International Journal of Systematic and Evolutionary Microbiology (2005), 55, 11–26 DOI 10.1099/ijs.0.63276-0

Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria)

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The heterocytous cyanobacteria form a monophyletic group according to 16S rRNA gene sequence data. Within this group, phylogenetic and morphological studies have shown that genera such as *Anabaena* and *Aphanizomenon* are intermixed. Moreover, the phylogeny of the genus *Trichormus*, which was recently separated from *Anabaena*, has not been investigated. The aim was to study the taxonomy of the genera *Anabaena*, *Aphanizomenon*, *Nostoc* and *Trichormus* belonging to the family Nostocaceae (subsection IV.I) by morphological and phylogenetic analyses of 16S rRNA gene, *rpoB* and *rbcLX* sequences. New strains were isolated to avoid identification problems caused by morphological changes of strains during cultivation. Morphological and phylogenetic data showed that benthic and planktic *Anabaena* strains were intermixed. In addition, the present study confirmed that *Anabaena* and *Aphanizomenon* strains were not monophyletic, as previously demonstrated. The evolutionary distances between the strains indicated that the planktic *Anabaena* and *Aphanizomenon* strains as well as five benthic *Anabaena* strains in cluster 1 could be assigned to a single genus. On the basis of the 16S rRNA, *rpoB* and *rbcLX* gene sequences, the *Anabaena*/*Aphanizomenon* strains (cluster 1) were divided into nine supported subclusters which could also be separated morphologically, and which therefore might represent different species. *Trichormus* strains were morphologically and phylogenetically heterogeneous and did not form a monophyletic cluster. These *Trichormus* strains, which were representatives of three distinct species, might actually belong to three genera according to the evolutionary distances. *Nostoc* strains were also heterogeneous and seemed to form a monophyletic cluster, which may contain more than one genus. It was found that certain morphological features were stable and could be used to separate different phylogenetic clusters. For example, the width and the length of akinetes were useful features for classification of the *Anabaena*/*Aphanizomenon* strains in cluster 1. This morphological and phylogenetic study with fresh isolates showed that the current classification of these anabaenoid genera needs to be revised.

Published online ahead of print on 6 August 2004 as DOI 10.1099/ijs.0.63276-0.

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour joining; PCA, principal-component analysis.

The GenBank/EMBL/DDBJ accession numbers are AJ630408–AJ630458 for the 16S rRNA gene sequences, AJ632022–AJ632070 for the *rbcLX* gene sequences and AJ628068–AJ628134 for *rpoB* gene sequences determined in this study.

A table of complete morphological characters is available as supplementary material in IJSEM Online.

INTRODUCTION

Heterocytous cyanobacteria consistently form a monophyletic cluster among cyanobacteria on the basis of their 16S rRNA gene sequences (Wilmotte, 1994; Turner, 1997, 1999; Wilmotte & Herdman, 2001; Lyra *et al.*, 2001), RFLP and genomic fingerprinting (Lyra *et al.*, 2001) and *nifD* sequences (Henson *et al.*, 2004). This monophyletic cluster contains the orders Nostocales and Stigonematales (Sections IV and V) (Rippka *et al.*, 1979), which were found to be intermixed (Turner *et al.*, 1997, 1999; Gugger & Hoffmann, 2004; Henson *et al.*, 2004). However, the genera *Anabaena* Born. et Flah., *Aphanizomenon* Born. et Flah., *Nostoc* Born. et Flah. and *Cylindrospermopsis* Seen. et Subba Raju clustered together within these orders in a 16S rRNA tree (Gugger & Hoffmann, 2004).

Currently, the genera *Anabaena*, *Aphanizomenon*, *Trichormus* (Born. et Flah.) Kom. et Anag. and *Nostoc* belong to order Nostocales, family Nostocaceae by traditional classification (Komárek & Anagnostidis, 1989) and subsection IV.I by bacteriological classification (Rippka *et al.*, 2001a). Identification of these genera is based on morphological features such as morphology of the filament, vegetative cells, heterocytes (heterocysts) and akinetes (Komárek & Anagnostidis, 1989). The form of the colony, shape of terminal cells, presence of sheath and gas vesicles, as well as life cycle, are additional features used for the identification of some genera. Recently, *Trichormus* was separated from the traditional genus *Anabaena* on the basis of akinete development and was transferred into the subfamily Nostocoidae. According to this revision, the species *Anabaena variabilis*, *Anabaena azollae* and *Anabaena doliolum* belong to the genus *Trichormus* (Komárek & Anagnostidis, 1989). The phylogeny of the *Trichormus* strains has not been studied. The assignment of some species either to *Anabaena* or to *Aphanizomenon* has been discussed (Komárek & Anagnostidis, 1989; Komárek & Kováčik, 1989). Also, the previous phylogenetic studies of Lyra *et al.* (2001), Gugger *et al.* (2002b) and Iteman *et al.* (2002) have shown that the genera *Anabaena* and *Aphanizomenon* are not monophyletic. Furthermore, the separation of the genera *Nostoc* and *Anabaena* has also been discussed in recent years (Henson *et al.*, 2002; Tamas *et al.*, 2000).

Phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometimes conflict with the morphological classification (Lyra *et al.*, 2001; Iteman *et al.*, 2002; Gugger & Hoffmann, 2004). The comparison of morphological and genetic data is hindered by the lack of cultures of several cyanobacterial morphospecies and inadequate morphological data of sequenced strains. Moreover, some strains may lose some important features such as gas vesicles (Lehtimäki *et al.*, 2000) or form of colony (Gugger *et al.*, 2002b) during long-term laboratory cultivation, which complicates identification. Komárek & Anagnostidis (1989) have estimated that more than 50 % of the strains in culture collections are misidentified. Therefore, new isolates should be studied by combined morphological and genetic approaches.

This study focused on the combined genetic and phenotypic relationships of the four genera *Nostoc*, *Trichormus*, *Anabaena* and *Aphanizomenon*. We isolated new *Anabaena*, *Aphanizomenon* and *Nostoc* strains. Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification. The phylogeny of the strains was investigated by sequencing two housekeeping genes, 16S rRNA and *rpoB*, as well as a carbon-fixation-associated gene, *rbcLX*.

METHODS

Strains and cultivation. The 51 *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* strains studied were unialgal, but not axenic (Table 1). The morphology of the strains with a few exceptions was analysed in this study. The morphology of *Nostoc muscorum*, *Nostoc calcicola*, *Nostoc ellipsosporum* and *Nostoc edaphicum* was described previously by Hrouzek *et al.* (2003) and *Anabaena compacta* ANACOM-KOR by Zapomělová (2004). The morphology of strain *Anabaena* sp. 277 (Lyra *et al.*, 2001; Gugger *et al.*, 2002a) was re-evaluated because of its clustering with *Aphanizomenon issatschenkoi* strains in the phylogenetic trees.

For morphological studies, the strains were cultivated in $BG11_0$ medium (Stanier *et al.*, 1971) at 18–22 °C under a light intensity of 30 μmol m^{-2} s^{-1}. For DNA extraction, the strains were cultivated in Z8 medium (Zehnder in Staub, 1961; Kotai, 1972) without nitrogen at 18·5–21·5 °C under a light intensity of 10 μmol m^{-2} s^{-1}.

Morphological study. The morphology of cells and filaments was studied using an Olympus CX 40 light microscope with a digital camera. Olympus DP SOFT version 4.0 software was used for image analysis. The following parameters were selected to describe the morphology of the studied strains: length and width of vegetative cells, heterocytes and akinetes; morphology of terminal cell; distance between heterocytes and distance between a heterocyte and the nearest akinete (counted as the number of cells); presence or absence of terminal heterocytes and gas vesicles; and shape of filament and its aggregation in colonies.

Statistical evaluation of morphological data. The mean values of measured morphological parameters were compared with one-way analysis of variance (ANOVA) followed by the Tukey honest significant difference (HSD) test in Statistica for Windows 4. To describe the variability of all morphological data and to evaluate the importance of measured morphological features, principal-component analysis (PCA) was carried out in Canoco for Windows 4.5 (ter Braak & Šmilauer, 1998). The program CanoDraw 4.5 was used for construction of the PCA plot.

DNA extraction. Cells were harvested by filtration through 1 or 5 μm Poretics filters (Osmonic) and stored at −20 °C. DNA was extracted by the modified CTAB method (Hönerlager *et al.*, 1995; Gkelis *et al.*, 2005). Filters containing cells were mechanically lysed using lysis matrix A and a Fast-prep instrument (Bio101) in 500 μl extraction buffer [100 mM Tris/HCl, pH 7·5, 1·5 % (w/v) SDS, 10 mM EDTA, 1 % (w/v) deoxycholate, 1 % (v/v) IGEPAL CA-630 (Sigma), 5 mM thiourea and 10 mM dithiothreitol, according to Hönerlager *et al.*, 1995] for 30 s at speed 5. The extracts were centrifuged at 10 000 ***g*** for 1 min and the DNA-containing supernatants were incubated in 5 M NaCl/10 % (w/v) CTAB at 65 °C for 20 min, followed by chloroform purification and ethanol precipitation.

