Biodiversity and Phylogeny of Planktic Cyanobacteria in Temperate Freshwater Lakes

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The author's contribution

Paper I
Pirjo Wacklin carried out the electron microscopy, sequencing of the 16S rRNA gene and phylogenetic analyses. She contributed to writing of the manuscript.

Paper II
Pirjo Wacklin contributed to designing of the study and the strain isolation. She designed the *rpoB* primers, performed sequencing, except for few 16S rRNA genes, and carried out all phylogenetic analysis. She interpreted the results and wrote the article.

Paper III
Pirjo Wacklin contributed to designing of the study, the strain isolation, sequencing of the 16S rRNA gene and morphological examination. She performed phylogenetic analysis, interpreted the results and wrote the article.

Paper IV
Pirjo Wacklin contributed to designing of the study, filtration and extraction of DNA. She carried out the DGGE analysis, interpreted the results and wrote the article.

Abbreviations

AAI	Average amino acid identity of genes, which two organisms share
ANI	Average nucleic acid identity of genes, which two organisms share
ARISA	Automated ribosomal intergenic spacer analysis
CARD-FISH	Catalysed reporter deposition fluorescence *in situ* hybridization
CCA	Canonical correspondence analysis
CCM	Carbon concentrating mechanisms
DGGE	Denaturing gradient gel electrophoresis
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
FISH	Fluorescence *in situ* hybridization
f.w.	Fresh weight
gvpA and *C*	Genes encoding gas vesicles proteins
gvpA-IGS	Non-coding intergenic spacer region between *gvpA1* and *gvpA2* genes
hetR	Gene needed for heterocyte differentiation
HGT	Horizontal gene transfer
ITS	Internal transcribed spacer region between 16S and 23S rRNA genes
LH-PCR	Length heterogeneity PCR
mcyA,B,D and *E*	Genes encoding for microcystin synthetase subunits
ME	Maximum evolution
ML	Maximum likelihood
MP	Maximum parsimony
Myr	Million years
N	Nitrogen
nifH	Gene encoding nitrogenase iron protein subunit
NJ	Neighbour Joining
OTU	Operational taxonomic unit
P	Phosphorus
PAR	photosynthetically active radiation
PCA	Principal component analysis
PC-IGS	Non-coding intergenic spacer region between phycocyanin genes *cpcA* and *cpcB*
PCR	Polymerase chain reaction
PEG	model of Sommer et al. (1986) describing the succession of phytoplankton in temperate lakes
psbA	Gene encoding D1 protein of photosystem II
rbcLX	Gene encoding ribulose-1,5-biphosphate carboxylase (RubisCO) large subunit and intergenic spacer region
RFLP	Restriction fragment length polymorphism
RING-FISH	Recognition of individual gene- FISH

rpoB	Gene encoding RNA polymerase β-subunit
rpoC1	Gene encoding RNA polymerase subunit C
sp.	species
SSCP	Single stranded conformation polymorphism
TGGE	Temperature gradient gel electrophoresis
TN	Total nitrogen
TP	Total phosphorus
T-RFLP	Terminal restriction fragment length polymorphism

Contents

ABSTRACT

Currently, the classification used for cyanobacteria is based mainly on morphology. In many cases the classification is known to be incongruent with the phylogeny of cyanobacteria. The evaluation of this classification is complicated by the fact that numerous strains are only described morphologically and have not been isolated. Moreover, the phenotype of many cyanobacterial strains alters during prolonged laboratory cultivation. In this thesis, cyanobacterial strains were isolated from lakes (mainly Lake Tuusulanjärvi) and both morphology and phylogeny of the isolates were investigated. The cyanobacterial community composition in Lake Tuusulanjärvi was followed for two years in order to relate the success of cyanobacterial phenotypes and genotypes to environmental conditions. In addition, molecular biological methods were compared with traditional microscopic enumeration and their ability and usefulness in describing the cyanobacterial diversity was evaluated.

The *Anabaena*, *Aphanizomenon,* and *Trichormus* strains were genetically heterogeneous and polyphyletic. The phylogenetic relationships of the heterocytous cyanobacteria were not congruent with their classification. In contrast to heterocytous cyanobacteria, the phylogenetic relationships of the *Snowella* and *Woronichinia* strains, which had not been studied before this thesis, reflected the morphology of strains and followed their current classification. The *Snowella* strains formed a monophyletic cluster, which was most closely related to the *Woronichinia* strain. In addition, a new cluster of thin, filamentous cyanobacterial strains identified as *Limnothrix redekei* was revealed. This cluster was not closely related to any other known cyanobacteria.

The cyanobacterial community composition in Lake Tuusulanjärvi was studied with molecular methods [denaturant gradient gel electrophoresis (DGGE) and cloning of the 16S rRNA gene], through enumerations of cyanobacteria under microscope and by strain isolations. *Microcystis*, *Anabaena*/*Aphanizomenon,* and *Synechococcus* were the major groups in the cyanobacterial community in Lake Tuusulanjärvi during the two-year monitoring period. These groups showed seasonal succession, and their success was related to different environmental conditions. The major groups of the cyanobacterial community were detected by all used methods. However, cloning gave higher estimates than microscopy for the proportions of heterocytous cyanobacteria and *Synechococcus*. The differences were probably caused by the high 16S rRNA gene copy numbers in heterotrophic cyanobacteria and by problems in the identification and detection of unicellular cyanobacteria.

TIIVISTELMÄ (Abstract in Finnish)

Syanobakteerit (sinilevät) ovat fotosyntesoivia bakteereita, joiden muodostamat massaesiintymät (kukinnat) ovat yleisiä loppukesäisin, erityisesti rehevissä järvissä. Useat yleisesti kukintoja muodostavista syanobakteereista, kuten osa *Anabaena*-, *Microcystis*- ja *Planktothrix*-sukujen kannoista, tuottavat myrkkyjä. Nämä myrkylliset kukinnat haittaavat vesien virkistyskäyttöä sekä aiheuttavat terveysriskin ihmisille ja eläimille.

Syanobakteerien tunnistus ja ekologinen tutkimus pohjautuu niiden luokitteluun, joka nykyisellään perustuu lähinnä syanobakteerien morfologiaan. Tämä luokittelu on monin osin ristiriidassa niiden geneettisen sukulaisuuden (fylogenian) kanssa. Luokittelun uudelleen arviointia on vaikeuttanut syanobakteerikantojen vähäisyys, tietämättömyys useiden sukujen/lajien geneettisistä sukulaisuussuhteista ja kantojen morfologian muuttuminen laboratoriokasvatuksessa. Tässä työssä yhdistettiin syanobakteerikantojen ja luonnonpopulaatioiden molekyylibiologista (geneettistä) ja morfologista tutkimusta. Tutkimusta varten eristettiin lukuisia syanobakteerikantoja, joiden geneettiset sukulaisuussuhteet ja morfologia selvitettiin. Ympäristötekijöiden vaikutusta syanobakteerilajien ja -genotyyppien runsastumiseen tutkittiin ja samalla vertailtiin eri menetelmien kykyä kuvata syanobakteeriyhteisön diversiteettiä.

Tutkimuksessa havaittiin että *Anabaena*-, *Aphanizomenon*-, *Trichormus*- ja *Limnothrix redekei*-kantojen geneettiset sukulaisuussuhteet eivät vastaa niiden nykyistä luokittelua. *Anabaena*-, *Aphanizomenon*- ja *Trichormus*- sukujen kannat, jotka muodostivat geneettisesti hajanaisia ryhmiä, voitaisiin luokitella jopa useampaankin sukuun. Sen sijaan *Snowella*- ja *Woronichinia*-kantojen sukulaisuussuhteet, joita ei ole aikaisemmin tutkittu, vastasivat niiden morfologiaa ja nykyistä luokittelua.

Tuusulanjärven syanobakteeripopulaatio muodostui kolmesta pääryhmästä, *Anabaena/Aphanizomenon*, *Microcystis*- ja *Synechococcus*-populaatiosta. Nämä ryhmät runsastuivat kesän eri aikoina ja erilaisissa ympäristöolosuhteissa. Elokuun loppupuolella lämpötilan, auringonsäteilyn ja ravinnepitoisuuksien laskiessa *Anabaena/Aphanizomenon*-populaatio syrjäytti *Microcystis*-populaation, joka oli runsaimmillaan heinä-elokuussa. Vertailemalla eri menetelmin saatuja tuloksia havaittiin, että vaikka kaikki kolme menetelmää (DGGE, 16S rRNA geenin kloonaus, mikroskopointi) havaitsivat pääryhmät, niin kloonaamalla *Anabaena/Aphanizomenon* ja *Synechococcus*-suvun osuudet arvioitiin suuremmiksi kuin mikroskopoimalla. Käyttämällä useita erilaisia menetelmiä saatiin monipuolisempi kuva syanobakteerien diversiteetistä.

Uusia tutkimustuloksia syanobakteerien sukulaisuussuhteista voidaan käyttää niiden luokittelun uudistamisessa. Tietoa syanobakteerilajien runsastumiseen johtavista ympäristötekijöistä sekä syanobakteeripopulaatioiden tutkimusmenetelmistä voidaan hyödyntää muun muassa järvien kunnostuksessa ja syanobakteerien monitorointimenetelmien kehittämisessä.

1 INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria (Cyanoprokaryota, Cyanophyta, blue-green algae) are photosynthetic prokaryotes, which possess mainly chlorophyll-*a* (Whitton and Potts 2000; Castenholz 2001a). Cyanobacteria are estimated to have occured as long ago as 2,600 to 3,500 million years (Myr), based on fossil records (Schopf 2000), organic biomarkers (Brocks et al. 1999), and genomic sequence analysis (Hedges et al. 2001). The earliest estimate of cyanobacterial occurrence (3,500 Myr) has nevertheless been questioned by Brasier et al. (2002). These earliest cyanobacteria are believed to have been capable of oxygen-evolving photosynthesis and are suspected to have played a major role in producing an oxygen-rich atmosphere on earth about 2,300 Myr ago (Blankenship 1992), although other theories explaining the rise of atmospheric oxygen have also been proposed (Catling et al. 2001; Kasting 2001). Endosymbiont event between a cyanobacteria and eukaryote gave rise to plastids (photosynthetic organelle), and consequently, algae and plants photosynthesise and possess chlorophyll-*a* (Bhattacharya et al. 2004).

Cyanobacteria are morphologically diverse (Fig. 1) (Whitton and Potts 2000). There are both filamentous and unicellular forms, which can aggregate as colonies. In colonies, cells and filaments may be arranged in different ways, e.g. radially, in strict planes, or irregularly. Filaments can be branching, coiled, or straight. Some cyanobacteria have evolved specialised cells for nitrogen fixation (heterocytes), survival in stressed conditions (akinetes), and dispersion (hormogonia). Cyanobacteria have many fascinating features, such as buoyancy, photosynthesis, fixation of atmospheric nitrogen (Castenholz 2001b), and production of a wide variety of bioactive compounds (Burja et al. 2001). In addition, cyanobacteria form symbiosis with several eukaryotic hosts such as plants, fungi, and protists (Adams 2000).

Probably owing to their physiological flexibility and long evolutionary history, cyanobacteria inhabit a large variety of terrestrial and aquatic habitats from deserts to lakes as well as hot springs and glaciers (Mur et al. 1999). Cyanobacteria form biofilms (microbial mats) on shores and on the surface of stones, plants, and artificial objects (Stal 2000). Planktic cyanobacteria, which are the main focus of this study, inhabit diverse aquatic environments from Antarctic lakes and nutrient-poor oceans to highly nutrient-rich lakes and ponds. They possess gas vacuoles, allowing buoyancy and facilitating the formation of blooms (mass-occurrences) (Walsby 1994). Cyanobacterial blooms occur commonly in many temperate lakes (Reynolds 1984) and were reported already 70 years ago in Lake Tuusulanjärvi, Finland (Järnefelt 1937). Cyanobacterial blooms are frequently toxic (Sivonen and Jones 1999) and thus pose a health risk for humans and animals, cause an aesthetic problem, and reduce the recreational value of water (Kuiper-Goodmann et al. 1999).

