap. flos-aquae</i> 326	689	468, 473	<u>267</u> , <u>269</u>		
<i>Ap. flos-aquae</i> PCC 7905	714	491		288	
<i>Ap. flos-aquae</i> PH83	682	471	269		
<i>Ap. flos-aquae</i> PH218	682	471	269		
<i>Ap. cf. flos-aquae</i> PMC9501	725	476, 485, 493	<u>273</u>	<u>282</u> , <u>289</u>	
<i>Ap. gracile</i> PH219*	725	475, 492	273		
<i>Ap. cf. gracile</i> PH271*	713	467	266		
<i>Ap. gracile</i> PMC9402	689	467, 502	266		

* Axenic culture.

lemmermannii PH262 and *An. spiroides* PMC9702 were sequenced to clarify the length difference between the short and long ITS1 PCR products. The ITS1-L sequences differed from ITS1-S by 201–228 bp, corresponding to the presence of tRNA^{Ile} and tRNA^{Ala} genes and to variations in the length of the regions

between the conserved domain D2 and the antiterminator box B. The ITS1-L products of strains *An. cf. lemmermannii* PH262 and *An. spiroides* PMC9702 also differed in the same region. It was the most variable region in ITS1-S. In total, 45 ITS1-S PCR products (249–300 bp) were sequenced from *Anabaena* and

Fig. 4. Alignment of nucleotide sequences of the ITS1-S regions of types α , β and γ from some representative *Anabaena* and *Aphanizomenon* strains. The conserved domains (D1, D1', D2, D4 and D5) and the antiterminator (boxes B and A) are located relative to the ITS1-S sequence from *Nostoc* PCC 7120 (AF180969). An., *Anabaena*; Ap., *Aphanizomenon*; Nos., *Nostoc*.

Aphanizomenon strains (Table 2). An alignment of some ITS1-S sequences is presented in Fig. 4. Three types of ITS1-S sequence were designated types α , β and γ (Table 2 and Fig. 4). The ITS1-S sequence of type α was present in all strains except *An. cf. cylindrica* PH133 and *Ap. flos-aquae* TR183 and PCC 7905 (Table 2). The β type was found in eight of the 45 ITS1-S sequenced (Table 2). It differed from the α type by the presence of 77 nucleotides and the absence of 61 nucleotides located between the two conserved domains D1 and D1' (Fig. 4). Both types of ITS1-S were observed in *An. cf. lemmermannii* PH262 and in *Ap. cf. flos-aquae* PMC9501 (Table 2, Figs 4 and 5). The γ type was found in the neurotoxic *An. cf. cylindrica* PH133 and the whole sequence differed from the α and β types by several polymorphic sites (Fig. 4).

The ITS1-S tree. The neighbour-joining and parsimony trees based on ITS1-S sequences were similar. Thus, only the parsimony tree is presented (Fig. 5). The ITS1-S sequences formed three main clusters. Cluster 1 was divided into two subclusters, 1a and 1b (Fig. 5). Subcluster 1a contained the type- α ITS1-S sequences of all anatoxin-a-producing *Anabaena* strains, 14, 37, 86, 123 and IC-1, the non-toxic *An. cf. lemmermannii* PH262 and the non-toxic *Ap. flos-aquae*/*Ap. gracile* strains PMC9501, PH219 and NIES81. Subcluster 1b contained all the type- β ITS1-S sequences, belonging to *Anabaena* strains PH57 and PMC9705 and *Aphanizomenon* strains PCC 7905, TR183 and 202 as well as *An. cf. lemmermannii* PH262 and *Ap. cf. flos-aquae* PMC9501. The ITS1-S sequence similarity values within subclusters 1a and 1b were respectively 95 and

Fig. 5. Consensus parsimony tree based on ITS1-S sequences (249–300 bp) of *Anabaena* and *Aphanizomenon* isolates. See legend to Fig. 3 for further details.