Table 1. Cyanobacterial strains used in this study, their origin and potential ability to produce microcystins

Taxonomic assignment	Strain	Geographical origin and year of isolation	*mcyE* PCR*
Anabaena			
An. augstumalis	SCMIDKE JAHNKE/4a	Rostock, Germany	−
An. cf. *circinalis* var. *macrospora*	1tu23s3	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu26s10	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu27s5	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu28s13	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	0tu25s6	Lake Tuusulanjärvi, Finland, 2000	−
An. cf. *crassa*	1tu27s7	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *cylindrica*	XP6B	Sediment, Porkkala, Helsinki, Gulf of Finland, Baltic Sea, 1999	−
An. circinalis	1tu34s5	Lake Tuusulanjärvi, Finland, 2001	−
An. circinalis	1tu30s11	Lake Tuusulanjärvi, Finland, 2001	−
An. circinalis	1tu33s12	Lake Tuusulanjärvi, Finland, 2001	+
An. compacta	ANACOM-KOR[a]†	Water reservoir, Kořensko, Czech Republic, 2002	−
An. flos-aquae	1tu31s11	Lake Tuusulanjärvi, Finland, 2001	+
An. flos-aquae	0tu33s15	Lake Tuusulanjärvi, Finland, 2000	−
An. flos-aquae	0tu33s2a	Lake Tuusulanjärvi, Finland, 2000	−
An. flos-aquae	1tu30s4	Lake Tuusulanjärvi, Finland, 2001	+
An. flos-aquae	1tu35s12	Lake Tuusulanjärvi, Finland, 2001	−
An. lemmermannii	1tu32s11	Lake Tuusulanjärvi, Finland, 2001	−
An. mucosa	1tu35s5	Lake Tuusulanjärvi, Finland, 2001	+
An. oscillarioides	BECID22	Epiphytic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	−
An. oscillarioides	BECID32	Epilithic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	−
An. oscillarioides	BO HINDAK 1984/43	Canada, 1984	−
An. planctonica	1tu33s10	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu28s8	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu30s13	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu33s8	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu36s8	Lake Tuusulanjärvi, Finland, 2001	−
An. sigmoidea	0tu36s7	Lake Tuusulanjärvi, Finland, 2000	−
An. sigmoidea	0tu38s4	Lake Tuusulanjärvi, Finland, 2000	−
An. smithii	1tu39s8	Lake Tuusulanjärvi, Finland, 2001	−
Anabaena sp.	1tu34s7	Lake Tuusulanjärvi, Finland, 2001	−
Anabaena sp.	0tu37s9	Lake Tuusulanjärvi, Finland, 2000	−
Anabaena sp.	0tu39s7	Lake Tuusulanjärvi, Finland, 2000	−
An. spiroides	1tu39s17	Lake Tuusulanjärvi, Finland, 2001	−
Aphanizomenon			
Ap. flos-aquae	1tu29s19	Lake Tuusulanjärvi, Finland, 2001	−
Ap. flos-aquae	1tu37s13	Lake Tuusulanjärvi, Finland, 2001	−
Ap. flos-aquae	1tu26s2	Lake Tuusulanjärvi, Finland, 2001	−
Ap. gracile	Heaney/Camb 1986 140 1/1	Freshwater, Lough Neagh, Ireland, 1986	−
Ap. gracile	1tu26s16	Lake Tuusulanjärvi, Finland, 2001	−
Ap. issatschenkoi	0tu37s7	Lake Tuusulanjärvi, Finland, 2000	−
Nostoc			
N. calcicola	III[b]	Field, České Budějovice, Czech Republic, 1989	−
N. calcicola	VI[b]	Field, Dobré Pole, Czech Republic, 1998	−
N. edaphicum	X[b]	Field, Chelčice, Czech Republic, 1989	−
N. ellipsosporum	V[b]	Field, Nezamyslice, Czech Republic, 1990	−
N. muscorum	I[b]	Field, Dlouhá Ves, Czech Republic, 1986	−
N. muscorum	II[b]	Field, Jevany, Czech Republic, 1985	−
Nostoc sp.	1tu14s8	Lake Tuusulanjärvi, Finland, 2001	−

Table 1. cont.

Taxonomic assignment	Strain	Geographical origin and year of isolation	*mcyE* PCR*
Trichormus			
T. azollae	BAI/1983	Unknown, 1983	−
T. doliolum	1	Unknown	−
T. variabilis	GREIFSWALD	Unknown, 1992	−
T. variabilis	HINDAK 2001/4	Soil, Dombay valley, Caucasus mountains, Russian Federation, 2001	−

*Determined by PCR with *mcyE* gene-specific primers.
†Strain described previously by Hrouzek *et al.* (2003) (*a*) or Zapomělová (2004) (*b*).

PCR and sequencing. The 16S rRNA gene and ITS region were amplified with primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards *et al.*, 1989) and B23S (5′-CTTCGCCTCTGTGTGCCT-AGGT-3′) (Lepère *et al.*, 2000) as described in Gkelis *et al.* (2005). The 16S rRNA gene (1432–1439 bp) was sequenced with internal sequencing primers 16S545R, 16S1092R and 16S979F. The *rbcLX* gene region was amplified and sequenced (782–1003 bp) with primers CX (5′-GGCGCAGGTAAGAAAGGGTTTCGTA-3′) and CW (5′-CGTAGCTTCCGGTGGTATCCACGT-3′) as described by Rudi *et al.* (1998). Despite several trials, amplification of *N. muscorum* strains was not successful and sequences were not obtained. Amplification of the partial *rpoB* gene was performed with primer pair rpoBF (5′-GTAGTTGTARCCNTCCCA-3′) and rpoBR (5′-RCMGCMGACGA-AGAAGACG-3′) or primer pair rpoBanaF (5′-AGCMACMGGTG-ACGTTCC-3′) and rpoBanaR (5′-CNTCCCARGGCATATAGGC-3′), which were designed in this study. For the rpoBF–rpoBR primer pair, amplification was carried out in 50 µl 1× DyNAzyme buffer containing 1·6 U DyNAzyme polymerase (Finnzymes), 0·2 mM each dNTP, 0·2 µM primers and 0·5 µl target DNA. PCR amplification consisted of initial denaturation for 5 min at 94 °C, 30 cycles of amplification: 1 min at 94 °C, 1·5 min at 50 °C and 2 min at 72 °C, and a final elongation for 7 min at 72 °C. For the rpoBanaF–rpoBanaR primer pair, 0·8 U Super Taq Plus polymerase (HT Biotechnology LTA) and 1× Super Taq Plus buffer (HT Biotechnology LTA) replaced DyNAzyme polymerase and buffer, 1 mg BSA ml^{-1} was added and elongation steps of PCR were performed at 68 °C. The *rpoB* fragments of strains 1tu28s8, 1tu33s10, 1tu30s13 and 1tu33s12 were cloned with InsT/Aclone PCR product cloning kit (Fermentas) in order to get high-quality sequences. The *rpoB* gene fragment (520–635 bp) was sequenced with the primers used in the amplification. In addition to studied strains, 13 reference strains, *Anabaena* sp. PCC 7108, 14, 123 and 277; *Anabaena flos-aquae* 202A1, *Anabaena lemmermannii* 66A, *Aphanizomenon* sp. TR183 and 202; *Aphanizomenon flos-aquae* PCC 7905, *Nostoc punctiforme* PCC 72102 and *Nodularia* sp. HEM, HKVV and PCC 7804, were amplified and sequenced. Sequencing of all genes was performed with an Applied Biosystems Big Dye Terminator cycle sequencing kit and 3700 sequencer at Genome Express (Meylan, France) or with an Applied Biosystems PRISM 310 sequencer according to the manufacturer's instructions. The potential microcystin production of strains was based on the detection of the *mcyE* gene in PCR with the specific primers mcyE-F2 and mcyE-R4 as described by Rantala *et al.* (2004).

Phylogenetic analysis. Sequences were aligned in the program ARB (http://www.arb-home.de). The alignment was edited manually and ambiguous bases and hypervariable regions were removed. The highly variable intergenic spacer region between *rbcL* and *rbcX* genes as well as the variable indel region in *rpoB* (positions 322–477 in the alignment) did not allow reliable alignment, and therefore these regions were excluded from the analysis. The *rbcLX* and *rpoB* sequences were studied based on three datasets: one containing all codon positions, one containing only the first and second codon positions of *rbcLX* or *rpoB* genes and a third containing translated amino acid sequences. Only minor differences were found in a comparison of the datasets and the few conflicting nodes had bootstrap support below 65 %. Resolution of clusters and bootstrap values were higher when the third codon positions were included. Therefore, the analysis of *rpoB* and *rbcLX* shown here included all codon positions of the genes. Altogether, 1393 bp of the 16S rRNA gene, 606 bp of *rbcLX* and 451 bp of *rpoB* were used for sequence analysis. Trees based on the 16S rRNA gene, *rbcLX* and *rpoB* were constructed by neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-parsimony (MP) algorithms in the program PAUP* v10b (Swofford, 2003) and by the maximum-likelihood (ML) algorithm in PHYLIP v3.6 (Felsenstein, 1993). For NJ, the evolutionary model of substitution was evaluated by the program MODELTEST v.3.06 (Posada & Crandall, 1998). The GTR+I+G, TrN+I+G and SYM+I+G evolutionary models of substitution were found to fit the data best for the 16S rRNA gene, *rbcLX* and *rpoB*, respectively. The parameters (base frequencies, rate matrix of substitution types and shape of gamma distribution) were estimated from the data. For NJ and MP analysis, 1000 bootstrap replicates were performed. For ML analysis, only 10 bootstrap replicates were performed due to limited computing power. In addition to these analyses, GTR+G+I evolutionary distances based on the 16S rRNA gene were analysed non-hierarchically with PCA in the program CAP (PISCES). Kishino–Hasegawa (Kishino & Hasegawa, 1989), Templeton (Templeton, 1983) and Winning-Sites tests (Prager & Wilson, 1988) were used to compare the alternative phylogenetic trees based on 16S rRNA gene sequences. The monophyly of planktic *Anabaena*/*Aphanizomenon*, only *Aphanizomenon* and only planktic *Anabaena* sequences as well as subclusters A, F and G were tested as implemented in PAUP* v10b (Swofford, 2003).