1.2 Classification of cyanobacteria

Classification is the arrangement of microbes into taxonomic groups (ranks) (Brenner et al. 2001), and it should reflect the evolutionary relationships between organisms (Wilmotte and Golubić 1991; Komárek 2003). In addition, classification guides the identification

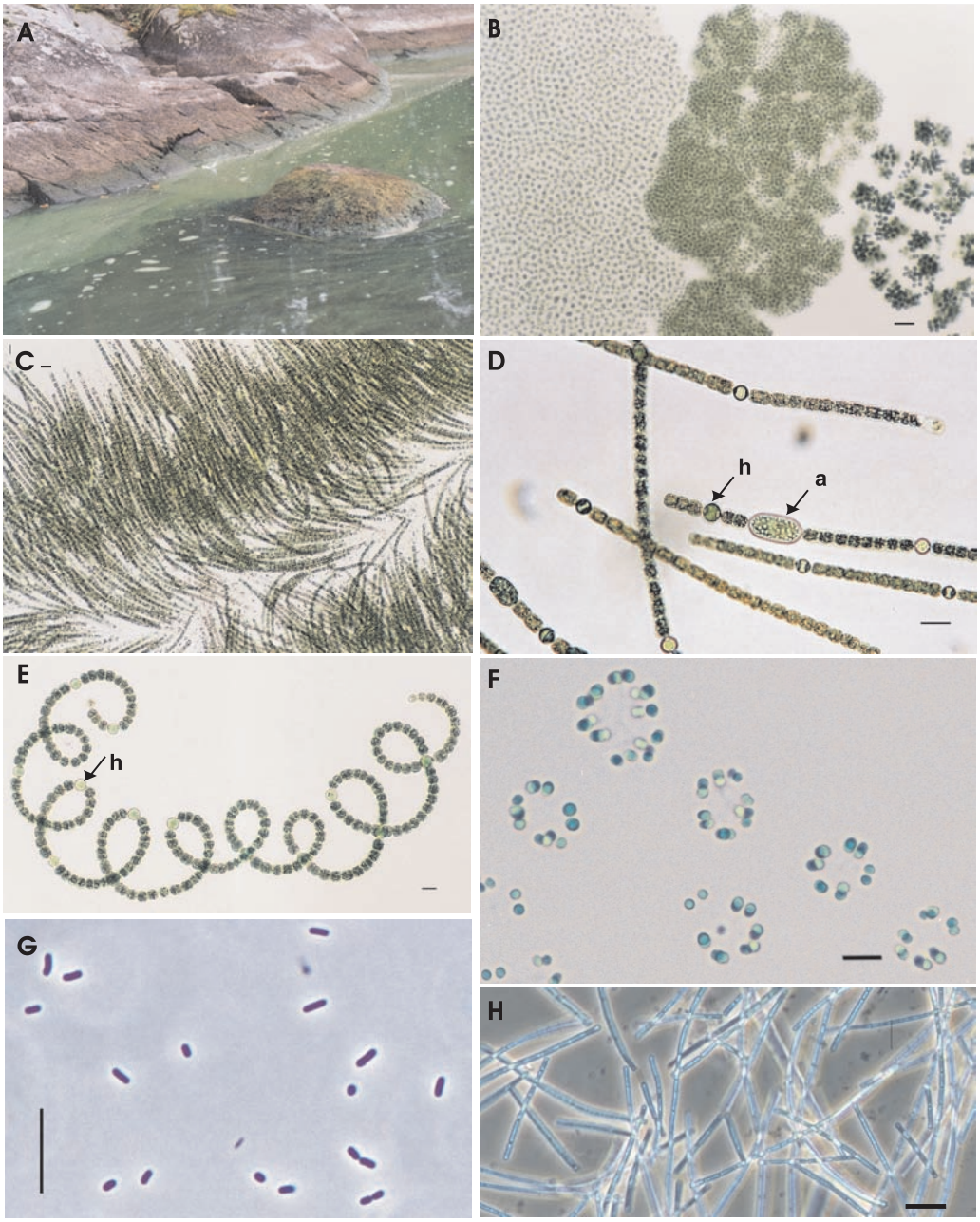

Fig. 1. Photograph of cyanobacterial bloom in Lake Tuusulanjärvi (A) and microphotographs of cyanobacterial colonies and strains showing some of their morphological diversity (B-F). (B) Colonies of *Microcystis* spp. in a sample taken from Lake Tuusulanjärvi July, 2000. (C) Colonies of *Aphanizomenon flos-aquae* 1tu29S19. (D) Straight trichomes of *Anabaena planctonica* 1tu28s8. (E) Coiled trichome of *Anabaena crassa* 1tu33S12. (F) Colonies of *Snowella litoralis* 0tu35S07. (G) Cells of *Synechococcus* sp. 0tu28S07 (phase contrast). (H) *Limnothrix redekei* 007a (phase contrast). Bars, 10 μm. Heterocytes (h) and akinetes (a) are indicated by arrows. (Photo A was taken by E. Kolmonen, photos B, F and G by A. Rantala, photo H by S. Gkelis, and photos C, D and E by P. Wacklin).

of bacteria and provides a common language to microbiologists (Brenner et al. 2001). Traditionally, cyanobacterial identification was based on morphology and they were classified as blue-green algae (Cyanophyta) among the eukaryotic algae under the botanical codes. During the turbulent history of cyanobacterial classification, several major revisions and changes have been proposed and more or less adopted. Anagnostidis and Komárek (1985), Wilmotte (1994), and Turner (1997) have reviewed the history of botanical classification extensively. Therefore an overview of the two most commonly adopted classification systems – the bacteriological approach in Bergey's Manual of Systematic Bacteriology (Boone and Castenholz 2001) and the botanical approach of Anagnostidis and Komárek (1985) – are explained here as is also the most recent proposal for cyanobacterial classification system (Hoffmann et al. 2005).

In the 1960s, cyanobacteria were found to have cellular features characteristics of prokaryotes, and consequently, Stanier et al. (1978) proposed including cyanobacteria in the bacteriological code. Rippka and co-workers (1979) created the bacteriological classification. Their scheme was adopted and modified in Bergey's Manual of Bacteriological Systematics (Boone and Castenholz 2001), the recognised authority on bacteriological classification. The bacteriological approach is based on genetic and phenotypic information about the cyanobacteria present in pure cultures (axenic strains) (Castenholz 2001b). Currently, the phylum cyanobacteria includes both oxygenic phototrophs, chlorophyll-*b*/*a*-containing prochlorales (prochlorophyta), and cyanobacteria (Castenholz 2001a). Cyanobacteria are divided into four subsections and further, into subgroups and genera (Castenholz 2001b) (see Table 1). The subsections and generic descriptions are still based mainly on morphology, due to the lack of genetically and phenotypically characterised isolates (Castenholz 2001b; Table 1).

Komárek and Anagnostidis (Anagnostidis and Komárek 1985; Komárek and Anagnostidis 1989, 1999, 2005) revised the classification of cyanobacteria under the botanical code. The classification of Anagnostidis and Komárek (1985) divides cyanobacteria (cyanoprokaryota) into four orders – Nostocales, Stigonematales, Chroococcales, and Oscillatoriales – which are further divided into families, subfamilies, genera, and species (see Table 1). This classification system emphasised morphological identification of species in natural samples and its use as a tool for ecologists to study the diversity of cyanobacteria (Anagnostidis and Komárek 1985).

Recently, botanical and bacteriological approaches have been converging; Botanical classification uses genetic information in addition to morphological, cytological, ecological, and biochemical features of cyanobacteria (Hoffmann et al. 2005; Komárek and Anagnostidis 2005). Botanical names are used in bacteriological classification, and the division of cyanobacteria into subsections mirrors the orders used in botanical classification (Table 1). Nevertheless, the nomenclature differs between these two classification systems, despite several proposals for their unification (Oren 2004; Oren and Tindall 2005; Hoffmann 2005). In addition, an isolated, living, pure culture of each described species is required in the bacteriological code, whereas preserved

Table 1. Classification of cyanobacteria according to bacteriological (Bergey's Manual of Systematic Bacteriology) and botanical systems (by Komárek and Anagnostidis; Hoffmann, Komárek and Kaštovský). Classification of the genera investigated in this study is shown.

Classification by Bergey's Manual of Systematic Bacteriology (Boone and Castenholz 2001)	**Classification by Komárek and Anagnostidis** (Anagnostidis and Komárek 1985; Komárek and Anagnostidis1988, 1999, 2005)	**Classification by Hoffmann, Komárek and Kaštovský** (2005) N.B.: Orders belong to four subclasses, which are not presented in correct order in this table
Subsection I: unicellular or colonial, division by binary fission in 1 to 3 planes or by budding E.g., Form-genus *Microcystis* Form-genus *Synechococcus* (*Snowella*, *Merismopedia* and *Woronichinia* not classified)	**Chroococcales:** Unicellular or colonial E.g., Family Merismopediaea Subfamily Gomphosphaeriaceae *Snowella, Woronichinia* Subfamily Merismopedioideae *Merismopedia* Family Microcystaceae *Microcystis* Family Synechococcaceae *Synechococcus*	**Gloeobacterales:** coccoid, lacking thylakoids **Synechococcales[2]:** thylakoids arrange parallel to cell surface, unicellular or colonial E.g., Family Merismopediaea *Merismopedia* Family Synechococcaceae *Synechococcus* **Chroococcales[1]:** radial arrangement of thylakoids, unicellular or colonial E.g., Family Gomphosphaeriaceae *Snowella, Woronichinia* Family Microcystaceae *Microcystis*
Subsection II: unicellular or colonial, division by multiple fission or in combination with binary fission		
Subsection III: filamentous, non-heterocytous E.g., Form-genus *Limnothrix*	**Oscillatoriales:** filamentous, non-heterocytous Family Pseudanabaenaceae Subfamily Pseudanabaenoideae *Limnothrix*	**Oscillatoriales[1]**: radial arrangement of thylakoids, large filamentous **Pseudoanabaenales[2]:** thylakoids arrange parallel to cell surface, thin filamentous Family Pseudanabaenaceae *Limnothrix*
Subsection IV: filamentous, heterocytous, non-branching E.g. Form-genus *Anabaena* Form-genus *Aphanizomenon*	**Nostocales:** filamentous, heterocytous, akinetes, false-branching Family Nostocaceae *Anabaena* *Aphanizomenon*	**Nostocales:** filamentous heterocytous cyanobacteria Family Nostocaceae *Anabaena* *Aphanizomenon*
Subsection V: filamentous, heterocytous, branching	**Stigonematales:** filamentous, heterocytous, akinetes, true-branching	

[1]Orders Chroococcales and Oscillatoriales form subclass Oscillatoriophycideae,
[2]Orders Synechococcales and Pseudanabaenales form subclass Synechococcophycidae.

specimens together with microphotographs or drawings are preferred in the botanical code (Oren 2004; Oren and Tindall 2005). To date, only five cyanobacterial species have valid descriptions according to bacteriological nomenclature (Oren 2004). The authors of both classification systems have emphasised that the current classification of cyanobacteria is temporary owing to inadequate genetic information and that major revisions will necessarily occur in the future (Castenholz 2001b; Komárek 2003). The classification of cyanobacteria and its revision are complicated by the presence of species based solely on morphology without any genetic information and by the sequences of cyanobacterial species in databases without morphological description (Komárek and Anagnostidis 1989; Wilmotte and Herdman 2001).

Recently, Hoffmann et al. (2005) proposed a revision to the cyanobacterial classification under the botanical code. Their proposed classification system was based on genetic relationships of cyanobacteria (mainly 16S rRNA gene sequences), morphology, and thylakoid arrangements. Three major changes were proposed: heterocytous cyanobacteria were unified into one subclass, prochlorophyta were included into the cyanobacterial classification system, and the distinction between coccoid and filamentous forms was no longer followed at the highest subclass level (Hoffmann et al. 2005). Instead, the division into subclasses was based on arrangements of thylakoids and the presence of differentiated cells. The coccoid and filamentous forms were separated at the order level (Hoffmann et al. 2005) (Table 1). The described classification systems are summarised and compared in Table 1. The classification of genera (*Anabaena*, *Aphanizomenon*, *Limnothrix*, *Merismopedia*, *Microcystis*, *Snowella*, *Synechococcus*, and *Woronichinia*), which are the main focus of this study, is shown in the different systems, and some of their morphological features are illustrated in Fig.1.

Simple identification of cyanobacterial species by microscopy without cultivation is practical and widely used, particularly in ecological studies. However, variability of morphological features in natural material complicates the identification of cyanobacteria under the microscope, in addition to problems caused by incorrect use of old or revised names and misidentification (Komárek and Anagnostidis 1989). Komárek and Anagnostidis (1989) estimated that a large number of the cyanobacterial strains in culture collections have been misidentified. Simple cyanobacteria such as *Synechoccous* and *Cyanoothece* are especially difficult to identify and classify (Castenholz 1992; Komárek et al. 2004). Recently, molecular biological methods (see the review of Gürtler and Mayall 2001) and cyanobacterial-specific primers (e.g., Urbach et al. 1992) have made it possible to study genetic relationships among non-axenic cyanobacteria and without cultivation of strains.

1.3 Phylogeny of cyanobacteria based on the 16S rRNA gene

Cyanobacteria form a monophyletic cluster among eubacteria (Woese 1987; Garrity and Holt 2001). The cyanobacterial cluster contains also the plastids of eukaryotes (Giovannoni et al. 1988; Wilmotte and Golubíc 1991; Turner 1997). The phylogenetic analysis of the 16S rRNA gene has revealed close relationships among cyanobacteria, indicating that the diversification of cyanobacteria happened within a short period of time

(Giovannoni et al. 1988; Wilmotte and Herdman 2001). Based on the phylogeny of the 16S rRNA genes, chlorophyll-*a*/*b* containing prochlorales (prochlorophyta) were shown to be polyphyletic (Urbach et al. 1992) and to cluster with cyanobacteria (Wilmotte 1994; Palenik and Swift 1996). This indicates that prochlorales shared a common ancestor with cyanobacteria, and prochlorales is not a valid phylogenetic group.