86%. The ITS1-S sequence similarity values within each subcluster were higher than between the sequences of the two subclusters, showing that there is more diversity in the different ITS1-S copies of a single strain (e.g. 80% sequence similarity between the α - and β -type sequences of *An. cf. lemmermannii* PH262) than between the homologous ITS1-S sequences of two strains. Cluster 2 contained the ITS1-S sequences of three non-toxic *Anabaena* strains (PMC9301, PMC9403 and PH215) and six non-toxic *Aphanizomenon* strains (PMC9401, PMC9402, PMC9707, 326, PH83 and PH218). The similarity of the ITS1-S sequences of the *Anabaena* and *Aphanizomenon* strains of cluster 2 was > 85%. Cluster 3 grouped all

hepatotoxic *Anabaena* strains (66A, NC83/1, 90, 202A1, 202A2 and PH256) and two non-toxic *Anabaena* strains, 299 and 82. The similarity values of the ITS1-S sequences in cluster 3 were > 82%, with the exception of the more distantly related ITS1-S sequence of *An. solitaria* 82, which shared only 72% similarity with the other sequences in cluster 3. Five separate branches contained the ITS1-S sequences of *Ap. cf. gracile* PH 271 and *Ap. flos-aquae* PMC9706 (branch 4), *An. compacta* strains PH118 and PH189 and *Anabaena* sp. PMC9701 (branch 5), *An. spiroides* PMC9702 and *An. planctonica* PH71 (branch 6) and *Anabaena* sp. 277 (branch 7). Branch 8 consisted of the type- γ ITS1-S sequence of the neurotoxic *An. cf.*

and a second intergenic spacer (IGS2, 77 bp). The length of the *rbcLX* sequences in most strains was 783 bp. *An. cf. cylindrica* PH133 had the longest sequence (793 bp), due to the insertion of 15 nucleotides in IGS1. *Anabaena* strain PMC9701 had the shortest *rbcLX* sequence (650 bp), due to the absence of 130 bp in the *rbcX* region. The parsimony and neighbour-joining trees of *rbcLX* were congruent (Fig. 6). Sequences of *Anabaena* and *Aphanizomenon* strains were intermixed in the *rbcLX* tree. Three main clusters, 1, 2 and 3, were observed (Fig. 6). Cluster 1 contained

all anatoxin-a-producing *Anabaena* strains, 14, 37, 86, 123 and IC-1, non-toxic *An. cf. lemmermannii* PH262 and non-toxic *Aphanizomenon* strains TR183, PCC 7905, PMC9501, NIES81 and PH219. The *Anabaena* and *Aphanizomenon* *rbcLX* sequences of cluster 1 shared similarity of > 99.6%. Cluster 2 was divided into two subclusters, 2a and 2b, respectively containing non-toxic *Anabaena* strains PH215, PMC9403, PMC9301, PH71, PMC9701 and PMC9702 and non-toxic *Aphanizomenon* strains 326, PMC9707, PMC9401 and PMC9402. The overall similarity value of the *rbcLX* sequences within cluster 2 was > 97%. Cluster 3 consisted of the hepatotoxic *Anabaena* strains 202A1, 202A2, PH256, NC83/1, 66A and 90 and non-toxic *Anabaena* strains 299, PH118, PH189, PH57 and PMC9705 and the non-toxic *Aphanizomenon* strains PH218, PH83 and 202. The sequence similarity in cluster 3 was > 97.6%. Branch 4 consisted of *Aphanizomenon* strains PMC9706 and PH271 and *An. solitaria* 82. The separated branches contained *Anabaena* sp. 277 (branch 5) and neurotoxic *An. cf. cylindrica* PH133 (branch 6). The *rbcLX* sequence of *An. cf. cylindrica* PH133 shared only 87% similarity with the other *Anabaena* strains.