RESULTS AND DISCUSSION

Phenotypic analysis of the strains

The 51 strains studied were morphologically heterogeneous. Most of them were planktic *Anabaena* and *Aphanizomenon* strains that were identified as belonging to three *Aphanizomenon* species, *Aphanizomenon flos-aquae* Ralfs ex Born. et Flah., *Aphanizomenon gracile* (Lemm.) Lemm. and *Aphanizomenon issatschenkoi* (Usač) Prošk.-Lavr., and 10 *Anabaena* species: *Anabaena crassa* (Lemm.) Kom.-Legn. et CronB., *Anabaena circinalis* Rabenh. ex Born.

Table 2. Selected morphological characteristics of the studied cyanobacterial strains

The morphology of *Nostoc muscorum*, *N. calcicola*, *N. ellipsosporum* and *N. edaphicum* was described previously by Hrouzek *et al.* (2003).

Strain	Gas vesicles	Shape of terminal cell	Trichome width (μm)*	Akinete			Akinete–heterocyte distance†
				Shape	Width (μm)*	Length (μm)*	
An. cf. *circinalis* var. *macrospora*							
1tu23s3	+	Rounded	5·0 (5·4, 4·4)	Cylindrical with rounded ends	6·9 (7·9, 4·5)	16·1 (21·9, 13·2)	ND
1tu26s10	+	Rounded	4·5 (4·9, 4·0)	Oval	6·3 (7·6, 4·5)	18·6 (26·6, 13·3)	ND
1tu27s5	+	Rounded	4·7 (5·1, 4·1)	Oval	5·9 (7·1, 5·2)	20·4 (24·6, 13·3)	ND
1tu28s13	+	Rounded	3·8 (5·4, 1·7)	Cylindrical	5·5 (8·5, 7·0)	23·0 (37·0, 9·9)	0–1
0tu25s6	+	Rounded	4·9 (5·4, 4·4)	Oval	6·5 (8·2, 5·8)	18·5 (24·3, 12·4)	ND
An. cf. *crassa* 1tu27s7	+	Rounded	6·8 (8·8, 5·0)	Not observed	–	–	–
An. circinalis							
1tu34s5	+	Rounded	8·0 (10·1, 7·8)	Oval	12·3 (14·0, 9·7)	21·0 (30·0,12·0)	0–2
1tu30s11	+	Rounded	8·0 (9·0, 7·4)	Not observed	–	–	–
1tu33s12	+	Rounded	8·2 (9·1, 7·3)	Not observed	–	–	–
An. compacta ANACOM-KOR‡	+	Rounded	4·6 (6·2, 2·9)	Rounded to oval	10·0 (14·8, 5·2)	11·5 (17·3, 5·2)	ND
An. flos-aquae							
1tu31s11	+	Rounded	4·9 (6·3, 4·0)	Rounded to oval	7·7 (8·5, 6·3)	13·9 (15·0, 10·0)	1–2
0tu33s15	+	Hyaline	6·1 (7·6, 3·2)	Oval, slightly curved	8·7 (11·7, 7·1)	17·6 (22·0, 13·5)	ND
0tu33s2a	+	Hyaline	5·6 (6·5, 5·2)	Oval	7·2 (7·9, 6·7)	15·9 (18·6, 11·6)	ND
1tu30s4	+	Hyaline	4·6 (5·3, 4·0)	Not observed	–	–	–
1tu35s12	+	Hyaline	5·1 (5·5, 4·7)	Not observed	–	–	–
An. lemmermannii 1tu32s11	+	Rounded	5·0 (5·0, 3·0)	Oval to cylindrical	6·3 (8·5, 7·0)	11·2 (16·3, 12·0)	ND
An. mucosa 1tu35s5	+	Rounded	9·0 (10·1, 8·2)	Spherical	14·1 (16·2, 12·5)	10·0 (18·0, 13·3)	ND
An. planctonica							
1tu33s10	+	Rounded	5·6 (7·1, 4·1)	Oval to rounded	10·9 (12·8, 9·7)	19·1 (23·5, 13·7)	ND
1tu28s8	+	Rounded	6·2 (6·8, 5·6)	Oval	11·4 (13·7, 10·0)	18·8 (23·7, 15·5)	ND
1tu30s13	+	Rounded	6·3 (11·0, 5·6)	Oval	12·3 (15·0, 9·1)	21·7 (27·0, 13·0)	0–6
1tu33s8	+	Rounded	6·5 (6·9, 5·9)	Not observed	–	–	–
1tu36s8	+	Rounded	5·4 (10·0, 5·2)	Oval to rounded	15·2 (19·1, 12·0)	17·6 (27·6, 12·0)	0–6
An. sigmoidea							
0tu36s7	+	Rounded	4·9 (5·8, 3·8)	Oval	8·5 (9·5, 7·6)	18·1 (20·2, 15·9)	ND
0tu38s4	+	Rounded	4·0 (4·3, 3·8)	Oval, slightly curved	6·0 (6·8, 4·3)	14·1 (15·5, 12·4)	ND
An. smithii 1tu39s8	+	Rounded	9·3 (12·0, 7·2)	Oval to rounded	17·4 (22·4, 13·3)	20·6 (34·2, 15·7)	0–6
Anabaena sp. 0tu37s9	+	Rounded	7·3 (8·4, 6·9)	Not observed	–	–	–
Anabaena sp. 0tu39s7	+	Rounded	7·3 (9·0, 6·5)	Not observed	–	–	–
An. spiroides 1tu39s17	+	Rounded	7·5 (8·4, 6·4)	Oval	11·8 (19·5, 9·5)	18·8 (23·0, 11·5)	ND
Ap. flos-aquae							
1tu29s19	+	Hyaline	4·2 (5·9, 2·9)	Cylindrical	5·9 (8·0, 4·3)	26·6 (39·0, 17·0)	3–12
1tu37s13	+	Hyaline	4·0 (5·4, 2·7)	Cylindrical	7·3 (9·2, 6·1)	62·5 (91·0, 37·0)	10–12
1tu26s2	+	Hyaline	4·5 (5·3, 3·3)	Cylindrical	6·1 (7·9, 4·9)	38·0 (78·2, 7·3)	10–12
Ap. gracile							
Heaney/Camb1986 140 1/1	+	Tapered	4·5 (7·5, 2·5)	Cylindrical	7·2 (12·5, 5·0)	39·5 (95·0, 20·0)	0
1tu26s16	+	Tapered	3·7 (4·4, 2·7)	Cylindrical	5·0 (5·0, 5·0)	11·6 (12·5, 11·3)	2–5
Ap. issatschenkoi 0tu37s7	+	Elongated, pointed	3·1 (4·0, 2·0)	Cylindrical	4·5 (5·5, 3·3)	17·7 (25·5, 10·9)	5–11

Table 2. cont.

Strain	Gas vesicles	Shape of terminal cell	Trichome width (μm)*	Akinete			Akinete–heterocyte distance†
				Shape	Width (μm)*	Length (μm)*	
An. augstumalis SCMIDKE JAHNKE/4a	–	Conical	3·0 (6·1, 1·5)	Oval	7·0 (9·2, 5·3)	18·3 (22·0, 13·0)	0–1
An. cf. *cylindrica* XP6B	–	Rounded	4·4 (5·4, 3·3)	Not observed	–	–	–
An. oscillarioides							
BECID22	–	Rounded	4·2 (5·4, 3·4)	Oval	6·6 (7·9, 5·7)	11·1 (21·0, 11·0)	0
BECID32	–	Rounded	5·5 (2·2, 3·3)	Oval	6·8 (7·9, 5·0)	15·0 (21·0, 11·3)	0
BO HINDAK 1984/43	–	Conical	3·8 (5·2, 2·3)	Not observed	–	–	–
Anabaena sp. 1tu34s7	–	Rounded	2·6 (4·5, 1·2)	Oval	4·5 (6·9, 2·1)	10·2 (15·5, 4·5)	2–10
Anabaena sp. 277	–	Rounded	5·1 (6·6, 3·6)	Not observed	–	–	–
Nostoc sp. 1tu14s8	–	Rounded	3·2 (4·2, 2·3)	Oval	4·1 (5·5, 3·4)	3·2 (4·3, 2·6)	2–10
T. azollae BAI/1983	–	Rounded	4·0 (5·3, 1·4)	Oval	7·5 (7·5, 7·5)	14·5 (15·0, 12·5)	0–14
T. doliolum 1	–	Conical	2·3 (3·0, 1·6)	Lenticular	2·7 (3·7, 1·9)	4·6 (6·5, 3·5)	4–10
T. variabilis							
GREIFSWALD	–	Conical	4·3 (7·7, 2·2)	Oval, slightly compressed in the middle	7·4 (7·4, 7·4)	13·1 (14·8, 12·3)	0–7
HINDAK 2001/4	–	Conical	6·7 (9·6, 2·1)	Oval, slightly compressed in the middle	6·9 (7·4, 5·0)	11·3 (14·8, 8·0)	0–10

*Numbers are means (maximum and minimum values).
†Presented as a number of cells between heterocyte and akinete.
‡Described by Zapomělová (2004).

et Flah., *Anabaena planctonica* Brunnth., *Anabaena mucosa* Kom.-Legn. et Eloranta, *Anabaena spiroides* Kleb., *Anabaena smithii* (Kom.) M. Watan., *Anabaena sigmoidea* Nyg., *Anabaena flos-aquae* [Lyngb.] Bréb. ex Born. et Flah., *Anabaena* cf. *circinalis* var. *macrospora* and *Anabaena lemmermannii* Richt., according to traditional morphological criteria (Geitler, 1932; Desikachary, 1959; Komárek & Anagnostidis, 1989). In addition, nine benthic strains were identified as *Anabaena oscillarioides* Bory ex Born. et Flah., *Anabaena* cf. *cylindrica* Lemm., *Anabaena augstumalis* Scmidle, *Trichormus variabilis* (Born. et Flah.) Kom. et Anag., *Trichormus azollae* (Strasb.) Kom. et Anag., *Trichormus doliolum* (Bharadw.) Kom. et Anag. and *Nostoc* sp. according to traditional morphological criteria. Heterocytes were present in all strains, whereas akinetes were not observed in 12 of the strains studied (Table 2). *Aphanizomenon flos-aquae* strains lost their fascicle-like colony structure during laboratory cultivation. The morphological characteristics of the strains are summarized in Table 2. Other morphological characters measured are available as supplementary material in IJSEM Online. Microphotographs of the selected strains and their important features are shown in Figs 1 and 2.