The cyanobacterial orders/subsections have not been supported by the 16S rRNA gene sequence analysis (Giovannoni et al. 1988; Turner 1997; Ishida et al. 2001; Gugger and Hoffmann 2004). Only heterocytous cyanobacteria belonging to the two orders/subsections appear to be monophyletic in the 16S rRNA gene analysis (Wilmotte and Herdman 2001; Gugger and Hoffmann 2004). This incongruence between phylogenetic analysis and the classification of cyanobacteria was taken into account in the most recent classification proposal (Hoffmann et al. 2005). In addition, the phylogenetic clustering of strains of several cyanobacterial genera seem to be incongruent with the cyanobacterial morphology and does not follow their current classification [e.g., *Anabaena* and *Aphanizomenon* (Lyra et al. 2001; Gugger et al. 2002a), *Oscillatoria* (Suda et al. 2002) and picocyanobacterial genera such as *Synechococcus* and *Synechocystis* (Wilmotte and Herdman 2001)]. In some cases strains of a genus or species formed a monophyletic cluster in the 16S rRNA gene analysis, for example *Planktothrix agardhii* (Lyra et al. 2001), *Nodularia* (Lyra et al. 2005), and *Microcystis* (Otsuka et al. 1998; Lyra et al. 2001). However, the morphologically distinguished *Microcystis* species were found to be genetically very closely related to each other (Otsuka et al. 1998). Unification of different *Microcystis* species into a single species has been proposed (Otsuka et al. 1998, 2001). Briefly summarised, the current classification of cyanobacteria does not follow their genetic relationships, and revisions are needed, as Castenholz (2001b) and Komárek (2003) concluded.

1.4 Phylogenetic marker genes and sequence analysis

1.4.1 The rRNA gene

The 16S rRNA gene, the most commonly used marker gene, has a central role in inferring phylogenetic relationships and in identification of bacteria. The 16S rRNA gene sequence similarities of bacteria were shown to correlate well with genome relatedness, expressed as DNA:DNA reassociation values (Stackebrandt and Goebel 1994) or as the average nucleotide or amino acid identity (ANI /AAI) of shared genes (Konstantinidis and Tiedje 2005a; 2005b). These correlations support the robustness of the 16S rRNA gene-based microbial phylogeny (Konstantinidis and Tiedje 2005b).

The 16S rRNA gene has a universal distribution in prokaryotes, functional consistency, both variable and conserved regions, and large size and thus, rather high information content - characteristics needed for a good phylogenetic marker gene (Woese 1987; Ludwig and Klenk 2001). In addition, the 16S rRNA gene sequences are relatively easy to align, and a large database has accumulated (currently over 6000 cyanobacterial sequences), allowing comparisons between strains (Ludwig and Klenk 2001). However, the resolution power of the 16S rRNA gene is at or above species level (Fox et al. 1992; Stackebrandt and Goebel 1994). The 23S rRNA gene is longer than

the 16S rRNA gene and consequently, contains more informative sites and leads to a better resolution, but the sequence database of the 23S rRNA gene is small in comparison to the 16S rRNA gene (Turner 1997; Ludwig and Klenk 2001).

Horizontal gene transfer (HGT) (e.g., Doolittle 1999) and the presence of multiple heterogeneous rRNA gene copies (Acinas et al. 2004) have raised concern about the reliability of relationships of bacterial strains determined on the basis of the 16S rRNA genes. The bacterial genome can contain up to 15 copies of 16S rRNA genes (Acinas et al. 2004). Although intragenomic divergence of the 16S rRNA genes can be as high as 11.6%, generally it seems to be low, less than 1% (Acinas et al. 2004). Among cyanobacteria, the observed intragenomic divergence of the 16S rRNA genes has been rather low (<1.3%) and related to heterocytous cyanobacteria (Table 2). A few heterocytous cyanobacterial strains, for which either information or whole genomes were available, contain several (4-5) copies of the 16S rRNA gene, whereas unicellular cyanobacteria have only one to two identical copies (Table 2).

HGT of the parts of the 16S rRNA gene has been reported in several closely related bacterial strains (Mylvaganam et al. 1992; Yap et al. 1999; Wang and Zhang 2000; van Berkum et al. 2003). In addition, Miyashita et al. (1996) and Miller et al. (2005) found that two chlorophyll-*d*-containing cyanobacterial strains have obtained a small part (14-18 nt) of the 16S rRNA gene from β-proteobacteria, which is only distantly related to cyanobacteria. The impact of HGT on the 16S rRNA genes seems to be a disputable issue (Doolittle 1999; Gogarten et al. 2002). Nevertheless, it has been suggested that

Table 2. The 16S rRNA gene copy numbers and sequence divergence in cyanobacteria[1]

Organism	No. of copies	No. of different copies	Divergence between copies %	Genome size[2]	Accession number	Reference
Heterocytous cyanobacteria						
Anabaena sp. PCC9302	5	2	1.3	?	AY038037	Iteman et al. 2002
Anabaena variabilis ATCC 29413[3]	4	1	0	7.07	NC_007413	DOE Joint Genome Inst.
Nostoc punctiforme PCC73102	4	2	0.1	9.06	NZ_AAAY000 00000	DOE Joint Genome Inst.
Nostoc sp. PCC 7120	4	2	0.07	7.21	NC_003272	Acinas et al. 2004
Non-heterocytous cyanobacteria						
Cyanobacteria Yellowstone A-Prime	2	1	0	2.93	NC_007775	TIGR
Cyanobacteria Yellowstone B-Prime	2	1	0	3.05	NC_007776	TIGR
Gloeobacter violaceus PCC 7421	1	1	-	4.66	NC_005125	Kazusa
Prochlorococcus marinus MIT 9312	1	1	-	1.71	NC_007577	DOE Joint Genome Inst.
Prochlorococcus marinus MIT 9313	2	1	0	2.41	NC_005071	DOE Joint Genome Inst.
Prochlorococcus marinus NATL2A	1	1	-	1.84	NC_007335	DOE Joint Genome Inst.
Prochlorococcus marinus CCMP1375	1	1	-	1.75	NC_005042	CNRS
Prochlorococcus marinus CCMP1986	2	1	0	1.66	NC_005072	DOE Joint Genome Inst.
Synechococcus elongatus PCC 6301	2	1	0	2.7	NC_006576	Nagoya Univ., Japan
Synechococcus elongatus PCC 7942	2	1	0	2.8	NC_007604	DOE Joint Genome Inst.
Synechococcus sp. CC9605	2	1	0	2.51	NC_007516	DOE Joint Genome Inst.
Synechococcus sp. CC9902	2	1	0	2.23	NC_007513	DOE Joint Genome Inst.
Synechococcus sp. WH 8102	2	1	0	2.43	NC_005070	DOE Joint Genome Inst.
Synechocystis sp. PCC 6803	2	1	0	3.95	NC_000911	Acinas et al. 2004
Thermosynechococcus elongatus BP-1	1	1	-	2.59	NC_004113	Kazusa

[1]Based on published genome sequences of cyanobacteria except *Anabaena* PCC9302.
[2]Genome sizes obtained from the NCBI genome database.
[3]The end of the 16S rRNA gene was incorrectly defined in two copies (the two last bases of the genes were missing) in Genebank.
?= not known.

conserved genes such as 16S rRNA are recalcitrant to transference in nature, and thus the impact of HGT on the phylogeny based on these genes is limited (Doolittle 1999; Philippe and Douady 2003; Woese 2004; Coenye et al. 2005).

1.4.2 Other marker genes

By comparing genome sequences, the number of genes fulfilling the criteria of good marker genes (i.e., universal distribution in all prokaryotes, in a single copy within a genome, and appropriate information content) has been found to be fewer than one hundred (Ludwig and Klenk 2001; Zeigler 2003; Santos and Ochman 2004). Based on a large set of genome sequences, Coenye et al. (2005) even concluded that a universal marker gene for all prokaryotes (similar to rRNA genes) might be difficult to find and that taxon–specific marker genes would be necessary.

The Ad Hoc Committee for the Re-evaluation of Species Definition in Bacteriology recommended the use of a minimum of five genes to obtain an adequate informative level of phylogenetic data (Stackebrandt et al. 2002). Actually, by analysing the bacterial genome sequences, Zeigler (2003) found that a small set of carefully selected marker genes could be used to discriminate among species equal to DNA:DNA reassociation. Evaluation of good marker genes for cyanobacteria has yet to be done.

HGT is common among prokaryotes (Doolittle 1999; Jain et al. 1999). HGT has commonly occurred between so-called housekeeping genes (e.g., operational genes coding for metabolic proteins and antibiotic resistances) (Rivera et al. 1998). Nevertheless, inferring phylogenetic relationships seems to be applicable to the core set of genes, which are involved in transcription, translation, and related processes (informational genes) and which seem to be only rarely transferred horizontally (Philippe and Douady 2003; Woese 2004; Ochman et al. 2005). Sánchez-Baracaldo et al. (2005) found that 33 out of the 36 studied operational and informational genes produced congruent trees with 14 cyanobacterial strains for which genome sequences are available. Only trees based on three metabolic genes (enolase, *uppS* and *hemB*) were incongruent with the other gene trees, probably due to HGT, gene duplication, or long branch attraction (Sánchez-Baracaldo et al. 2005). The main disadvantage of the marker genes other than 16S rRNA genes is that their sequence databases are currently rather small (Ludwig and Klenk 2001).

Konstantinidis and Tiedje (2005a, 2005b) used a measure of average amino acid or nucleotide identity of all shared genes between two bacterial strains as an alternative method to estimate their relatedness. This approach avoids the problem of finding common marker genes that have a reasonable resolution even between close relatives (i.e., below the species level) and which are conserved enough to allow primer design (Konstantinidis and Tiedje 2005b).

1.4.3. Phylogenetic sequence analysis

Phylogenetic analyses are used to estimate the evolutionary relationships of bacteria. The sequence analyses usually include alignment of sequences, construction of a phylogenetic tree, and testing the reliability of the constructed tree, e.g., with bootstrapping (Ludwig and Klenk 2001). Aligning of sequences is a crucial step in phylogenetic analysis, since only the positions with a common ancestor (homologous positions) can be used in

phylogenetic analysis (Swofford et al. 1996). In alignment, the sequences from different strains are organised by inserting gaps so that homologous positions of the sequences are placed in the same columns of the data matrix. Several computer programs [e.g. ClustalW (Chenna et al. 2003) and ARB (Ludwig et al. 2004)] have been created for aligning the sequences.

The relationships of the aligned sequences are usually shown as a tree, in which the branching pattern of the tree (topology) displays the evolutionary relationships of the strains (Nei and Kumar 2000). The most commonly applied tree construction methods are distance, maximum parsimony (MP), and maximum likelihood (ML) (Nei and Kumar 2000; Ludwig and Klenk 2001). Distance methods such as neighbour joining (NJ) (Saitou and Nei 1987) use pair-wise distances (i.e. the number of base differences between two sequences), calculated from aligned sequences and usually corrected to evolutionary distances within a substitution model (Nei and Kumar 2000). The sequences with the shortest distances are clustered together in a tree, where the tree length is optimised to correspond to the distance matrix (Nei and Kumar 2000). The MP method uses the actual sequence data instead of distances and searches for the tree(s) with minimum length, i.e., topology of the tree can be explained with a minimum number of transformations from one character state to another (Swofford et al. 1996; Nei and Kumar 2000). ML method estimates the likelihood for tree topology that could have resulted in the sequence alignment under the given model of evolution and searches for the tree with maximum likelihood (Swofford et al. 1996; Nei and Kumar 2000). Mathematical background and more detailed discussion of tree construction methods are presented in Swofford et al. (1996) and Nei and Kumar (2000).

1.5 The species concept for cyanobacteria

Species expresses the membership of organisms in a taxonomic rank (Stackebrandt and Goebel 1994). Species forms the basic unit of classification systems and is a tool for describing diversity. The species name should tell the reader about the phenotypic features of an organism and about the relationships to other organisms. The species concept for cyanobacteria, while are a part of phylum eubacteria, should be similar to the bacterial species concept. However, cyanobacteria are morphologically highly divergent in comparison to most other bacteria, and consequently, it has been suggested that morphological features be given more weight in the species definition (Castenholz and Norris 2005).

Species definition for cyanobacteria and for prokaryotes in general is a controversial issue among taxonomists and no general agreement exists (e.g., Ward 1998; Castenholz and Norris 2005; Komárek 2003). Currently, prokaryotic species definition relies on DNA:DNA relatedness, which is measured as the relative binding ratio (RBR) and/or the difference in the thermal denaturation midpoint (ΔT_m) between DNAs from two organisms (heteroduplex DNAs) (Rosselló-Mora and Amann 2001). The genomes of two strains have to share above 70% RBR or less than 5°C ΔT_m to be considered members of the same species (Wayne et al. 1987). In addition, the phenotypic characteristics of the species should agree with the DNA:DNA reassociation results (Wayne et al. 1987).