Comparison of the clustering in the trees

The strain composition of the three main clusters (1, 2 and 3) was generally the same in the 16S rDNA, ITS1-S and *rbcLX* trees (Figs 3, 5 and 6). In cluster 1, the *Anabaena* strains producing anatoxin-a were always grouped with the same non-toxic *Anabaena* and *Aphanizomenon* strains (PH262, PH219, TR183, PCC-7905, PMC9501 and NIES81). In cluster 2, the same non-toxic *Anabaena* strains (PH215, PMC9301, PMC9403 and PMC9701) and *Aphanizomenon* strains (326, PMC9401, PMC9402 and PMC9707) were clustered together in all the trees. Finally, cluster 3 always contained all the microcystin-producing *Anabaena* strains and the non-toxic *Anabaena* sp. 299. However, there were also differences between the trees and groupings. In the 16S rDNA tree, *Anabaena mendotae* PH57 and *Ap. flos-aquae* PMC9706 were found together in cluster 1 (Fig. 3). In contrast, *An. mendotae* PH57 was associated with *An. cf. lemmermannii* PH262 in cluster 1 of the ITS1-S tree (Fig. 5) and with *An. cf. cylindrica* PMC9705 in cluster 3 of the *rbcLX* trees (Fig. 6). The *An. compacta* strains PH189 and PH118 were situated in cluster 5 in the 16S rDNA and ITS-S trees but in cluster 3 in the *rbcLX* tree. *An. planctonica* PH71 was grouped differently in all three trees.

DISCUSSION

Morphological observations of the *Anabaena* and *Aphanizomenon* cultures showed that some diacritical features used for species and genus determination were lost during isolation and cultivation, such as the ability to differentiate akinetes or heterocysts and to form the fascicle-like colonies typical of *Ap. flos-aquae*. Without

these characters, species determination in the two genera is not feasible. In addition, the cell dimensions of the cultured strains did not correspond to the size range usually observed in the original species designation. Similar changes have been observed previously for *Anabaena* (Stulp, 1982) and *Nodularia* strains (Lehtimäki *et al.*, 2000; Laamanen *et al.*, 2001). At the genus level, determination of the trichome structure (e.g. metameric or subsymmetric) was not possible for several strains. Nevertheless, general features such as bead-like cells in *Anabaena* were always distinguishable from the vegetative cells of *Aphanizomenon*, which had slightly constricted cell walls between two adjacent cells. Moreover, vegetative cells, heterocysts and akinetes were wider in *Anabaena* isolates than in *Aphanizomenon* isolates. Thus, *Anabaena* and *Aphanizomenon* strains were distinguishable on the basis of morphological criteria.

In contrast to the morphological classification, analyses of the 16S rDNA, ITS1-S and *rbcLX* sequences of *Anabaena* and *Aphanizomenon* strains showed high similarity between *Anabaena* and *Aphanizomenon* strains and did not confirm the taxonomic validity of the two genera. The 16S rDNA sequence similarity between certain *Anabaena* and *Aphanizomenon* strains was higher than the similarity observed between strains belonging to the genus *Anabaena*. The three analyses revealed that the genera *Anabaena* and *Aphanizomenon* are not monophyletic. Several previous studies based on 16S rDNA (Lyra *et al.*, 1997, 2001; Rudi *et al.*, 1998; Iteman *et al.*, 1999) and DNA-dependent RNA polymerase (*rpoC1*) (Fergusson & Saint, 2000) have shown that *Anabaena* and *Aphanizomenon* strains clustered together. It was also reported that genus-specific probes for 16S rDNA designed for laboratory strains could not discriminate between *Anabaena* and *Aphanizomenon* in natural samples (Rudi *et al.*, 2000).