Aphanizomenon and *Anabaena* strains differed significantly by the mean width of vegetative cells, although the width of vegetative cells of some *Anabaena* (e.g. *Anabaena* cf. *circinalis* var. *macrospora* 1tu28s13) and *Aphanizomenon* strains was overlapping. Generally, the variability in width and length of vegetative cells, heterocytes and akinetes was high (Table 2), which complicates the use of these characters for identification and separation of *Anabaena* and *Aphanizomenon*. These genera were also distinguished by the morphology of the end cells of the trichome which was rounded to oval in planktic *Anabaena* strains and elongated-hyaline to tapered in *Aphanizomenon* strains.

Aphanizomenon strains had more or less straight trichomes, slightly constricted at the cross-walls, and their vegetative cells were from barrel-shaped to cylindrical (Fig. 1). The *Aphanizomenon issatschenkoi* strain was clearly distinguishable from the other *Aphanizomenon* strains by elongated and pointed terminal cells (Table 2; Fig. 1j, k). *Aphanizomenon flos-aquae* strains were characterized by hyaline end cells, cylindrical and long akinetes (up to 91 μm) and a long distance between the heterocytes and akinetes (Table 2; Fig. 1d, e). *Aphanizomenon gracile* trichomes were slightly tapered, but not pointed, and differed from *Aphanizomenon flos-aquae* by the absence of long hyaline end cells and shorter akinetes (Table 2; Fig. 1i, h).

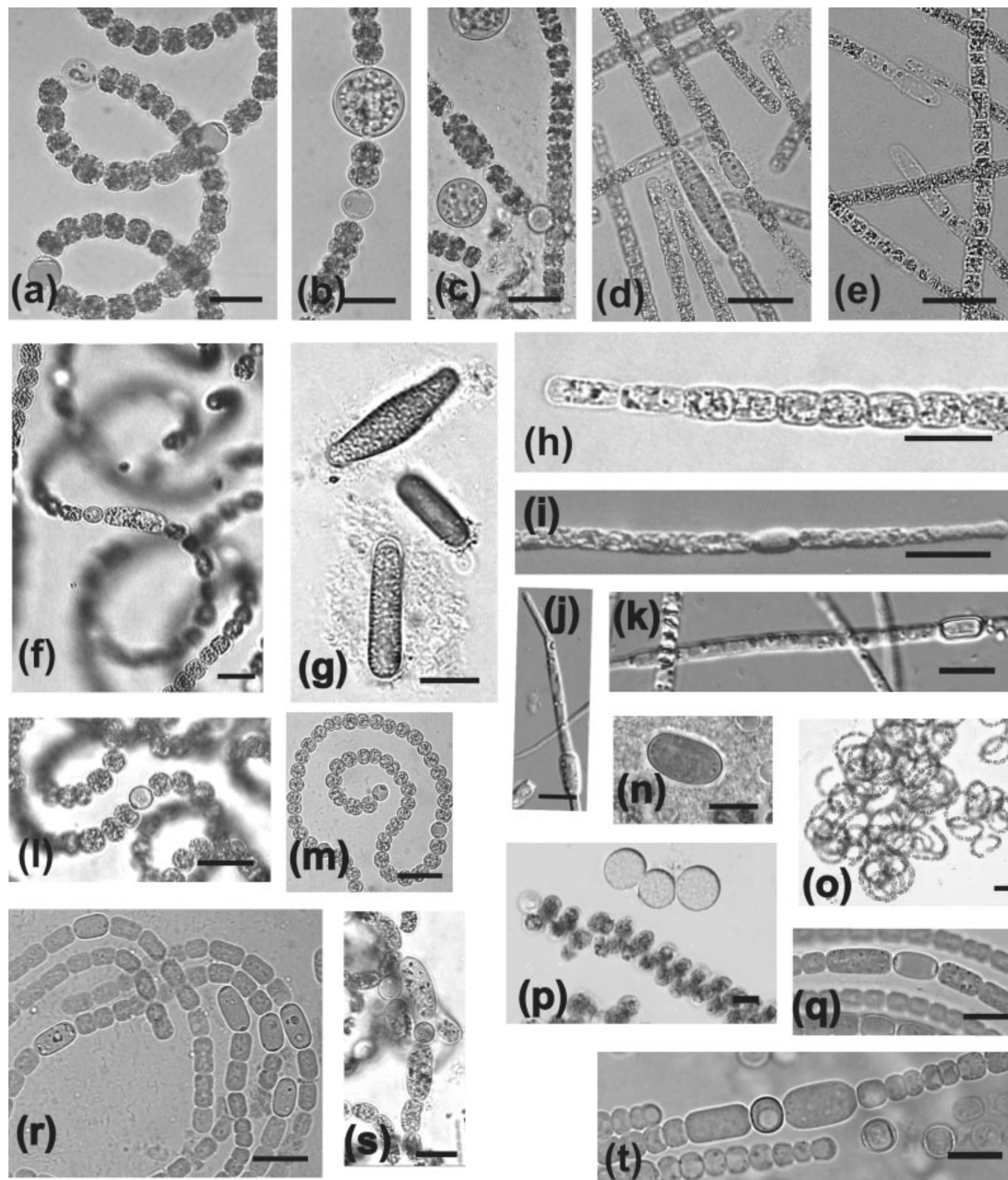

Fig. 1. Microphotographs of some *Anabaena* and *Aphanizomenon* strains in phylogenetic cluster 1, showing important features of the strains. (a) *Anabaena* cf. *crassa* 1tu27S7; (b) *Anabaena planctonica* 1tu30s13; (c) *Anabaena smithii* 1tu39s8; (d) *Aphanizomenon flos-aquae* 1tu26s2; (e) *Aphanizomenon flos-aquae* 1tu37S13; (f) *Anabaena* cf. *circinalis* var. *macrospora* 1tu28s13; (g) akinetes of *Anabaena* cf. *circinalis* var. *macrospora* 1tu23s3; (h) *Aphanizomenon gracile* 1tu26s16; (i) *Aphanizomenon gracile* 1tu26s16; (j) akinete of *Aphanizomenon issatschenkoi* 0tu37s7; (k) *Aphanizomenon issatschenkoi* 0tu37s7; (l) *Anabaena flos-aquae* 1tu33s2a; (m) *Anabaena circinalis* 1tu30s11; (n) akinete of *Anabaena flos-aquae* 1tu33s2a; (o) *Anabaena lemmermannii* 1tu32s11; (p) *Anabaena compacta* ANACOM-KOR; (q) *Anabaena oscillarioides* BECID23; (r) *Anabaena* cf. *cylindrica* XP6B; (s) akinete of *Anabaena lemmermannii* 1tu32s11; (t) *Anabaena oscillarioides* BECID22. Bars, 10 µm.

Anabaena strains can be divided into two groups according to their habitats (Table 2). Planktic species had gas vesicles, which were absent in benthic species. *Anabaena* sp. 1tu34s7, which was isolated from the plankton, did not have gas vesicles and we suspect that it was of benthic origin. In planktic *Anabaena* strains, trichomes varied from coiled to straight (Fig. 1) and two morphological groups were recognized according to the trichome width. Strains of *Anabaena* cf. *crassa*, *Anabaena circinalis*, *Anabaena planctonica*, *Anabaena spiroides*, *Anabaena smithii*, *Anabaena mucosa* and *Anabaena sigmoidea* had significantly ($P<0{\cdot}05$) wider trichomes, heterocytes and akinetes than strains of *Anabaena flos-aquae*, *Anabaena lemmermannii* and *Anabaena* cf. *circinalis* var. *macrospora* (Table 2).

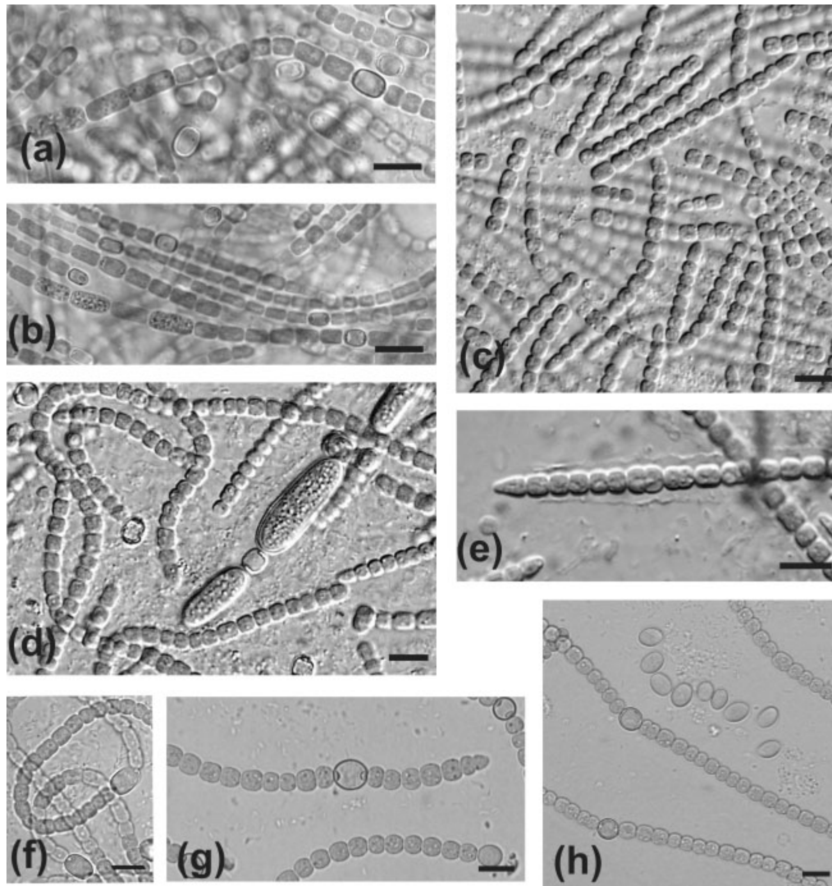

Fig. 2. Microphotographs of some *Anabaena* and *Trichormus* strains in phylogenetic clusters 2–6, showing important features of the strains. (a) *T. variabilis* HINDAK 2001/4; (b) *T. variabilis* GREIFSWALD; (c) *Anabaena* cf. *oscillarioides* BO HINDAK 1984/43; (d) and (e) *Anabaena augstumalis* SCHMIDKE JAHNKE/4a; (f) *T. azollae* BAI/1983; (g) and (h) *T. doliolum* 1. Bars, 10 μm.