In RBR and ΔT_m determination, denatured DNAs of two organisms are mixed, allowed to reassociate, and form hybrid molecules in controlled experimental conditions (Vandamme et al. 1996). The more similar are the DNAs of two organisms, the more hybridisation occurs. The RBR and ΔT_m are determined by comparing the results from the heteroduplex DNA to the results obtained from homoduplex DNAs (Rosselló-Mora and Amann 2001).

This species concept has been criticised as being arbitrary, underestimating diversity, and not universal for all organisms (Rosselló-Mora and Amann, 2001; Brenner et al. 2001). In addition, DNA:DNA relatedness determinations are time-consuming and allow only pair-wise comparisons of closely related organisms (Rosselló-Mora and Amann 2001). As a result, only a small set of cyanobacterial strains has been studied with the DNA: DNA reassociation method (Stam 1980; Lachance 1981; Stulp and Stam 1984; Wilmotte and Stam 1984; Kondo et al. 2000; Otsuka et al. 2001; Suda et al. 2002). Owing to these problems related to the DNA:DNA reassociation method, a wide variety of different molecular methods has been developed and are commonly used to study the genetic relationships of strains (see the reviews of the methods by Vandamme et al. 1996 and Gürtler and Mayall 2001).

The 16S rRNA gene sequencing is probably the method most commonly used to study genetic relationships of bacteria. By comparing DNA:DNA reassociation values and 16S rRNA gene similarities, Stackebrandt and Goebel (1994) found that bacterial species having a 16S rRNA gene similarity of less than 97.5% most likely belong to different species. However, species having a 16S rRNA similarity of more than 97.5% might have either low or high DNA:DNA relatedness and could belong either to the same or to different species (Stackebrandt and Goebel 1994). Owing to the limited resolution between closely related species, species definition cannot be based solely on the 16S rRNA gene sequences (Stackebrandt and Goebel 1994; Ludwig et al. 1998).

The genome sequences have provided new insight into the prokaryotic species definition. “Species genome concept” (Lan and Reeves 2000) and “pan-genome concept” (Medini et al. 2005) have been suggested to describe the species. The species genome/pan-genome contains all the genes present in any strain of a species. Species genome/pan-genome is divided into a core set of genes, which are found in most (95%) or all strains, and an auxiliary/dispensable set of genes, found only in some strains of the species (Lan and Reeves 2000; Tettelin et al. 2005; Medini et al. 2005). Thus, pan-genome might be orders of magnitude larger than any genome of a single organism when strains of the species have a large number of unique dispensable genes (Medini et al. 2005).

Konstantinidis and Tiedje (2005b) compared the gene contents of 70 closely related bacterial genomes (>94% 16S rRNA gene sequence similarity). The measure of relatedness of genomes of two strains, the average nucleotide identity (ANI), correlated well with the 16S rRNA sequence similarity (r=0.79) and the DNA:DNA reassociation values (r=0.93) (Konstantinidis and Tiedje 2005b). The DNA:DNA reassociation value of 70% – the current species definition – corresponded to circa 94% ANI (Konstantinidis and Tiedje 2005b). However, Konstantinidis and Tiedje (2005b) also found that strains of the

same species can vary up to 35% in their protein-coding gene content, and the variation reflected the ecological niches of those strains. In contrast, species defined as having ANI as high as 98-99%, or having lower ANI but overlapping ecological niches, showed minimum gene differences (<5% of the well-characterised genes differ) (Konstantinidis and Tiedje 2005b). Konstantinidis and Tiedje (2005b) concluded that the current species definition is liberal, but the change to a more stringent definition (e.g., ANI > 98%) would result in a drastically higher number of species and would therefore be impractical (Konstantinidis and Tiedje 2005b).

1.6 Diversity of planktic cyanobacteria in temperate lakes

1.6.1 Planktic cyanobacteria in temperate lakes

Cyanobacterial blooms are common in temperate eutrophic lakes during the warm periods of summer (Reynolds 1984). These blooms are commonly formed by gas-vacuolated genera such as *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Planktothrix* (Oliver and Ganf 2000), all of which are known to contain toxic strains (Sivonen and Jones 1999). In addition, unicellular (e.g., *Synechococcus*) and colonial picocyanobacteria (e.g., *Snowella* and *Merismopedia*) can be abundant in freshwater bodies, although they do not commonly form blooms (Stockner et al. 2000; Callieri and Stockner 2002). Nevertheless, picocyanobacteria can contribute significantly to primary production, especially in oligotrophic freshwater lakes (Stockner et al. 2000). In eutrophic lakes, the biomass and abundance of picocyanobacteria increase with increasing total phytoplankton biomass, although their relative importance decreases (Bell and Kalff 2001; Stockner et al. 2000).

Some cyanobacteria seem to be restricted to certain environments (Komárek and Anagnostidis 1999). For example, *Prochlorothrix hollandica* is the only prochlorales (prochlorophyta) species that has been reported to occur in freshwater lake (Burger-Wiersma et al. 1986; Callieri and Stockner 2002), while other prochlorales species, *Prochlorococcus*, is abundant in marine environments (Giovannoni and Stingl 2005). Based on the available 16S rRNA gene sequences, Zwart et al. (2002) identified several bacterial clusters (including cyanobacterial clusters *Microcystis, Aphanizomenon flos-aquae*, and *Planktothrix agardhii*) characteristic of freshwater lakes (i.e., no sequences of marine origin existed). However, one genotype of *Aphanizomenon flos-aquae* is known to be abundant in the brackish Baltic Sea (Barker et al. 2000b; Laamanen et al. 2002). *Cylindrospermum raciborskii* has fairly recently spread to temperate lakes from tropical ones (Padisák 1997).

1.6.2 Ecotypes of planktic cyanobacteria

Cyanobacteria have been divided into different groups (called ecostrategists, ecotypes, or functional groups) according to their physiological characteristics, mainly buoyancy, colony formation, and nitrogen fixation (Mur et al. 1983 in Mur et al. 1999; Hyenstrand et al. 1998; Schreurs 1992; Reynolds 1984; Reynolds et al. 2002). Mur et al. (1999) classified planktic cyanobacteria into bloom-forming, homogenously dispersed, stratifying, nitrogen-fixing, and small colonial ecostrategists, whereas Hyenstrand et al. (1998) formed three ecotypes based on

nitrogen fixation and buoyancy: ecotypes containing nitrogen fixing and buoyant cyanobacteria, buoyant non-nitrogen fixers, and non-buoyant non-nitrogen fixers. Different ecotypes/ecostrategists are thought to respond differently to environmental factors such as light and nutrient availabilities (Mur et al. 1999).

Molecular biological methods have made it possible to study the diversity of cyanobacterial populations and genotypes in more detail, and thus, have expanded the ecotype concept. Hayes et al. (2002) have studied the genetic population structure of *Planktothrix* in Lake Zürich and *Nodularia* populations in the Baltic Sea by diagnostic PCR (Table 3). They concluded that these populations were not clonal but showed spatial and temporal variation in their genetic community structure and that genotypes having different alleles for example, gas vesicle coding genes, were adapted to different environmental conditions (Hayes et al. 2002). Postius and Ernst (1999) showed that a morphologically similar freshwater *Synechococcus* (*Cyanobium*) population contained several genotypes, which responded differently to factors such as nutrient deprivation, light intensity and predation. These co-existing *Synechococcus* ecotypes generated a physiologically highly variable population and were adapted to different ecological niches (Postius and Ernst 1999). In addition, marine *Prochlorococcus* strains have been divided into low- and high-light adaptive ecotypes (Ferris and Palenik 1998; Rocap et al. 2003), and marine *Synechococcus* strains were found to be adapted to different light conditions and to mixing or stratification periods (Giovannoni and Stingl 2005).

1.6.3 Seasonal dynamics of planktic cyanobacteria in eutrophic lakes

General seasonal succession of phytoplankton in temperate lakes has been described by Reynolds (1984) and in a model called PEG by Sommer et al. (1986). Eutrophic lakes generally have two biomass maxima, one formed by diatoms in spring and the other formed by *Microcystis* and/or *Ceratium* in late summer (Reynolds 1984). Between these maxima, pulses of biomass dominated by green algae and later by the filamentous cyanobacteria *Anabaena* and *Aphanizomenon* are formed (Reynolds 1984). According to the PEG model, non-nitrogen fixing cyanobacteria become abundant in summer when silica and phosphorus become depleted; later, during nitrogen deficiency, nitrogen-fixing cyanobacteria dominate (Sommer et al. 1986). The PEG model was based on the deep, stratifying Lake Constance, and therefore it may apply less well to non-stratified and shallow lakes that are easily mixed by wind (Sommer et al. 1986). Picocyanobacteria, which were not included in the PEG model, seem to have one or two maxima, one in spring and the other in late summer (Callieri and Stockner 2002).

1.6.4 Planktic cyanobacteria in Finnish lakes

Cyanobacteria are usually a significant phytoplankton group in eutrophic Finnish lakes (Lepistö 1999; Lepistö and Rosenström 1998), in which species belonging to the genera *Aphanizomenon*, *Anabaena*, and *Microcystis* are present in the highest biomass levels during the summer (Lepistö 1999). The genus *Anabaena* seems to be the most common bloom-forming genus in Finnish lakes, accounting for 60% of the reported water blooms (Lepistö 1999). Lepistö (1999)

concluded that in Finnish mesotrophic and eutrophic lakes, diatoms dominated in spring, while cyanobacteria increased in abundance towards the end of the summer and dominated in the late summer (Lepistö 1999). In hypereutrophic lakes, cyanobacterial dominance was more intensive and continued into the autumn (Lepistö 1999).

Finnish lakes are commonly coloured brownish by humic substances (dystrophic lakes) (Ilmavirta 1982). Very humic lakes are demanding environments for phototrophs because of the light restriction to the thin surface layers, and the deficiency of nutrients due to stratification and unavailability of humus-bound nutrients. Cyanobacteria generally contribute little to the phytoplankton biomass in dystrophic and oligotrophic lakes (Lepistö 1999; Lepistö and Rosenström 1998). Phycocyanin-containing picocyanobacteria are common in humic lakes, whereas phycoerythrin-containing picocyanobacteria are found in oligotrophic and less humic waters (Kukkonen et al. 1997; Jasser and Arvola 2003).

1.7 Life style and the ecological role of planktic cyanobacteria in temperate freshwater lakes

1.7.1 Characteristics of cyanobacteria

Buoyancy

Gas vacuoles consisting of gas vesicles provide buoyancy to cyanobacteria (Walsby 1972). Gas vacuoles allow cyanobacteria to migrate vertically and to gain access to spatially separated resources (nutrient and light), to avoid sinking, and to escape high irradiances that damage the cells (Waslby 1994; Oliver and Ganf 2000). Migration in the water column is dependent on the size of the cells or colonies; large colonial cyanobacteria such as *Anabaena circinalis* and *Microcystis* sp. can migrate tens or hundreds of metres per day, whereas unicellular cyanobacteria migrate only a few centimetres per day (Walsby 1994).

Buoyancy is regulated in cyanobacteria by accumulation or use of carbohydrates and other dense cell components, by collapsing gas vesicles, and by molecular controlling of gas vesicle production (Walsby 1994). Generally, buoyancy in cyanobacterial cells is reduced in high irradiance, whereas low irradiance increases buoyancy (Walsby 1994). However, many environmental factors, such as nutrient availabilities, affect buoyancy regulation (Walsby 1994; Brookes and Ganf 2001). The failure of buoyancy regulation or the overlapping replacement of old colonies by new ones (succession) has been suggested as the cause of persistent cyanobacterial blooms (Walsby 1994).

The width of gas vesicles has been shown to vary within *Nodularia* (Barker et al. 1999) and within *Planktothrix* populations (Bright and Walsby 1999). Narrow gas vesicles tolerate higher pressures better than wider gas vesicles (Hayes and Walsby 1986; Bright and Walsby 1999). The narrower and stronger gas vesicles can be advantageous to cyanobacteria in deep lakes, because they can resist irreversible collapse of gas vesicles at greater depths during mixing of the water column (Walsby 1994; Walsby et al. 1998). On the other hand, narrow gas vesicles are more costly to produce, because more gas vesicle proteins are needed to make the cells float (Walsby 1994).

Nitrogen fixation

The fixing of N_2, the most common form of nitrogen on earth, confers a major advantage on nitrogen-fixing cyanobacteria during periods of nitrogen deficiency in the water column (e.g., reviews of Hyenstrand et al. 1998 and of Oliver and Ganf 2000). The amount of fixed nitrogen varies among lakes; fixed nitrogen can account for a large part (6-82%) of the total nitrogen load in eutrophic freshwater lakes, whereas the amount of fixed nitrogen is considerably smaller (<1%) in oligotrophic and mesotrophic lakes (Howarth et al. 1988a). Several environmental factors have been found to affect the nitrogen fixation rates in lakes (Howarth et al. 1988b; Vitousek et al. 2002). According to field and laboratory experiments, low dissolved inorganic nitrogen (DIN) levels (<50-100 mg m^{-3}) are needed to induce the nitrogenase activity and nitrogen fixation in cyanobacteria (Horne and Commins 1987). The trace elements iron and molybdenum are also required for nitrogenase activity. Light availability has a major role in controlling nitrogen fixation, which is an energy-demanding process. In addition to availability of nutrients and light, concentration of oxygen, turbulence, grazing, and temperature have been reported to affect nitrogen fixation (Howarth et al. 1988b; Vitousek et al. 2002).