The number, size or sequence of the ITS1 products, used for the classification of closely related cyanobacterial strains (Nelissen *et al.*, 1994; Lu *et al.*, 1997; Neilan *et al.*, 1997b; Otsuka *et al.*, 1999; Schedelman *et al.*, 1999), did not allow the delineation of *Anabaena* and *Aphanizomenon* strains. However, the sizes and numbers of ITS products distinguished the two genera from other genera of the Nostocales such as *Cylindrospermopsis*, *Raphidiopsis* (M. Gugger, unpublished), *Nodularia* (Laamanen *et al.*, 2001) and *Nostoc* (Iteman *et al.*, 2000). The numbers of ITS1 products also indicate that *Anabaena* and *Aphanizomenon* strains have several different ribosomal operons. Three types of ITS1-S sequence were found in our isolates. The α and β types of ITS1-S were found in the majority of our *Anabaena* and *Aphanizomenon* strains. The high similarity between these two types of sequence and their simultaneous presence within single strains of the two genera (e.g. *Anabaena* PH262 and *Aphanizomenon* PMC9501) revealed the recent divergence of the α and β sequences. The ITS1-S sequence of type γ found in *An. cf. cylindrica* PH133 was comparable to the α and β types only in the conserved domains of the ITS1

locus and was therefore highly divergent from the two other types of ITS1-S found in *Anabaena* and *Aphanizomenon* strains.

Stulp & Stam (1985) found the genus *Anabaena* to be very homogeneous by morphological and DNA–DNA hybridization analyses. In contrast, this study and the previous studies of Lyra *et al.* (1997, 2001) have shown that the genus *Anabaena* is heterogeneous. Strain *An. cf. cylindrica* PH133 did not contain gas vesicles, indicating that the strain is probably periphytic or benthic. Based on genotypic (16S rDNA, ITS1-S and *rbcLX*) and physiological (production of an unknown neurotoxin) criteria, we found *An. cf. cylindrica* PH133 to be considerably different from planktic *Anabaena* strains. Similarly, 16S rDNA studies on *Anabaena* sp. PCC 7108, originating from the intertidal zone in the USA (Rippka *et al.*, 1979; Lyra *et al.*, 2001), and *Anabaena* sp. NIES 19, originating either from Japan (see Beltran & Neilan, 2000) or from pond water in the UK (synonymous to PCC 73105; see Neilan *et al.*, 1995, 1999), and *rpoC1* of *Anabaena bergii* ANA283A, originating from Australia (Wilson *et al.*, 2000; Fergusson & Saint, 2000), showed that these strains are more related to other Nostocales isolates than to the cluster consisting of planktic *Anabaena* strains. The strains *An. cf. cylindrica* PH133, *Anabaena* sp. PCC 7108 (Lyra *et al.*, 2001) and *Anabaena* sp. NIES19 (Beltran & Neilan, 2000) shared < 95% 16S rDNA sequence similarity with the planktic *Anabaena* strains. This difference between the planktic *Anabaena* isolates and other *Anabaena* isolates cannot be explained by morphological misidentification but, instead, suggests that a re-evaluation of the genus *Anabaena* is needed. A recent study based on 16S rDNA sequences of seven *Aphanizomenon* strains demonstrated that the genus was monophyletic and in agreement with the morphological classification of the strains (Li *et al.*, 2000). In this study, we compared *Anabaena* and *Aphanizomenon* strains and found that *Aphanizomenon* can not be regarded as monophyletic.

Phylogenies based on *rbcLX* have been found to be incongruent with 16S rDNA phylogenies within the cyanobacteria and purple bacteria (Watson & Tabita, 1997; Rudi *et al.*, 1998). Study of three different loci of the same strains showed overall similarity of the *rbcLX*, 16S rDNA and ITS-S trees, with three main clusters and two branches containing *Anabaena* sp. 277 and *An. cf. cylindrica* PH133 always near the root of the tree. Comparison of the 16S rDNA and *rbcLX* phylogenies revealed that 11 of 14 strains contained in cluster 1, 9 of 11 strains of cluster 2 and 7 of 8 strains of cluster 3 in the 16S rDNA tree were grouped similarly in the *rbcLX* tree. However, a few differences were also found, such as the number of strains contained in cluster 3, but this cluster in the *rbcLX* tree is not supported by its bootstrap percentage. Our strains could be placed in the cluster 'Nostoc lineage I' described by Rudi *et al.* (1998). Compared with the latter study, less variability was observed in the *rbcLX* nucleotide sequences of *Anabaena* and *Aphanizomenon*