However, some transition types with overlapping values were found (e.g. *Anabaena circinalis* and *Anabaena flos-aquae*) (Table 2). Benthic *Anabaena* strains had flexuous trichomes with a diffuse mucilaginous sheath and typical oval morphology of akinetes and terminal heterocytes (Fig. 2).

The genus *Trichormus* was morphologically variable. The special lenticular-shaped akinetes separated *T. doliolum* from all other investigated strains (Fig. 2h). Both strains of *Trichormus variabilis* were morphologically similar, with long wavy filaments and apoheterocytic development of akinetes (Fig. 2a, b). *Trichormus azollae*, a cyanobiont of *Azolla* fern, was morphologically related to the *Nostoc* strains. Similarly to some *Nostoc* strains (*N. muscorum*, *N. ellipsosporum*), it formed long, irregularly coiled trichomes surrounded by a diffuse mucilaginous envelope (Fig. 2f). In addition, the type of akinete development (starting from the middle of the filament between two heterocytes) was similar to that of several *Nostoc* strains. Morphology of the *Nostoc* strains was previously described by Hrouzek *et al.* (2003).

Our results indicated that parameters of akinetes were important taxonomic characters. PCA of all morphological characters showed that most of the total variance is attributable to variance in width and length of akinetes (Fig. 3). Moreover, the shape of the akinetes was quite

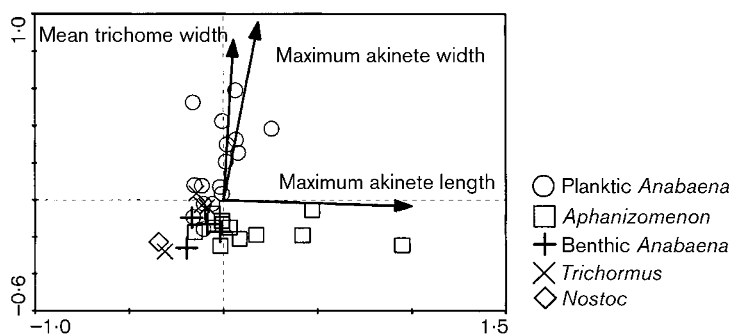

Fig. 3. PCA plot based on the morphological characteristics of studied cyanobacterial strains. The most variable characteristics were length and width of akinetes as well as width of trichomes (shown by the arrows). The first and second principal components accounted for 99 % of the total variance.

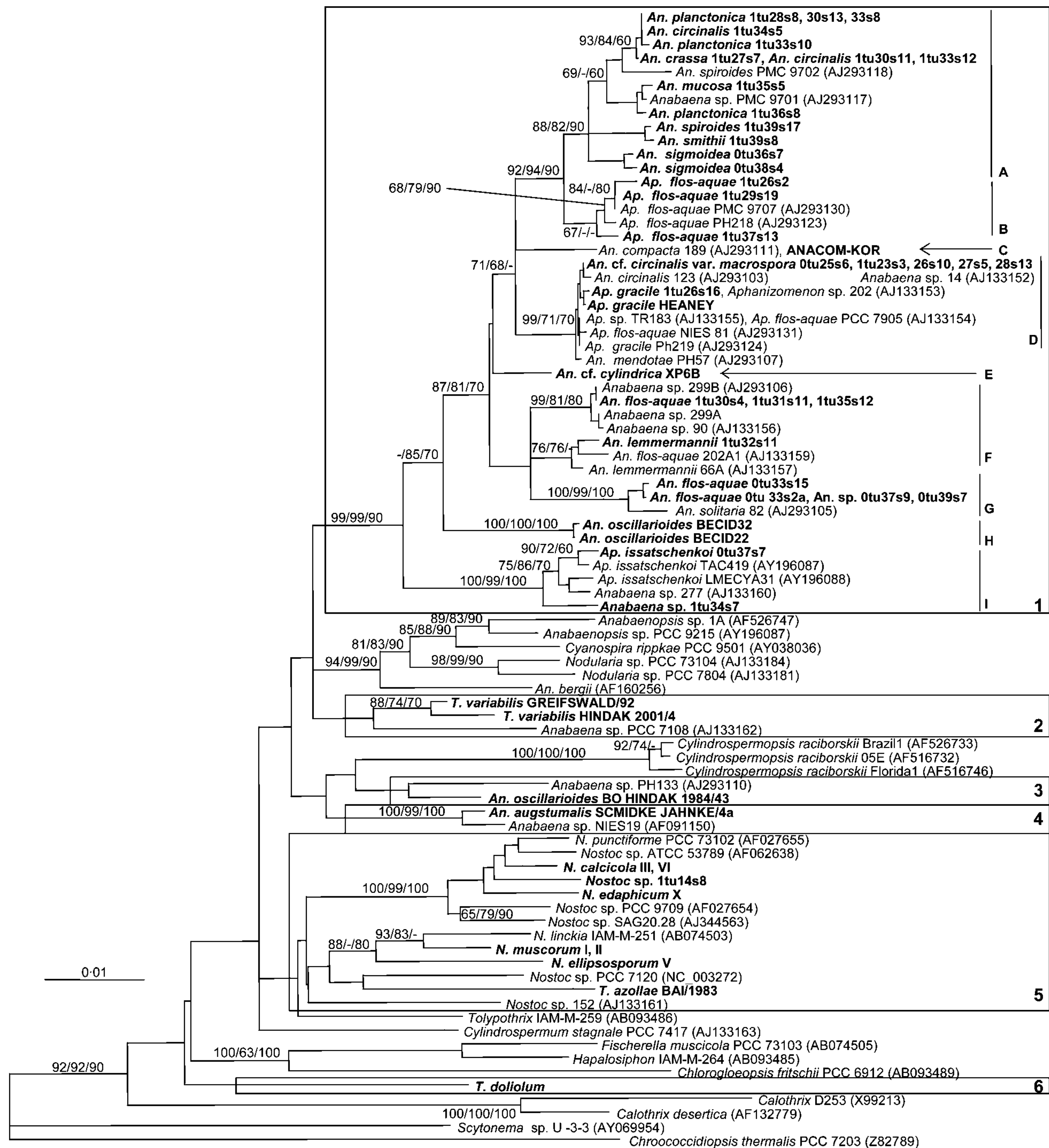

Fig. 4. Neighbour-joining tree based on 16S rRNA gene sequences (1393 bp) showing the clustering of studied *Anabaena* (abbreviated as *An.*), *Aphanizomenon* (*Ap.*), *Trichormus* (*T.*) and *Nostoc* (*N.*) strains (in bold). Numbers near nodes indicate bootstrap values over 65 % for NJ, MP and ML analyses.

stable. Previously, Stulp & Stam (1982, 1985) found the position of akinetes, shape of terminal cells and width of vegetative cells to be useful taxonomic characters for *Anabaena*. These morphological features were retained in different light and temperature conditions (Stulp & Stam, 1985) and even brackish water conditions (Stulp & Stam, 1984a).

Genetic relationships of the studied strains

Six clusters were consistently formed in the analysis of 16S rRNA, *rpoB* and *rbcLX* genes: cluster 1 contained all planktic *Anabaena* and *Aphanizomenon* strains as well as five benthic *Anabaena* strains; clusters 2, 3, 4 and 6 contained benthic strains *T. variabilis*, *Anabaena oscillarioides*