Nitrogen fixation is restricted to bacteria and has not been found in eukaryotic phytoplankton (Oliver and Ganf 2000). Heterocytous cyanobacteria have been thought to be mainly responsible for the planktic nitrogen fixation in lakes (Howarth et al. 1988a). Recently, nitrogenase genes originating from picocyanobacteria were found to be present and expressed in freshwater lakes by *nifH*-based PCR (Zani et al. 2000; MacGregor et al. 2001) indicating that cyanobacteria other than the heterocytous cyanobacteria are also potential nitrogen-fixers in lakes. In oceans, the rate of picocyanobacterial nitrogen fixation can be equal to the nitrogen fixation rate of *Trichodesmium*, the major nitrogen-fixer in tropical seas (Falcón et al. 2004; Montoya et al. 2004).

Stored nutrients

Nutrient reserves enable cyanobacteria to maintain growth during periods of nutrient depletion (Allen 1984; Oliver and Ganf 2000). In cyanobacteria, cyanophycin and phycocyanin function as nitrogen reserves, polyphosphate as phosphorus storage compound and glycogen as carbon and energy reserve (Kromkamp 1987).

The cyanophycin nitrogen reserve seems to be unique to cyanobacteria (Allen 1984; Oliver and Ganf 2000). Cyanophycin is thought to serve the only function of nitrogen storage (Simon 1971), whereas phycocyanin functions also as a pigment component in light-harvesting antennae (Allen 1984). Both cyanophycin and phycocyanin are degraded during nitrogen limitation (Tandeu de Marsac and Houmard 1993). Kinetics for phosphorus uptake and accumulation differ among cyanobacterial species. An extreme example is the storage capacity of *Gloeotrichia echinulata*, which seems to store all needed phosphorus in sediment prior to migration to the surface water layer (Pettersson et al. 1993).

1.7.2 Physical factors

Temperature

Cyanobacterial blooms usually occur during warm periods, at temperatures above 20°C (Robarts and Zohary 1987).

Both field and laboratory experiments (Reynolds 1984; Robarts and Zohary 1987) have supported the hypothesis that elevated temperatures favour cyanobacteria over other phytoplankton (Tilman et al. 1986; McQueen and Lean 1987). Cyanobacteria have generally higher temperature optima (>25 °C) for growth, photosynthesis, and respiration than have green algae and diatoms (Robarts and Zohary 1987). However, blooms formed by *Woronichinia naegeliana* (Lepistö 1999) and *Planktothrix rubescens* (Keto and Sammalkorpi 1988) have been reported to occur under ice. Elevated temperature may also have indirect effects on the abundance of cyanobacteria through water column stability and stratification (see section Turbulence and mixing below) (Robarts and Zohary 1987; Hyenstrand et al. 1998; Oliver and Ganf 2000).

Response to temperature varies among cyanobacterial genera and strains. *Microcystis* has been observed to be more temperature sensitive in comparison to *Anabaena*, *Aphanizomenon*, and *Planktothrix* (Robarts and Zohary 1987; Schreurs 1992; Oliver and Ganf 2000), and its growth was found to decline sharply at temperatures below 15°C (Robarts and Zohary 1987). *Planktothrix* tolerated the widest range of temperatures, and one strain of *P. rubescens* grew well even at 4°C (Robarts and Zohary 1987).

Turbulence and mixing

Turbulence and mixing, which are related to temperature and stratification, have been suggested to play a role in the success of cyanobacteria (Steinberg and Hartmann 1988). Stratification leads to decreased nutrient availability in surface layers and thus favours migrating species (Oliver and Ganf 2000). Cyanobacteria are known to be sensitive to turbulence, which breaks down filaments and decreases growth, nitrogen fixation, and photosynthesis as well as hinders buoyant cyanobacteria from keeping an optimal vertical position (Paerl et al. 2001; Moisander et al. 2002). Even a small-scale shear, which corresponds to turbulence caused by moderate or high wind in the surface water layer, has been shown to decrease the nitrogen and carbon fixation of heterocytous cyanobacteria and to cause fragmentation of their filaments (Moisander et al. 2002). However, gentle stirring of laboratory cultures accelerates growth, and in nature, it might delocalise nutrients and trace metals and thus promote growth (Paerl et al. 2001). Paerl et al. (2001) suggested that the sensitivity of heterocytous cyanobacteria to turbulence could restrict their occurrence in environments such as oceans.

Different cyanobacterial morphotypes have been reported to inhabit waters with varying degrees of turbulence: larger cells and colonial forms such as *Microcystis* and *Anabaena* are generally favoured in lakes with longer periods of stratification, while smaller forms (single filaments) occur in easily mixed lakes with reduced importance of buoyancy (Oliver and Ganf 2000). Migration of cyanobacteria, which do not have gas vacuoles, is dependent on turbulence of water column (Oliver and Ganf 2000).

Light

Compared to the other phytoplankton, cyanobacteria have been reported to benefit from lower light intensities of photosynthetically active radiation (PAR) (Mur et al. 1978 in Mur et al. 1999; Scheffer 1998). By forming surface blooms, cyanobacteria can also shade the water column beneath (Oliver and Ganf 2000). Laboratory experiments and field studies have shown that light

requirements of cyanobacteria vary among species (Oliver and Ganf 2000). For example, *Planktothrix agardhii* has been shown to adapt to low light (Mur et al. 1999; Scheffer 1998), whereas *Microcystis* photosynthesises at optimal rates and resists photoinhibition in high surface irradiances (Paerl et al. 1985). Genotypes adapted to low and high light have been observed among freshwater *Synechococcus* (Postius and Ernst 1999) and among marine *Prochlorococcus* populations (Ferris and Palenik 1998).

In addition to light intensity, light quality (changes in spectral distribution) may play a major role in structuring the community composition of phytoplankton (Oliver and Ganf 2000; Callieri and Stockner 2002). Cyanobacteria have phycobiliproteins, and therefore they can absorb radiation over a wider range of wavelengths than can eukaryotic microalgae (Tandeau de Marsac and Houmard 1993). This flexibility is advantageous when underwater light is reduced to longer wavelengths or light quality fluctuates (Oliver and Ganf 2000). Dissolved and suspended organic compounds absorbing blue light cause a shift towards the longer wavelengths in deeper water layers and thus benefit phycocyanin-containing cyanobacteria (Vörös et al. 1998; Oliver and Ganf 2000). Some cyanobacteria are also capable of changing the content of light-harvesting pigments (phycocyanin and phycoerythrin) complementary to available light wavelengths (so-called complementary chromatic adaptation) (Tandeau de Marsac and Houmard 1993). Light spectrum competition experiments have shown that the divergence of light-harvesting pigment composition in picocyanobacteria allows efficient utilisation of light energy and the co-existence of different species (Stomp et al. 2004).

1.7.3 Nutrients

Phosphorus

It is generally accepted that phosphorus limitation commonly occurs in temperate lakes (Schindler 1977; Smith 1982), whereas nitrogen and carbon can be fixed from the air (Schindler 1977). Increased total phosphorus concentration has been shown to increase the cyanobacterial and phytoplankton biomass (Schindler 1977; Trimbee and Prepas 1987; Watson et al. 1997). Several authors have concluded that total phosphorus concentrations above 50 – 100 mg m^{-3} increase the likelihood of cyanobacterial dominance in lakes (Schreurs 1992; Watson et al. 1997; Downing et al. 2001; Jeppesen et al. 2005). However, even in the low phosphate concentrations (picomolar concentrations), rapid cycling of phosphate by planktic microbes can support high rates of production in lakes (Hudson et al. 2000). The biological processes (the uptake and regeneration of phosphorus) as well as abiologic removal of phosphorus (e.g., absorption into clay particles and sediment) regulate phosphate concentrations in water (Karl 2000).

Differences in phosphorus requirements and utilisation between cyanobacterial genera/genotypes play a role in their success (Huisman and Hulot 2005). Among marine *Prochlorococcus* and *Synechococcus* genotypes, differences in phosphorus utilisation and alkaline phosphatase activities under phosphorus-limited conditions determined their occurrence in marine environments (Moore et al. 2005).

Nitrogen

Although nitrogen limitation in lakes is not as common as phosphorus limitation, it does occur occasionally, for example in the surface layer during stratification of the water (Wetzel 1983). Some cyanobacteria can fix nitrogen, which gives them a major advantage at times of nitrogen deficiency (see above). In addition, buoyant cyanobacteria can benefit from the migration to ammonium sources in deeper water (hypolimnia), and picocyanobacteria compete over larger species for ammonium with high surface-to-volume ratio during nitrogen deficiency (Hyenstrand et al. 1998). Nitrogen deficiency affects not only the growth of cyanobacteria, but also the buoyancy of gas-vacuolated cyanobacteria, as nitrogen is essential for synthesis of gas vesicles (Oliver and Ganf 2000).

Blomqvist et al. (1994) and Hyenstrand et al. (1998) proposed that nitrogen-fixing cyanobacteria are favoured by low dissolved inorganic nitrogen (DIN) levels, non-nitrogen fixing cyanobacteria by ammonium, and eukarotic algae by nitrate. However, from the studies investigating combination effects of nitrogen source and light (Ward and Wetzel 1980; Garcia-Conzales et al. 1992), Oliver and Ganf (2000) concluded that instead of nitrate favouring eukaryotic algae, cyanobacteria were rather disadvantaged at utilising nitrate under low light.

Nitrogen: phosphorus (N:P) ratio.

It has been suggested that a low N:P ratio favours cyanobacteria over other phytoplankton (Schindler 1977; Smith 1983; Levine and Schindler 1999; Smith and Bennet 1999). The N:P ratio hypothesis has its basis in the observed stoichiometry of C:N:P (106:16:1, the so-called Redfield ratio) and the resource-competition theory of Tilman et al. (1982), which describes the effect of the supply ratio of resources (e.g,. nitrogen and phosphorus) on species composition.

The hypothesis of low N:P ratio on cyanobacterial success has gained great deal of attention, although its role is still depated (Smith and Bennet 1999, Reynolds 1999; Downing et al. 2001). The low N:P hypothesis has not taken into account that not all cyanobacteria fix nitrogen (Huisman and Hulot 2005). Several authors have argued that instead of the N:P ratio, individual concentrations of resources (nutrients) are crucial for species competition, and cyanobacterial dominance is more associated with increase in nutrient concentrations (especially P) and phytoplankton biomass than with N:P ratio (Shapiro et al. 1990; Scheffer 1998; Reynolds 1999; Downing et al. 2001). In eutrophic lakes, some factor other than nitrogen or phosphorus (e.g., light or trace metals) may also limit the growth of cyanobacteria, and the N:P ratio would not have an effect on cyanobacterial success (Reynolds 1999; Paerl et al. 2001; Huisman and Hulot 2005).

Carbon

In addition to nitrogen, cyanobacteria can fix carbon from air. Cyanobacteria have perhaps the most effective inorganic carbon concentrating mechanisms (CCM) of all photosynthetic organisms (Badger and Price 2003). CCMs containing the carboxysome, Rubisco-enzyme and a variable array of inorganic carbon transporters elevate the CO_2 levels up to 1000-fold around the Rubisco, the key enzyme in carbon fixation (Badger and Price 2003). On the basis of comparative genomics, all freshwater cyanobacteria (five strains studied to date) have both high- and low-affinity CO_2 transporters

and, in contrast to marine cyanobacteria, high affinity HCO_3^- transporters (Badger et al. 2006). Thus, freshwater cyanobacteria can induce a high-affinity inorganic carbon uptake system when inorganic carbon is limited, for example, during heavy blooms or in cyanobacterial mats (Badger and Price 2003). Environmental factors such as light, salinity, temperature, and nutrients regulate the activity of CCMs and inorganic carbon transporters, and thus the ecological advantage of the CCM and transporters also depends on other environmental factors besides CO_2 and pH (Beardall and Giordano 2002).

The better adaptations of cyanobacteria to low CO2 concentrations at high pH have been suggested as helping to continue photosynthesis and outcompete other phytoplankton (Shapiro 1990). More recently, Shapiro (1997) concluded that the low CO_2/high pH conditions, which cyanobacteria have created, help cyanobacteria keep their dominance rather than allow them to initiate it.

Metabolic coupling and release of organic matter

Phytoplankton including cyanobacteria are also responsible for production of organic matter in the pelagic zone of lakes (Münster and Chróst 1991). They release dissolved organic matter (DOM) into the water by active excretion, and via leakage from damaged cells or after cell lysis, which are important DOM sources for heterotrophic bacteria in freshwater lakes (Jüttner 1981; Lovell and Konopka 1985; Münster and Chróst 1991 and references therein). The released organic compounds are hydrolysed and rapidly taken up by heterotrophic bacteria (Lovell and Konopka 1985, Münster and Chróst 1991) and channelled into the microbial loop (Azam et al. 1983; Hagström et al. 1988) and further to higher food web levels by grazing (Münster and Chróst 1991). The amount of released DOM varies, being highest during high solar radiation and more important in oligotrophic lakes than in eutrophic lakes (Münster and Chróst 1991 and references therein).