isolates (as shown by the high sequence similarities in each cluster). This could be related to the larger number of strains in this study. According to the results of Rudi *et al.* (1998), this observation suggested that there might be lateral gene transfer between *Anabaena* and *Aphanizomenon* strains.

Anabaena strains that produce different types of toxin clustered separately from each other in the 16S rDNA, ITS1-S and *rbcLX* trees. The first cluster contained anatoxin-a-producing *Anabaena* strains and non-toxic strains from both genera. The second cluster contained non-toxic *Anabaena* strains and non-toxic *Aphanizomenon* strains and the third cluster contained microcystin-producing and non-toxic *Anabaena* strains. Despite their diverse geographical origins, *Anabaena* strains that produce similar toxic compounds were clustered together in the 16S rDNA, ITS1-S and *rbcLX* trees, highlighting that these strains have evolved from the same ancestor. In the order Nostocales, corresponding results have been found within the genera *Anabaena* (Lyra *et al.*, 1997, 2001; Beltran & Neilan, 2000) and *Nodularia* (Lehtimäki *et al.*, 2000; Laamanen *et al.*, 2001). *Anabaena* strains producing anatoxin-a, anatoxin-a(S), microcystins, saxitoxin and an unknown neurotoxin grouped separately based on 16S rDNA sequences (Lyra *et al.*, 1997, 2001; Beltran & Neilan, 2000; this study). Each cluster of strains that produce a specific toxin also contained non-toxic strains. These clusters are noticeable within the *Anabaena* strains that produce microcystins or anatoxin-a (Lyra *et al.*, 2001; this study) and for the one that produces saxitoxin (Beltran & Neilan, 2001), but also amongst clusters of other genera that produce the same type of hepatotoxin, microcystins, such as *Microcystis* or *Planktothrix* (Neilan *et al.*, 1997a; Otsuka *et al.*, 1999; Lyra *et al.*, 2001). Whether these non-toxic strains have toxin genes at all or whether they contain inactivated toxin genes remains to be studied.

The 16S rDNA sequence similarity of *Anabaena* and *Aphanizomenon* strains was higher than 97.5%, which indicates that the strains of the two genera belong to the same species (Stackebrandt & Goebel, 1994). Previously, the study of Palinska *et al.* (1996) revealed 16S rDNA similarity of morphologically different isolates from the genera *Merismopedia* and *Synechocystis*. Polyphasic studies on morphology, 16S rDNA sequences, polymorphic loci or DNA hybridization have revealed genetic similarity of several *Microcystis* species (Otsuka *et al.*, 1998, 1999, 2001; Kondo *et al.*, 2000). In addition, *Planktothrix* strains with different pigments were genotypically similar (Humbert & Leberre, 2001; Lyra *et al.*, 2001). Recent studies of Nübel *et al.* (2000) and Rippka *et al.* (2000) have also emphasized the need for multidisciplinary studies to understand the diversity of the cyanobacteria. On the basis of the congruent results obtained by genetic (Lyra *et al.*, 2001; this study), whole-cell protein (Lyra *et al.*, 1997) and fatty acid (Li *et al.*, 1998; Gugger *et al.*, 2002) analyses, the taxonomic validity of the genera

Aphanizomenon and *Anabaena* seems to be questionable, even though morphological and morphometric characterizations support the distinction made between these two genera. The differences between molecular and morphological results may reflect the existence of species variants in populations or ecotypes adapted to different environmental conditions.

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