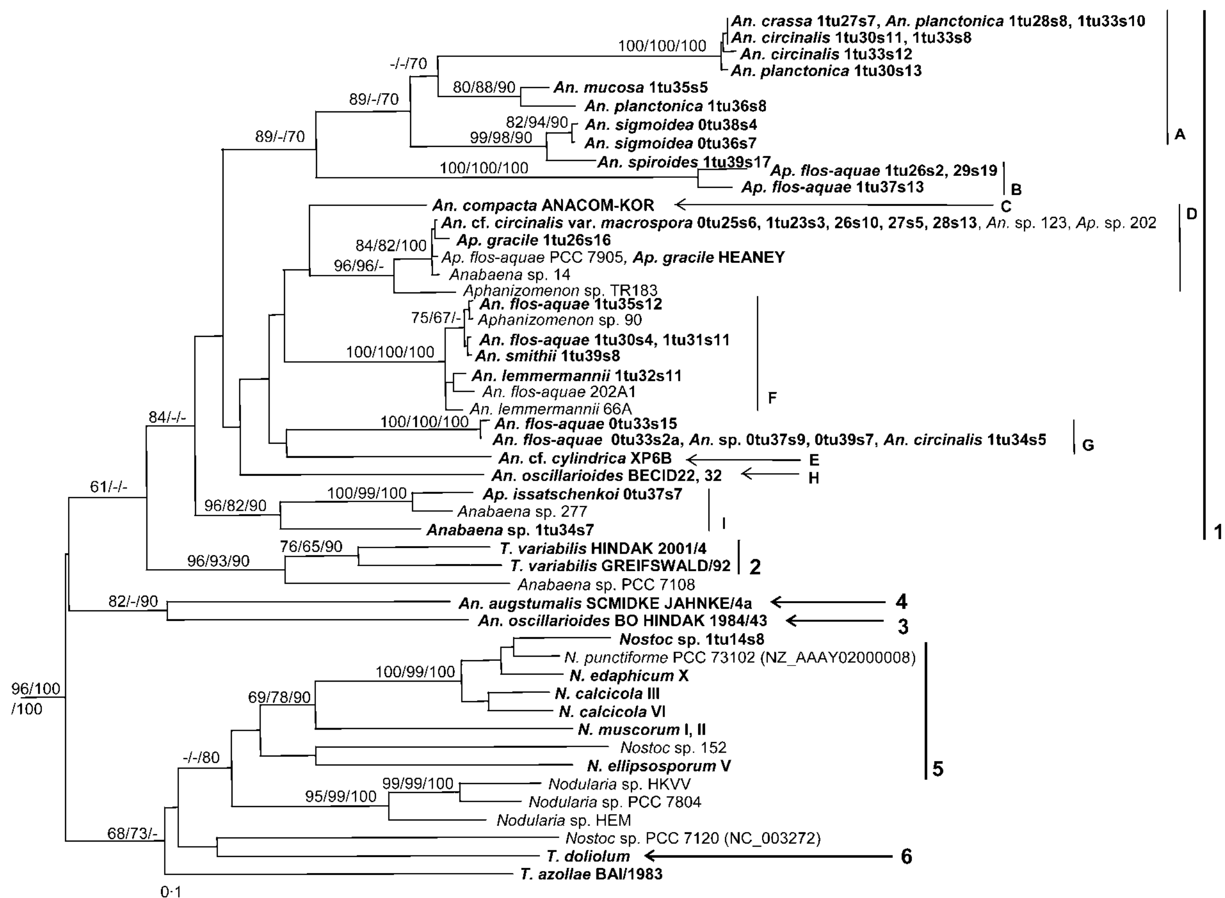

Fig. 5. Neighbour-joining tree based on *rpoB* sequences (451 bp) showing the clustering of studied *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* strains (in bold). Numbers near nodes indicate bootstrap values over 65 % for NJ, MP and ML analyses. The outgroup taxa, *Microcystis* sp. 130 and *Planktothrix* NIVA-CYA126, are not shown.

BO HINDAK 2001/4, *Anabaena augstumalis* and *T. doliolum*, respectively; and cluster 5 contained all *Nostoc* strains and *T. azollae.* The tree topologies were similar with all tree-constructing methods, and therefore only the NJ tree is presented for each gene (Figs 4, 5 and 6). The overall topology was in agreement for the 16S rRNA gene, *rbcLX* and *rpoB* trees. However, within closely related *Anabaena* strains, some differences were found: *Anabaena smithii* 1tu39s8 clustered with hepatotoxic *Anabaena* strains in the *rpoB* tree and *Anabaena circinalis* 1tu34s5 with *Anabaena flos-aquae* strains in the *rpoB* and *rbcLX* trees instead of subcluster A (Figs 4, 5 and 6). Otherwise, the few conflicting nodes between gene trees received only low bootstrap support. These were generally lower in *rbcLX* and *rpoB* trees than in the 16S rRNA gene tree, probably because of a smaller number of variable bases. Rudi *et al.* (1998) also found that the topologies of the 16S rRNA gene and *rbcLX* trees were not congruent for genetically closely related *Nostoc* and *Anabaena* strains and stated that this was due to lateral gene transfer between the strains. Nevertheless, in the present study, lateral gene transfer did not seem to play a major role in determining the topologies of gene trees.

Since hierarchical clustering of 16S rRNA, *rpoB* and *rbcLX* gene sequences did not receive high bootstrap support for all the clusters and subclusters of all gene trees, PCA was performed. All clusters were also found in PCA, confirming the validity of the hierarchical clustering (data not shown). Principal components 1 and 2 together accounted for 84·6 % of the total variance in the 16S rRNA distance matrix.

Genetic data did not support the distinction of planktic *Anabaena* and *Aphanizomenon* from benthic *Anabaena* strains as did the morphological data. The benthic *Anabaena* strains BECID22, BECID32, XP6B, 1tu34s7 and 277, which lack visible gas vesicles, were intermixed with planktic *Anabaena* and *Aphanizomenon* strains in 16S rRNA, *rpoB* and *rbcLX* gene trees with high bootstrap support (cluster 1 in Figs 4, 5 and 6). Moreover, a 16S rRNA gene tree with the forced monophyly of planktic *Anabaena*/*Aphanizomenon* strains was significantly worse ($P = 0{\cdot}0010\text{–}0{\cdot}0013$) than the original tree in all tests performed. Thus, these tests did not support the separation of planktic and benthic *Anabaena*/*Aphanizomenon* strains. This finding contradicts

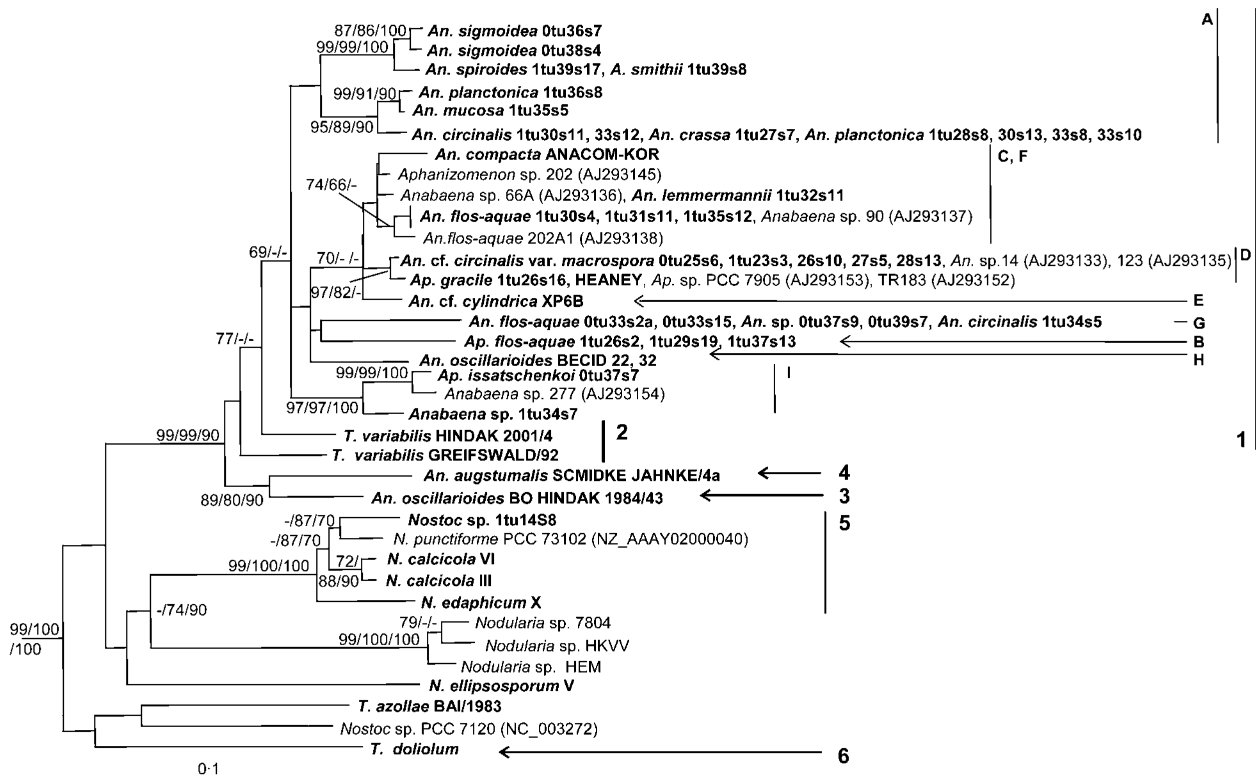

Fig. 6. Neighbour-joining tree based on *rbcLX* sequences (606 bp) showing the clustering of studied *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* strains (in bold). Numbers near nodes indicate bootstrap values over 65 % for NJ, MP and ML analyses. The outgroup taxa, *Microcystis* sp. 130 (Z94894) and *Planktothrix* NIVA-CYA126 (Z94873), are not shown.

Iteman *et al.* (2002), who found a distinct subcluster of planktic heterocytous cyanobacteria with the exception of *Cylindrospermopsis*. Other benthic *Anabaena*/*Trichormus* strains were placed outside cluster 1, which contained all planktic and five benthic *Anabaena* as well as all *Aphanizomenon* strains.

Anabaena and *Aphanizomenon* strains in cluster 1 were genetically heterogeneous (16S rRNA gene sequence similarity >94·8 %) and intermixed in all gene trees (cluster 1 in Figs 4, 5 and 6), confirming the results of earlier studies with other *Anabaena* and *Aphanizomenon* strains (Lyra *et al.*, 2001; Gugger *et al.*, 2002b; Iteman *et al.*, 2002). In addition, the monophyly of *Aphanizomenon* or planktic *Anabaena* was rejected in all statistical tests ($P=0{\cdot}0001$). The monophyly of *Aphanizomenon* strains was proposed in a recent paper of Li *et al.* (2003), but in that study planktic *Anabaena* sequences were not included in the analysis.