Organic compounds are commonly present in freshwater systems; however, a major fraction of DOM is not readily utilisable for microbes (Münster and Chróst 1991). When easily available nutrients are depleted, the production of ectoenzymes and the utilisation of polymeric organic compounds are beneficial for microorganims (Chróst 1991). Cyanobacteria are capable to utilise a wide range of dissolved organic phosphorus (Whitton et al. 1991) and nitrogen compounds (Berman and Bronk 2003) by producing extracellular enzymes such as phosphatases (Chróst 1991; Whitton et al. 1991) and aminopeptidases (Martinez and Azam 1993). DON can be a preferred nitrogen source for cyanobacteria over the fixed nitrogen in lakes, probably due to high-energy demand of nitrogen fixation (Berman and Bronk 2003). A batch culture experiment with the axenic *Anabaena* strain showed increasing phosphatase activity with decreasing total phosphorus concentrations (Vaitomaa et al. 2002).

Trace elements

Cyanobacteria require a variety of trace metals such as iron, molybdenum, and copper for key enzymes, growth, photosynthesis, and nitrogen metabolism (Rueter and Petersen 1987; Tandeau de Marsac and Houmard 1993). Iron and molybdenum limitation occur in lakes (Rueter and Peterson 1987 and Paerl et al. 2001). In humic lakes, trace metals can be tightly bound to humus, which

decreases their availability and promotes their deficiency (Paerl et al. 2001). Cyanobacteria have siderophores to chelate iron, which can benefit cyanobacteria during restricted availability of iron (Paerl et al. 2001).

In addition to chemical and physical factors, biological factors such as grazing and food-web interactions (cascading trophic interactions) (Carpenter et al. 1985) affect cyanobacterial occurrences and abundance in freshwater systems (Scheffer et al. 1998; Paerl et al. 2001). Cyanobacteria are a diverse group of bacteria, and thus, cyanobacterial species or genotypes can be successful in a wide variety of environmental conditions (Paerl et al. 2001; Huisman and Hulot 2005).

1.8. Isolation and cultivation of cyanobacteria

Castenholz (1992) estimated that less than 5% of cyanobacterial "species" have been isolated. Cyanobacterial strains can be isolated by direct picking of filaments or colonies under a light microscope with a Pasteur micropipet (see Rippka 1988) as well as by common microbiological isolation methods, liquid enrichment, and streaking/spreading on plates (Rippka 1988). The ability of motile cyanobacteria to glide towards light, centrifugation for buoyant cyanobacteria, and washing by filtaration can all be utilised to isolate cyanobacteria and especially in obtaining axenic (pure) cultures (Castenholz 1988 and Rippka 1988). Rouhiainen et al. (1995) used a soft-agarose layer on plates of solid Z8 medium to prevent drying of planktic *Anabaena* colonies and to isolate and purify *Anabaena* strains successfully. Antibiotic or phenol treatments in darkness can be useful for obtaining axenic strains of obligate phototrophic cyanobacteria. Antibiotic/phenol treatment eliminates heterotrophic bacteria, which commonly adhered to the cyanobacterial cells or in the mucilage or sheaths of cyanobacteria and which are difficult to remove by other methods (Castenholz 1988). The most commonly applied culture media for cyanobacteria and for comprehensive presentation of culturing conditions and isolation methods are reported by Rippka (1988) and Castenholz (1988).

Some cyanobacteria cannot be isolated easily (Castenholz 1988), at least not by current cultivation methods. For example, the most common *Aphanizomenon flos-aquae* genotype in the Baltic Sea has not been isolated despite several attempts (Laamanen et al. 2002). The reasons for failure in the cultivation of cyanobacteria could be their sensitivity to high nutrient concentrations (Castenholz and Waterbury 1989; Ernst et al. 2005), to high light conditions, or to contaminants in the chemicals of the cultivation media (Castenholz and Waterbury 1989). Cyanobacteria grow slowly, and it may take a long time (even months) to produce a visible growth (Castenholz 1988).

Cyanobacterial strains in culture collections are usually stored as living cultures, by transferring cells to fresh media when needed. The living cultures and frequent transfers increase the risk of mixing cultures as well as possibility for the phenotypic changes of cyanobacterial isolates, which seem to be common during prolonged cultivation (Castenholz and Waterbury 1989). However, some cyanobacterial strains such as *Oscillatoria* do not survive in preservation methods commonly used for other bacteria such as lyophilisation or storage in liquid nitrogen (Castenholz and Waterbury 1989).

1.9 Molecular methods for studying cyanobacterial community composition

Molecular biological methods have revealed a large number of new bacterial lineages and higher microbial diversity in natural environments than was previously thought to exist by cultivation-based methods (Pace 1997; Giovannoni et al. 1990; Ward et al. 1990, Venter et al. 2004). The diversity of cyanobacteria in nature has traditionally been studied by microscopy, which usually allows identification at the species level in contrast to many other bacteria. However, the development of molecular biological methods has revealed that in some cases microscopic enumeration underestimated the diversity of cyanobacteria in nature, especially the diversity of simple unicellular forms (Giovannoni et al. 1990). Moreover, microscopic identification is not suited for recognition of different genotypes, for example, separation of toxic strains from non-toxic ones (Sivonen and Jones 1990).

The most commonly used marker gene in microbial and cyanobacterial ecology is the 16S rRNA gene. Cyanobacterial specific primers have been designed for the 16S rRNA gene (Urbach et al. 1992; Nelissen et al. 1996; Nübel et al. 1997; Lepère et al. 2000), and a large database (currently over 6000 sequences) allows comparison of the newly obtained 16S rRNA gene sequences. In addition to the 16S rRNA gene, other genes and intergenic spacer regions (Table 3) have been used to study different aspects of cyanobacterial diversity. The choice of primers/probes and the method applied determine the resolution at the different taxonomic levels, from strain to domain level.

The principles of the molecular biological methods applied to cyanobacteria are shown in Table 3. Most of the molecular biological methods are based on extraction of total DNA or RNA from the environmental sample and PCR amplification of target genes. In theory, PCR amplifies targeted templates in their original proportions in the samples. However, DNA extraction and PCR amplification-related biases, such as the success of DNA extraction among variable species as well as differential amplification, the presence of PCR inhibitors, PCR artefacts, and primer specificity and efficiency all can skew the results of the community composition (von Wintzingeroda et al. 1997).

1.9.1 Cloning of DNA fragments

In cloning, the target region is amplified by PCR, a clone library is created, and clones are usually screened by a fingerprinting method such as RFLP and ARDRA and/or sequencing. Cloning provides detailed genetic information about the community members and can be applied to different target regions; moreover, the use of general primers does not presume prior knowledge of community composition. For diverse environments, a high number of clones has to be studied, increasing the cost and making the method tedious to perform. This also makes the analysis of numerous samples costly and time-consuming. Quantification of bacteria by cloning has been shown to depend greatly on the primer pair used, on the diversity of the templates, on the number of PCR cycles, and on the cloning vector (von Wintzingeroda et al. 1997; Wilson 1997). The cloning of 16S rRNA genes and the subsequent sequencing of the clones have been applied to planktic cyanobacterial communities, e.g., in the Sargasso Sea (Giovannoni et al. 1990) and in freshwater lakes (Boutte et al. 2005), as well as to

benthic cyanobacteria (e.g., Taton et al. 2003).

1.9.2 Community fingerprinting

In contrast to cloning, fingerprinting methods allow rapid screening of a large number of samples. In addition to investigations of bacterial diversity, many of the fingerprinting methods (e.g., RFLP) are commonly used in the characterisation of bacterial strains. In the fingerprinting methods, the PCR-amplified fragments of the target region are separated in gel or in capillary electrophoresis based on their length (T-RFLP, LH-PCR and ARISA), melting (DGGE/TGGE), or conformational (SSCP) differences (for abbreviations and principles of the methods, see Table 3). By comparing the similarities of banding patterns, fingerprinting methods can be used to estimate diversity changes or similarities of community structure between samples.

The length variation of the fragments generated by T-RFLP, LH-PCR, and ARISA can be accurately determined by fluorescent labels and automated capillary electrophoresis (Liu and Stahl 2002), which allow quantification of the bacteria. In contrast, gel-to-gel variation causes problems in quantification of DGGE and SSCP data (see Table 3) (Liu and Stahl 2002). However, in some studies, DGGE band intensities have been used to estimate the abundance of cyanobacterial genotypes and good correlations between DGGE band intensities and cyanobacteria present in the sample have been detected (Nübel et al. 2000; Kolmonen et al. 2004; Casamayor et al. 2000). Muyzer et al. (1993) and Casamayor et al. (2000) have estimated that a subpopulation contributing more than 1% to the total population can be detected by DGGE. The main advantage of DGGE and SSCP is that they allow the affiliation of bands with bacterial sequences in the databases by band excision, re-amplification, and sequencing (Ferris et al. 1996) or by hybridisation with probes (Muyzer et al. 1993).

A common limitation of all the fingerprinting methods is that different sequences can migrate to the same position in a gel (Liu and Stahl 2002). Crosby and Criddle (2003) found by *in silico* comparison of the different fingerprinting methods (in which biases related to PCR amplification or DNA extraction were excluded) that ITS-based ARISA caused overestimation of the bacterial diversity whereas 16S rRNA-based fingerprint methods, LH-PCR and to a lesser extent DGGE and RFLP, underestimated the bacterial diversity. T-RFLP seemed to be more suitable for environments with low bacterial diversity (Engebretson and Moyer 2003). More detailed discussion of the limitations and applicability of fingerprinting methods can be found for DGGE in Muyzer and Smalla (1998), and for SSCP, RFLP and LH-PCR and ARISA in Liu and Stahl (2002).

1.9.3 Other PCR-based methods

In addition to cloning and fingerprinting methods, several other techniques that apply PCR have been developed and successfully applied in assessing cyanobacterial diversity. Diagnostic PCR introduced by Hayes and Barker (1997) involves picking filaments or colonies of cyanobacteria, direct cell lysis, and PCR followed by sequencing one or more target regions or electrophoretic separation of genotypes. Diagnostic PCR has been successfully applied to study genetic diversity of *Aphanizomenon* (Barker et al. 2000b, Laamanen et al. 2002), *Planktothrix* (Beard et al. 1999),

Table 3. Molecular biological tools for studying the diversity of cyanobacteria without cultivation. Studies that apply methods in microbial ecology and in particular for cyanobacteria were selected.

Method	Principle of method	Used target region(s)	PCR	References
Cloning	Cloning of amplified gene fragments and screening of clones by, e.g., sequencing of clones or fingerprinting analysis of fragments.	E.g., 16S rRNA gene, *nifH*, *rpoC1*, rbcL, *psbA1*	+	Giovannoni et al. 1990; Kirshtein et al. 1991; Palenik, 1994; Pichard et al.1997; Zeidner et al. 2003
Quantitative PCR	Real-time monitoring of PCR amplification at each cycle based on laser detection and fluorescent dyes or probes.	*mcyE ,mcyD, mcyA mcyB*, ITS, PC-IGS	+	Rinta-Kanto et al. 2001; Foulds et al. 2002; Becker et al. 2002; Vaitomaa et al. 2003; Kurmayer et al. 2003
Diagnostic PCR	Direct amplification of target region from picked colonies or filaments. Discrimination of different alleles by presence/absence of PCR product, variability of length of target region, or sequencing.	PC-IGS, *gvpA*-IGS, ITS, *gvpA/C*, *mcyA*, *mcyB*	+	Hayes and Barker, 1997; Beard et al. 1999; Barker et al. 2000a, 2000b; Laamanen et al. 2001, 2002; Kurmayer et al. 2002; Via-Ordorika et al. 2004
DGGE /TGGE	Fingerprinting method. Electrophoretic separation of amplified fragments based on their melting behaviour. Melting caused by temperature or denaturant gradient in gel electrophoresis. Phylogenetic affiliation of bands by excision and sequencing or by hybridisation with probe.	16S rRNA gene, *nifH, hetR*, ITS, *psbA*	+	Muyzer et al. 1993; Rosado et al. 1998; Nübel et al. 1999; Becker et al. 2004; Zeidner and Béjà, 2004; Nilsson et al. 2005
SSCP	Fingerprinting method. Separation of ssDNA fragments based on their three-dimensional conformation differences in gel electrophoresis. Phylogenetic affiliation of bands by excision and sequencing.	16S rRNA gene	+	Lee et al. 1996
ARISA	Fingerprinting method. Separation of amplified ITS fragments on the basis of their length in capillary electrophoresis. Detection based on fluorescent labelled forward primer.	ITS	+	Borneman and Triplett 1997; Fischer and Triplett 1997
LH-PCR	Fingerprinting method. Separation of 16S rRNA genes based on their length in capillary electrophoresis. Detection based on fluorescent labelled primers.	16S rRNA gene	+	Suzuki et al. 1998
RFLP	Fingerprinting method applying restriction enzymes prior to separation of amplified fragments on the basis of their length in gel electrophoresis.	16S rRNA gene, *mcyA*	+	Martínez-Murcia et al. 1995; Hisbergues et al. 2003;
T-RFLP	Fingerprinting method applying restriction enzymes prior to separation of amplified fragments on the basis of their length in capillary electrophoresis. Detection based on flourescent labelled primer.	16S rRNA gene	+	Liu et al. 1997; Redfield et al. 2002
Microarray	Hybridisation of amplified fragments with fluorescent probes spotted to the slide or with probes linked to the slide via universal address sequence (universal array).	16S rRNA gene, *nifH*	+	Rudi et al. 2000; Castiglioni et al. 2004; Jenkins et al. 2004

Table 3. continued

FISH	Permabilisation of cells by fixative and hybridisation of fluorescent labelled probes with ribosomal rRNA *in situ*. Detection under microscopy or by flow-cytometry.	16S rRNA	-	Delong et al. 1989; Schönhuber et al. 1999, West et al. 2001; Pernthaler et al. 2002; Zwirglmaier et al. 2004
Metagenomics	Cloning of total environmental DNA and large-scale sequencing of clones.	whole genome	-	Tyson et al. 2004; Venter et al. 2004 ; DeLong et al. 2006

Abbreviations for methods: ARISA, automatedribosomal intergenic spacer analysis; DGGE/TGGE, denaturing /temperature gradient gel electrophoresis; FISH; fluorescence in-situ hybridisation; LH-PCR, length heterogeneity PCR; SSCP, single-stranded conformation polymorphism; T-RFLP, terminal restriction fragment length polymorphism. Abbreviations for target regions: *gvpA* and *C*, genes encoding the proteins of gas vesicles; *hetR*, gene needed for heterocyte differentiation; ITS, internal transcribed space region between 16S and 23S rRNA genes*; nifH* gene encoding nitrogenase iron protein subunit; *mcyA,B,D* and *E* genes encoding for microcystin synthetase subunits; PC-IGS, non-coding intergenic spacer region of phycocyanin operon; *psbA*, gene encoding D1 protein of photosystem II; *rbcL*, gene encoding ribulose-1,5-biphosphate carboxylase large subunit; *rpoC1*, gene encoding RNA polymerase subunit C.

Microcystis (Kurmayer et al. 2002; Via-Ordorika et al. 2004), and *Nodularia* (Hayes and Barker 1997; Barker et al. 2000a, Laamanen et al. 2001) populations. This method allows analysis of a large number of samples and quantitative measurements of different genotypes and thus studies of spatial and temporal variations in population genetic structure (Hayes et al. 2002). However, it is labour-intensive and not suited for non-colonial cyanobacteria.

Quantitative PCR (real time PCR) detects and quantifies copy numbers of target genes present in microbial population by online monitoring of the amplification process (Zhang and Fang 2006). Quantitative PCR is a sensitive method, that allows rapid analysis of a large number of samples. It has been applied to investigate, for example, toxic cyanobacteria, by quantifying *mcy*-genes (Rinta-Kanto et al. 2001; Foulds et al. 2002; Kurmayer et al. 2003; Vaitomaa et al. 2003).

In DNA microarrays (DNA chips), large numbers of probes spotted on a slide are hybridised with DNA or in the case of environmental applications commonly with PCR-amplified gene fragments. Microarrays allow rapid detection of bacteria in natural samples. From ten to several thousand probes specific for the strain to phylum level can be included in the microarray. The 16S rRNA gene-based microarray has been developed and applied to the detection of planktic cyanobacteria (Rudi et al. 2000; Castiglioni et al. 2004) and to detection of functional genes such as *nifH* in marine environment (Jenkins et al. 2004). These microarray methods developed for detection of cyanobacteria from environmental samples require PCR amplification of templates to increase sensitivity (Rudi et al. 2000; Castiglioni et al. 2004; Jenkins et al. 2004). Currently, environmental applications of the microarray technology are challenged by sensitivity, specificity, and quantification (Zhou 2003).

1.9.4 Non-PCR-based methods

Bacterial cells in complex samples can be identified without cultivation by fluorescence *in situ* hybridisation (FISH) (Table 3) (DeLong et al. 1989; Amann et al. 1990a, 1995; Wagner et al. 2003). In FISH, the cells are made permeable to the fluorescent-labelled probes by a fixative, the probes are hybridised under stringent conditions to ribosomal RNA, and fluorescent signals are detected by an epifluorescence microscope (Amann et al. 1995) or flow-cytometry (Amann et al. 1990b; Sekar et al. 2004). The main advantage of FISH is that it allows detection and quantification of intact cells. Probes detecting all cyanobacteria and specifically certain cyanobacterial groups have been designed (Loy et al. 2003), and the applicability of FISH has been demonstrated for cyanobacteria in studies of microbial mats (Schönhuber et al. 1999; Abed et al. 2002) and of picocyanobacteria in marine environments (West et al. 2001; Worden et al. 2000). One of the major limitations of the standard FISH has been its applicability only for ribosomal rRNA and difficulties of optimising the permeability protocol for diverse bacteria population. In addition, low signal intensity, low ribosomal content of a cell, or autofluorescence of organisms decrease the sensitivity of FISH (Wagner et al. 2003 and Zwirglmaier 2005). Many of these limitations may be overcome by recent developments of FISH (Wagner et al. 2003 and Zwirglmaier 2005) such as *in situ* recognition of low copy number genes by FISH (RING-FISH) (Zwirglmaier et al.

2004), increasing signal intensity by, e.g., CARD-FISH (Zwirglmaier 2005), and new permeability protocols (Pernthaler et al. 2002).

The developments of sequencing techniques and the sequence analysis methods have paved the way for metagenomic research. In metagenomics, large fragments of environmental DNA are cloned and the clones sequenced, or screened for genetic markers, and sequenced on a large scale. This method circumvents the limitations of PCR and culturing (Handelsman 2004; Tringe and Rubin 2005). The major advantage of the metagenomic method is the oppurtunity to assemble whole genomes directly from DNA extracted from environmental samples, thereby providing insight into the genomics of uncultivable organisms (Tyson et al. 2004; Venter et al. 2004). This technique has been applied to microbial communities in acid mine drainage biofilm (Tyson et al. 2004), soil (Tringe et al. 2005), marine water (Venter et al. 2004; Delong et al. 2006), and whale fall (Tringe et al. 2005).

2 AIMS OF THE STUDY

1. To isolate planktic cyanobacterial strains from freshwater lakes (Papers I, II, III, and IV)

2. To characterise the isolated cyanobacterial strains morphologically and study their phylogeny (Papers I, II, and III)

3. To explore the utility of *rpoB* gene sequences for phylogeny of cyanobacteria (Paper II)

4. To investigate the occurrence and abundance of cyanobacterial genotypes and/or morphotypes in relation to environmental conditions (Papers III and IV).

5. To compare the usefulness of molecular biological methods and microscopic counting in describing cyanobacterial community composition in Lake Tuusulanjärvi (Paper IV)

3 MATERIALS AND METHODS

The techniques and materials used in this study are listed in Table 4 and described in more detail in the respective papers I-IV. The strains used, their origin, and the accession numbers of sequences obtained in this study are listed in Table 5.

Table 4. The methods used in the study. The Roman numerals refer to the papers in which each method was applied and described in more detail.

Method	**Paper**
Sampling	I-IV
Determination of physicochemical parameters from water samples	III, IV
Strain isolation	I-IV
DNA extraction and purification	I-IV
PCR amplification	I-IV
16S rRNA gene sequencing	I-IV
rpoB and *rbcLX* gene sequencing	II
mcyE gene detection by PCR	I-IV
Morphological characterisation of strains by light microscopy	I-III
Electron microscopy of cyanobacterial cells	I, III
Microscopic counting of cyanobacterial species	III, IV
DGGE with cyanobacteria-specific primers	II, III
Cloning of the 16S rRNA gene	II, IV
Phylogenetic sequence analysis (NJ, ME, ML, MP)	I-IV
Image analysis of DGGE gels	IV
Ordination (PCA, CCA) and variance analysis	III, IV

Table 5. Cyanobacterial strains used, their origins, and the accession numbers of sequences determined in this study. Strains isolated in this study are in bold.

Taxonomic assignment	Strain	Geographical origin and isolation year	Accession numbers for 16S rRNA/ rpoB/ rbcLX genes	*mcyE*-PCR[a]	Paper
Anabaena					
An. augstumalis	SCMIDKE JAHNKE/4a	Rostock, Germany, unknown	AJ630458/ AJ628118/ AJ632057	-	II
An. cf. *circinalis var. macrospora*	**1tu23s3**	Lake Tuusulanjärvi, Finland, 2001	AJ630408/ AJ628068/ AJ632032	-	II, IV
An. cf. *circinalis var. macrospora*	**1tu26s10**	Lake Tuusulanjärvi, Finland, 2001	AJ630409/ AJ628069/ AJ632033	-	II
An. cf. *circinalis var. macrospora*	**1tu27s5**	Lake Tuusulanjärvi, Finland, 2001	AJ630410/ AJ628070/ AJ632036	-	II
An. cf. *circinalis var. macrospora*	**1tu28s13**	Lake Tuusulanjärvi, Finland, 2001	AJ630411/ AJ628071/ AJ632038	-	II
An. cf. *circinalis var. macrospora*	**0tu25s6**	Lake Tuusulanjärvi, Finland, 2000	AJ630412/ AJ628072/ AJ632022	-	II
An. cf. *crassa*	**1tu27s7**	Lake Tuusulanjärvi, Finland, 2001	AJ630413/ AJ628073/ AJ632037	-	II
An. cf. *cylindrica*	**XP6B**	Sediment, Porkkala, Helsinki, Gulf of Finland, Baltic Sea, 1999	AJ630414/ AJ628074/ AJ632062	-	II
An. circinalis	**1tu34s5**	Lake Tuusulanjärvi, Finland, 2001	AJ630415/ AJ628075/ AJ632049	-	II
An. circinalis	**1tu30s11**	Lake Tuusulanjärvi, Finland, 2001	AJ630416/ AJ628076/ AJ632041	-	II
An. circinalis	**1tu33s12**	Lake Tuusulanjärvi, Finland, 2001	AJ630417/ AJ628077/ AJ632042	-	II
An. compacta	ANACOM-KOR	Water reservoir, Kořensko, Czech Republic, 2002	AJ630418/ AJ628078/ AJ632031	-	II
An. flos-aquae	**1tu31s11**	Lake Tuusulanjärvi, Finland, 2001	AJ630419/ AJ628079/ AJ632045	+	II
An. flos-aquae	**0tu33s15**	Lake Tuusulanjärvi, Finland, 2000	AJ630420/ AJ628080/ AJ632023	-	II, IV
An. flos-aquae	**0tu33s2a**	Lake Tuusulanjärvi, Finland, 2000	AJ630421/ AJ628081/ AJ632024	-	II
An. flos-aquae	**1tu30s4**	Lake Tuusulanjärvi, Finland, 2001	AJ630422/ AJ628082/ AJ632044	+	II, IV
An. flos-aquae	**1tu35s12**	Lake Tuusulanjärvi, Finland, 2001	AJ630423/ AJ628083/ AJ632051	+	II, IV
An. lemmermannii	**1tu32s11**	Lake Tuusulanjärvi, Finland, 2001	AJ630424/ AJ628084/ AJ632046	+	II, IV
An. mucosa	**1tu35s5**	Lake Tuusulanjärvi, Finland, 2001	AJ630425/ AJ628085/ AJ632052	-	II, IV
An. oscillarioides	**BECID22**	Epiphytic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	AJ630426/ AJ628086/ AJ632060	-	II
An. oscillarioides	**BECID32**	Epilithic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	AJ630427/ AJ628087/ AJ632061	-	II
An. oscillarioides	BO HINDAK 1984/ 43	Canada, 1984	AJ630428/ AJ628088/ AJ632059	-	II
An. planctonica	**1tu33s10**	Lake Tuusulanjärvi, Finland, 2001	AJ630429/ AJ628089/ AJ632047	-	II
An. planctonica	**1tu28s8**	Lake Tuusulanjärvi, Finland, 2001	AJ630430/ AJ628090/ AJ632039	-	II
An. planctonica	**1tu30s13**	Lake Tuusulanjärvi, Finland, 2001	AJ630431/ AJ628091/ AJ632043	-	II
An. planctonica	**1tu33s8**	Lake Tuusulanjärvi, Finland, 2001	AJ630432/ AJ628092/ AJ632048	-	II, IV
An. planctonica	**1tu36s8**	Lake Tuusulanjärvi, Finland, 2001	AJ630433/ AJ628093/ AJ632053	-	II
An. sigmoidea	**0tu36s7**	Lake Tuusulanjärvi, Finland, 2000	AJ630434/ AJ628094/ AJ632025	-	II, IV
An. sigmoidea	**0tu38s4**	Lake Tuusulanjärvi, Finland, 2000	AJ630435/ AJ628095/ AJ632028	-	II, IV
An. smithii	**1tu39s8**	Lake Tuusulanjärvi, Finland, 2001	AJ630436/ AJ628096/ AJ632056	-	II
An. sp.	**1tu34s7**	Lake Tuusulanjärvi, Finland, 2001	AJ630437/ AJ628097/ AJ632050	-	II
An. sp.	**0tu37s9**	Lake Tuusulanjärvi, Finland, 2000	AJ630438/ AJ628098/ AJ632027	-	II
An. sp.	**0tu39s7**	Lake Tuusulanjärvi, Finland, 2000	AJ630439/ AJ628099/ AJ632029	-	II, IV
An. spiroides	**1tu39s17**	Lake Tuusulanjärvi, Finland, 2001	AJ630440/ AJ628100/ AJ632055	-	II
Aphanizomenon					
Ap. flos-aquae	**1tu29s19**	Lake Tuusulanjärvi, Finland, 2001	AJ630441/ AJ628101/ AJ632040	-	II
Ap. flos-aquae	**1tu37s13**	Lake Tuusulanjärvi, Finland, 2001	AJ630442/ AJ628102/ AJ632054	-	II
Ap. flos-aquae	**1tu26s2**	Lake Tuusulanjärvi, Finland, 2001	AJ630443/ AJ628103/ AJ632035	-	II, IV
Ap. gracile	Heaney/Camb 1986 140 1/1	Freshwater, Lough Neagh, Ireland, 1986	AJ630444/ AJ628104/ AJ632058	-	II
Ap. gracile	**1tu26s16**	Lake Tuusulanjärvi, Finland, 2001	AJ630445/ AJ628105/ AJ632034	-	II, IV
Ap. issatschenkoi	**0tu37s7**	Lake Tuusulanjärvi, Finland, 2000	AJ630446/ AJ628106/ AJ632026	-	II