Cluster 1, containing all planktic and five benthic *Anabaena* as well as all *Aphanizomenon* strains, was divided into nine subclusters, which mostly received high bootstrap support in all the gene trees (subclusters A–I in Figs 4, 5 and 6). Morphologically, the strains in this cluster were separated from the other strains including benthic strains in clusters 2–4 by absence of terminal heterocysts. The cut-off points 97·5 and 95 % 16S rRNA gene sequence similarity have been suggested for bacterial species and genus definition, respectively (Stackebrandt & Goebel, 1994; Ludwig *et al.*, 1998). According to those definitions, the evolutionary distances of this study suggested that the strains in cluster 1 could be divided into two to three species belonging to a single genus (Table 3). Cluster 1 could also be divided into more than three species (up to nine) according to the

Table 3. Matrix showing *P*-distances based on the 16S rRNA gene (1386 bp) between the subclusters of cluster 1

Subcluster	A	B	C	D	E	F	G	H	I
A	98·6								
B	99·1	99·4							
C	98·3	98·2	100						
D	98·4	98·8	98·5	99·8					
E	98·0	98·0	98·9	98·6	–				
F	97·7	98·0	98·8	98·1	99·2	98·8			
G	96·8	97·0	97·4	97·6	97·9	98·4	99·4		
H	96·5	96·8	97·4	96·9	98·2	98·0	98·0	99·9	
I	95·4	95·7	96·3	96·3	96·6	97·1	96·7	97·0	98·8

subclustering of strains in phylogenetic trees and morphological data presented here, although the evolutionary distances between subclusters were $>97 \cdot 5\%$. Most of the phylogenetic subclusters found in all the gene trees were also defined by morphological characters. In addition, the evolutionary distances between the subclusters were discontinuous (Table 3). The subclustering was also reflected in the length and the sequence of the variable indel region of the *rpoB* gene and was confirmed in the PCA of the 16S rRNA gene (data not shown). In addition, the strains in each subcluster shared a similar denaturing-gradient gel electrophoresis (DGGE) pattern of 16S rRNA gene fragments (450 bp) (unpublished results). The studied strains had from one to six different copies of the 16S rRNA gene according to DGGE analysis. These findings further support the subclustering of *Anabaena*/*Aphanizomenon* strains. However, the clustering of the studied *Anabaena* and *Aphanizomenon* strains did not follow the current classification of the genera. In future, DNA–DNA reassociation studies will be able to give more evidence for relationships of studied strains at the species level. Previously, Stam & Stulp (1984b, 1985) found in DNA–DNA reassociation studies that the morphology of *Anabaena* strains was reflected in their genetic relationships. However, our studies indicated that the classification of *Anabaena* strains might be more complicated.

The highly supported subcluster A contained various planktic *Anabaena* strains, *Anabaena planctonica*, *Anabaena crassa*, *Anabaena mucosa*, *Anabaena spiroides*, *Anabaena smithii* and *Anabaena sigmoidea* (Figs 4, 5 and 6), which shared relatively high 16S rRNA gene sequence similarity ($>98 \cdot 6\%$). Both irregularly coiled *Anabaena* strains such as *Anabaena circinalis* and straight *Anabaena* species such as *Anabaena planctonica* strains were included in subcluster A, indicating that coiling of the trichome was not useful for classification of *Anabaena* morphotypes. The strains were otherwise morphologically relatively similar to each other and to *Anabaena* strains in subcluster F and G. Nevertheless, a few morphological differences were found between these subclusters. Strains in subcluster A had significantly wider heterocytes, akinetes and trichomes than other *Anabaena* strains in cluster F and G. Thus, the close relationship and shared morphological features suggest that the strains in subcluster A might be related at the species level.

Aphanizomenon flos-aquae strains were included in subcluster B in all the gene trees, although the bootstrap support was low in MP and ML analysis of the 16S rRNA gene (Figs 4, 5 and 6). These strains were closely related and shared identical *rbcLX* gene sequences and highly similar 16S rRNA ($>99 \cdot 4\%$) and *rpoB* ($>99 \cdot 8\%$) genes. Also, in the study of Gugger *et al.* (2002b) a cluster of mainly *Aphanizomenon flos-aquae* sequences was found in 16S rRNA gene and *rbcLX* trees, but it was not resolved in the ITS tree. The studied *Aphanizomenon flos-aquae* strains shared several morphological features, e.g. hyaline end cells, long akinetes and originally fascicle-like colonies, which separate these strains from the other *Anabaena* and also *Aphanizomenon* strains. The proposed type strain of *Aphanizomenon flos-aquae*, PCC 7905, did not cluster with the *Aphanizomenon flos-aquae* strains of this study. However, Rippka *et al.* (2001b) pointed out that the morphology of the PCC 7905 now in culture does not correspond well with the description of *Aphanizomenon flos-aquae*. The *Aphanizomenon* strains TR183 and 202 in cluster D, described previously as *Aphanizomenon flos-aquae* (Gugger *et al.*, 2002b), are called *Aphanizomenon* sp. in this study, because the original identification of these strains was problematic (Lyra *et al.*, 2001). No morphological description of strain NIES81 was found. *Aphanizomenon flos-aquae* seems to lose its typical colony structure in laboratory cultivation, which might complicate its identification (Gugger *et al.*, 2002b; this study). However, phylogenetic and morphological data from this study suggest that *Aphanizomenon flos-aquae* might form its own species.

Anabaena compacta ANACOM-KOR from the Czech Republic shared an identical 16S rRNA gene sequence with the previously described *Anabaena compacta* strain 189 from Denmark (Gugger *et al.*, 2002b), and these strains formed a distinct cluster, C, in the16S rRNA and *rpoB* trees (Figs 4 and 5). In the *rbcLX* analysis, *Anabaena compacta* ANACOM-KOR was clustered together with hepatotoxic *Anabaena* strains in cluster F (Fig. 6). *Anabaena compacta* was easily identified by its solitary, densely and regularly coiled trichomes (Fig. 1) and thus the morphology was in accordance with genetic data. The dense coiling of *Anabaena compacta* ANACOM-KOR was stable in culture and seems to be characteristic for this species, although the coiling was not a useful feature for the classification of other *Anabaena* species.

Aphanizomenon gracile and *Anabaena* cf. *circinalis* var. *macrospora* strains were closely related and intermixed in all the gene trees (cluster D in Figs 4, 5 and 6; Table 3). The cluster also included neurotoxic *Anabaena* strains described by Gugger *et al.* (2002b) and Lyra *et al.* (2001). This close relationship between neurotoxic *Anabaena* and *Aphanizomenon* strains (PCC 7905, TR183 and 202) had been previously revealed by 16S rRNA-RFLP, the 16S rRNA gene (Lyra *et al.*, 2001; Iteman *et al.*, 2002), *rbcLX* and ITS sequencing (Gugger *et al.*, 2002b), as well as by the analysis of cellular fatty acids (Gugger *et al.*, 2002a). The morphological similarity of *Aphanizomenon gracile* to some *Anabaena* strains has been discussed previously by Komárek & Anagnostidis (1989). These strains grow in solitary trichomes which do not have elongated, hyaline or pointed end cells. Surprisingly, all studied strains (*Anabaena* and *Aphanizomenon*) included in cluster D had very similar morphology and development of akinetes. They all form cylindrical akinetes by fusion of several vegetative cells next to or distant from a heterocyte. This type of akinete development separates it from other subclusters including planktic *Anabaena* strains and could

be used for classification of *Anabaena/Aphanizomenon* species. Previously, the development of akinetes has been found to be a stable character and suggested to have important taxonomic value for *Anabaena* classification (Stulp & Stam, 1982, 1985). The morphological similarities and close evolutionary distances suggest that *Aphanizomenon gracile* and *Anabaena* strains in cluster D might be assignable to a single species.

Benthic *Anabaena cylindrica* XP6B was the only representative of subcluster E (Figs 4, 5 and 6). XP6B was actually closely related to the hepatotoxic strains of subcluster F (16S rRNA sequence similarity >99·2 %). Morphologically this strain was separated from strains in subcluster F by a lack of gas vesicles, elliptical vegetative cells and heterocytes as well as presence of diffusive mucilage around trichomes. The benthic strains *Anabaena oscillarioides* BECID22 and BECID32 of cluster 1 formed subcluster H in all the gene trees (Figs 4, 5 and 6) and shared <98·2 % 16S rRNA gene sequence similarity with the other subclusters. These BECID strains were morphologically separable from other subclusters by the absence of gas vesicles, cylindrical akinetes (Fig. 1t) and trichomes arranged in fascicle-like formation with diffusive mucilage. More strains closely related to subclusters E and H are needed in order to conclude their morphological similarities and classification. Nevertheless, the close relationship between these benthic *Anabaena* and planktic *Anabaena/Aphanizomenon* strains indicates that gas vesicles are not a useful feature for classification of *Anabaena*.

In all gene trees, subcluster F consists of *Anabaena flos-aquae* and *Anabaena lemmermannii* strains, which were potential microcystin producers according to *mcyE*-PCR. These strains were closely related (16S rRNA gene sequence similarity >98·8 %) to each other, but also to non-toxic *Anabaena flos-aquae* strains of cluster G (16S rRNA gene sequence similarity >98·4 %) (Fig. 4; Table 3). Cluster F received high bootstrap support only in the *rpoB* analysis (Fig. 5). In the 16S rRNA gene tree, the hepatotoxic strains were divided into two highly supported clusters and in the *rbcLX* analysis also *Aphanizomenon* sp. 202 and *Anabaena compacta* ANACOM-KOR were placed in this cluster (Fig. 6). The close similarity within subcluster F and between it and non-hepatotoxic *Anabaena flos-aquae* strains indicated that the latter might have recently lost their ability to produce microcystins (Rantala *et al.*, 2004). We were not able to find any morphological criteria to distinguish strains from subclusters F and G, which mostly contained strains identified as *Anabaena lemmermannii* and *Anabaena flos-aquae*. These subclusters could be separated only by the phylogenetic analysis and by the potential hepatotoxin production of the strains in subcluster F with the exception of a non-toxic strain, *Anabaena* sp. 299A (this study; Gugger *et al.*, 2002b). Thus, the delineation of morphotypes *Anabaena lemmermannii* and *Anabaena flos-aquae* remains to be confirmed.

Interestingly, planktic *Aphanizomenon issatschenkoi*, 'benthic' *Anabaena* sp. 1tu34s7 and *Anabaena* sp. 277 were grouped together (subcluster I in Figs 4, 5 and 6) and were clearly separated from the other planktic *Anabaena* and *Aphanizomenon* strains in all gene trees as well as in PCA. This cluster shared <96·4 % 16S rRNA gene sequence similarity with other clusters/subclusters, indicating that the *Aphanizomenon issatschenkoi* strains, *Anabaena* sp. 277 and 1tu34s7 belong to different species from *Anabaena/Aphanizomenon* strains in cluster 1. The cluster was quite heterogeneous morphologically, containing both benthic and planktic strains. The *Anabaena* strains 1tu34s7 and 277 in this subcluster were isolated from plankton (this study; Lyra *et al.*, 2001) and they might have lost their gas vesicles during cultivation, which has been reported by Rippka *et al.* (2001b) in the case of PCC 7905. However, gas vesicles were not found in 1tu34s7 soon after isolation, and thus at least 1tu34S7 is probably benthic. It is evident that *Aphanizomenon issatschenkoi* strains characterized by solitary trichomes with pointed end cells are both morphologically and genetically clearly separated from the other *Aphanizomenon* strains as well as from typical planktic *Anabaena* strains. The only observed morphological similarity of strains in cluster I was the shape of akinetes, which were cylindrical in *Aphanizomenon issatschenkoi* and *Anabaena* sp. 1tu34s7. Akinetes were not found in *Anabaena* sp. 277.

Trichormus strains, which were separated morphologically from *Anabaena* and *Aphanizomenon* strains by akinete development, were divided into three well-separated clusters, 2, 5 and 6 (Figs 4, 5 and 6), and were not monophyletic. These *Trichormus* strains were found to be morphologically heterogeneous. Two benthic strains with wavy filaments and apoheterocytic akinete formation were assigned as *T. variabilis*. These *T. variabilis* strains GREIFSWALD and HINDAK/2001/4 also shared a high 16S rRNA gene sequence similarity (99·2 %) and formed cluster 2, which was loosely grouped with *Anabaena* PCC 7108 (Figs 4 and 5). Also, DNA–DNA reassociation studies of Stulp & Stam (1984b, 1985) with several *Anabaena* species support our results that *Anabaena* (*Trichormus*) *variabilis* strains were clearly separated from other *Anabaena* species. Stulp & Stam (1984b, 1985) found that relative binding values between *Anabaena varibilis* and other *Anabaena* species ranged from 31 to 39 %. Interestingly, *T. doliolum*, which was morphologically separated from other *Trichormus* strains by shape of akinete, shared less than 95·3 % 16S rRNA gene sequence similarity with any other cyanobacterial sequence and formed cluster 6 (Figs 4, 5 and 6). This indicates that *T. doliolum* might not be related to *Anabaena*, *Trichormus* or *Nostoc* strains at the species or even genus level if the suggested cut-off point for genus definition, 95 % 16S rRNA gene sequence similarity, is followed (Ludwig *et al.*, 1998). *T. azollae* BAI/1983 clustered with heterogeneous *Nostoc* strains (Figs 4, 5 and 6) and might actually belong to the genus *Nostoc* rather than to *Trichormus* or *Anabaena*. Similar results were found in analysis of restriction sites in the *nif* region (Meeks *et al.*,

1988). Also, morphological features such as a mucilaginous envelope support the transfer of *T. azollae* to the genus *Nostoc*.

The benthic *Anabaena oscillarioides* BO HINDAK 1984/43 and *Anabaena augstumalis* JAHNKE/4a formed clusters 3 and 4 in the 16S rRNA gene tree (Fig. 4) and these clusters shared 16S rRNA gene sequence similarity $<95·5$ %. In the *rpoB* and *rbcLX* gene trees, *Anabaena oscillarioides* BO HINDAK 1984/43 and *Anabaena augstumalis* JAHNKE/4a were grouped together, probably because no other closely related sequences of these genes were available (Figs 5 and 6). These *Anabaena* strains were morphologically separated from other *Anabaena* strains in cluster 1 by conical end cells and the presence of terminal heterocytes (Table 2). Among other features, conical end cells were also suggested by Lachance (1981) to separate *Anabaena* and *Nostoc* strains. The benthic *Anabaena*/*Trichormus* clusters 2–4 were grouped with planktic *Anabaena*/*Aphanizomenon* rather than with *Nostoc* strains (Figs 4, 5 and 6). However, the clustering of all *Anabaena*/*Aphanizomenon* and *T. variabilis* received high bootstrap support only in the *rbcLX* tree (Fig. 6). The evolutionary distances between the benthic *Anabaena* strains BO HINDAK 1984/43, JAHNKE/4a and *T. variabilis* as well as *Anabaena*/*Aphanizomenon* strains in cluster 1 were $>95·8$ %, indicating that these clusters 1–4 are not related at the species level. However, more closely related sequences of benthic *Anabaena* and *Trichormus* are needed before their phylogenetic position and classification can be resolved.

Nostoc strains were separated from genera *Anabaena* and *Trichormus* with the exception of *T. azollae* in all gene trees (Figs 4, 5 and 6) and thus supported the distinction between the genera *Nostoc* and *Anabaena*, as shown previously in studies of 16S rRNA gene sequences (Wilmotte & Herdman, 2001) and the *nifD* gene (Henson *et al.*, 2002). This was opposite to the studies of Tamas *et al.* (2000), which was based on a short fragment of *nifH*. *Nostoc* sequences were heterogeneous (sequence similarity 93·9 % for the 16S rRNA gene) at the bases of all the gene trees. This is in agreement with the DNA–DNA reassociation studies of Lachance (1981). Therefore, the studied *Nostoc* strains may actually represent two different genera. The *Nostoc* strains were clustered together in the 16S rRNA gene tree with low bootstrap support (Figs 4, 5 and 6), and did not form clusters in the *rbcLX* and *rpoB* trees. However, within the *Nostoc* cluster, sequences of *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 shared high 16S rRNA sequence similarity (97·7 %) and clustered together with high bootstrap values in all the gene trees (Figs 4, 5 and 6). These *Nostoc* strains also shared many common features such as terminal conical heterocytes and narrow, straight hormogonia and were morphologically differentiated from the other studied *Nostoc* strains (Hrouzek *et al.*, 2003; this study). The high sequence and morphological similarity suggest that *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 could be assigned to a single species. In addition, *N. muscorum* and *N. ellipsosporum* were morphologically (Hrouzek *et al.*, 2003) and genetically more closely related to each other than to the other studied *Nostoc* strains. However, the 16S rRNA gene sequence similarity of these two *Nostoc* strains and *T. azollae* to any other strains was $<96·7$ %, indicating that these strains are not related to each other or to other *Nostoc* strains at the species level.

Conclusion

This study indicates that planktic *Anabaena*/*Aphanizomenon* and benthic *Anabaena* were not monophyletic, since the planktic *Anabaena* and *Aphanizomenon* as well as five benthic strains in cluster 1 were intermixed. Strains in cluster 1 could be assigned to a single genus according to the genetic data. Cluster 1 could be divided into several (eight or nine) species based on genetic and morphological data, although the 16S rRNA gene sequence similarity between the *Anabaena*/*Aphanizomenon* subclusters was above 97·5 %. Most of these supported phylogenetic *Anabaena*/*Aphanizomenon* subclusters (A–E and H) were found in all the gene trees. Moreover, the strains within the subclusters shared certain morphological features that might be used in classification of the strains at the species level. The subclusters were morphologically separable from each other mainly on the basis of akinete parameters, sometimes in combination with the width of the trichome or potential hepatotoxicity. In contrast, coiling of trichomes, distance between heterocytes or the length of vegetative cells seem not to be useful criteria for separation of the subclusters. *Trichormus* strains were not monophyletic. *T. azollae* might belong to the genus *Nostoc* rather than to *Anabaena*. *T. doliolum* possibly forms a separate genus according to evolutionary distances. *T. variabilis* strains were more closely related to the benthic *Anabaena* strains than *Nostoc* strains, although *T. variabilis* and benthic *Anabaena* strains in clusters 2–4 were genetically relatively heterogeneous. Therefore, more benthic *Anabaena* and *Trichormus* strains need to be studied to confirm their phylogenetic positions. Nevertheless, the shape of end cells and presence of terminal heterocytes were found to be important for discriminating between the benthic *Anabaena* and *T. variabilis* in clusters 2–4 from the *Anabaena* strains in cluster 1. The phylogenetic relationship of the studied strains did not follow the current taxonomic classification of Komárek & Anagnostidis (1989) or that of *Bergey's Manual of Systematic Bacteriology* (Rippka *et al.*, 2001c) and therefore a revision of the taxonomy of these anabaenoid strains is needed.

ACKNOWLEDGEMENTS

This study was funded by the MIDI-CHIP project (EKV2-CT-1999-00026), the Enste Graduate School and the Academy of Finland (grant 201576). We thank Dr Jaana Lehtimäki and Matti Wahlsten for isolating strains XP6B, BECID22 and BECID32, Luydmila Saari for purifying and maintaining the strains during the study and Dr Stefano

Ventura for providing internal 16S rRNA primer sequences. Drs Christina Lyra and David Fewer are thanked for critical reading of the manuscript and helpful comments.

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