Table 5. Continued

Taxonomic assignment	Strain	Geographical origin and isolation year	Accession numbers for 16S rRNA/ *rpoB*/ *rbcLX* genes	*mcyE*-PCR[a]	Paper
Nostoc					
N. calcicola	III	Field, České Budéjovice, Czech Republic, 1989	AJ630447/ AJ628107/ AJ632063	-	II
N. calcicola	VI	Field, Dobré Pole, Czech Republic, 1998	AJ630448/ AJ628108/ AJ632064	-	II
N. edaphicum	X	Field, Chelčice, Czech Republic, 1989	AJ630449/ AJ628109/ AJ632065	-	II
N. ellipsosporum	V	Field, Nezamyslice, Czech Republic, 1990	AJ630450/ AJ628110/ AJ632066	-	II
N. muscorum	I	Field, Dlouhá Ves, Czech Republic, 1986	AJ630451/ AJ628111/ -	-	II
N. muscorum	II	Field, Jevany, Czech Republic, 1985	AJ630452/ AJ628112/ -	-	II
N. sp.	**1tu14s8**	Lake Tuusulanjärvi, Finland, 2001	AJ630453/ AJ628113/ AJ632030	-	II, IV
Limnothrix					
L. redekei	**007a**	Lake Kastoria, Greece, 1999	AJ505941/ - /-	-	I
L. redekei	**165a**	Lake Kastoria, Greece, 1999	AJ505942/ - / -	-	I
L. redekei	**165c**	Lake Kastoria, Greece, 1999	AJ505943/ - /-	-	I
Merismopedia					
M. glauca	**0BB39S01**	Bubano Basin, Italy, 2000	AJ781044/ -/-	-	III
Microcystis					
M. sp.[b]	**1tu31s06**	Lake Tuusulanjärvi, Finland, 2001	AM259270/-/-	-	IV
Pseudanabaena					
P. sp. [b]	**0tu30s18**	Lake Tuusulanjärvi, Finland, 2000	AM259268/-/-	-	IV
Snowella			AM259269/-/-		
S. rosea	**1LM40S01**	Lake Maggiore, Italy, 2001		-	III
S. litoralis	**1LT47S05**	Lake Trasimeno, Italy, 2001	AJ781040/ -/-	-	III
S. litoralis	**0tu35s07**	Lake Tuusulanjärvi, Finland, 2000	AJ781039/ -/-	-	III
S. litoralis	**0tu37S04**	Lake Tuusulanjärvi, Finland, 2000	AJ781041/ -/-	-	III
Synechococcus			AJ781042/ -/-		
S. sp. [b]	**0tu28s07**	Lake Tuusulanjärvi, Finland, 2000		-	IV
S. sp. [b]	**0tu30s01**	Lake Tuusulanjärvi, Finland, 2000	AM259221/-/-	-	IV
S. sp. [b]	**1tu14s11**	Lake Tuusulanjärvi, Finland, 2001	AM259220/-/-	-	IV
S. sp. [b]	**1tu21s05**	Lake Tuusulanjärvi, Finland, 2001	AM259272/-/-	-	IV
S. sp. [b]	**1tu39s01**	Lake Tuusulanjärvi, Finland, 2001	AM259271/-/-	-	IV
Trichormus					
T. azollae	BAI/1983	Unknown, 1983	AJ630454/ AJ628114/ AJ632067	-	II
T. doliolum	1	Unknown	AJ630455/ AJ628115/ AJ632068	-	II
T. variabilis	GREIFSWALD	Unknown, 1992	AJ630456/ AJ628116/ AJ632069	-	II
T. variabilis	HINDAK 2001/4	Soil, Mts. Caucasus, USSR, 2001	AJ630457/ AJ628117/ AJ632070	-	II
Woronichinia					
W. naegeliana[c]	**0LE35S01**	Reservoir Letovice, Czech Republic, 2000	AJ781043 / -/-	nd	III

[a] +, product in PCR with *mcyE*-specific primers (Rantala et al. 2004) indicating potential hepatotoxicity, –, no product in *mcyE*-PCR;
[b] identification of strains based on the 16S rRNA gene sequence analysis;
[c] *W. naegeliana* strain was lost during the study; nd = not determined

4 RESULTS

4.1 Isolation and identification of planktic cyanobacterial strains

Four *Snowella* strains from Finland and Italy as well as a *Woronichinia* strain from the Czech Republic were isolated (III). Two of the *Snowella* strains were made axenic (III). Three green *Snowella* strains were identified as *S. litoralis* (Häyrén) Komárek et Hindák (Komárek and Anagnostidis 1999), and the red strain was identified as *S. rosea* (Snow) Elenkin (Komárek and Hindák 1988), according to colony structure, colour, and cell morphology (Fig. 1 and Table 3 in III). The *Woronichinia* strain was identified as *W. naegeliana* (Unger) Elenkin according to Komárek and Anagnostidis (1999) (Fig. 2 in III). Unfortunately, the *Woronichinia* strain died after some months of cultivation.

Cyanobacterial strains 007a, 165a, and 165c were isolated from plankton in Lake Kastoria (Greece). These thin, filamentous strains had polar gas vacuoles typical of *Limnothrix* at the beginning of the isolation process (Fig. 1 and Table 1 in I). Thylakoids of the strains were arranged parallel to the cell surface as determined by electron microscopy (Fig. 1 in I). The strains were identified as *L. redekei* (I).

Numerous morphologically heterogeneous *Anabaena* (28 strains) and *Aphanizomenon* (5 strains) were isolated from water samples of Lake Tuusulanjärvi, Finland (Table 5). In addition, three strains were isolated from benthic environments of the Baltic Sea (Table 5). The strains were identified in 30 cases at the species level and assigned to ten *Anabaena* and three *Aphanizomenon* species according to botanical criteria (see Table 2 in II).

Anabaena and *Aphanizomenon* isolates represented all 16S rRNA gene clusters of heterocytous cyanobacteria, which were commonly present in Lake Tuusulanjärvi during the two-year monitoring of the lake with DGGE and cloning of 16S rRNA genes (Fig. 5 in IV). Four strains representing the most common S*ynechococcus* genotypes and a strain representing the single homogeneous *Microcystis* genotype in lake were also isolated (Table 5; IV). These genotypes formed the main cyanobacterial community in the Lake Tuusulanjärvi (IV). In addition, strains belonging to genera *Snowella*, *Nostoc*, and *Pseudanabaena*, which were less abundant in Lake Tuusulanjärvi during the monitoring period, were isolated (Table 5; IV).

Several isolates changed their phenotypic features during laboratory cultivation. Many strains of heterocytous cyanobacteria did not produce akinetes after being cultivated for long periods in the laboratory (II). *Limnothrix redekei* strains did not produce polar gas vesicles (I), and *Snowella* and *Ap. flos-aquae* strains lost their colony structure (II; III) during laboratory cultivation.

All the strains (Table 5) were clonal isolates and either axenic or unicyanobacterial. Isolates were obtained by several transfers of colonies into a new solid Z8 medium with or without nitrogen (I-IV).

4.2 Phylogeny of heterocytous cyanobacteria

The studied *Anabaena*, *Aphanizomenon*, *Nostoc*, and *Trichormus* strains formed six clusters in 16S rRNA, *rpoB*, and *rbcLX* gene trees (Fig. 2; Fig. 4-6 in II). *Aphanizomenon* as well as all planktic and five benthic *Anabaena* isolates (BEDIC22, BECID32, XP6B, 1tu34s7, 277) clustered together (cluster 1) in all gene trees (Fig

2; Fig. 4-6 in II). The rest of the benthic *Anabaena* strains as well as the *Nostoc* and *Trichormus* strains formed clusters 2-6 (Fig 2; Fig. 4-6 in II). Clustering and subclustering within cluster 1 remained the same in all gene trees, with the exception of two strains (1tu34s5 and 1tu39s8) within cluster 1, and the different tree-constructing methods gave congruent results (Fig. 4-6 in II).

The planktic and benthic strains in cluster 1 were genetically heterogeneous (the 16S rRNA gene sequence similarity could be as low as 94.8%) and were divided into several (8-9) stable subclusters in the gene trees (Fig 2; Fig. 4-6 in II). The

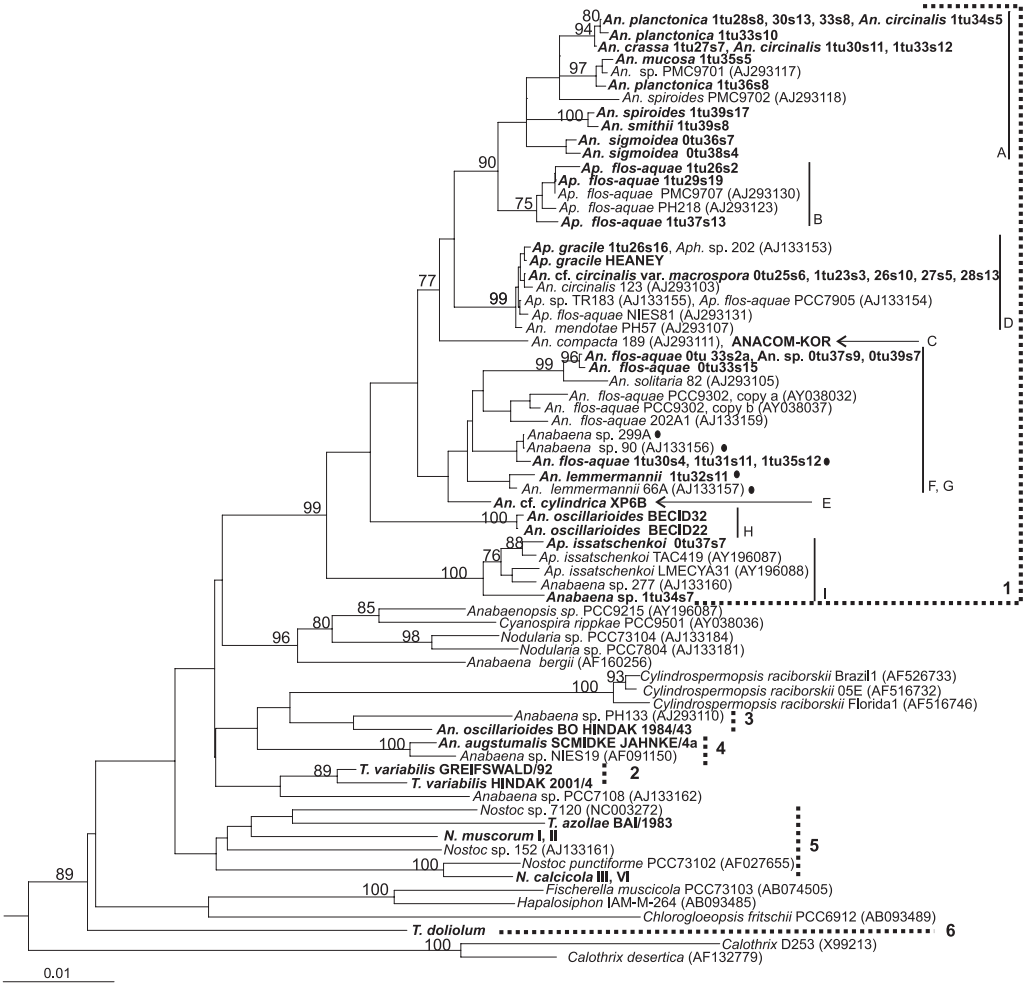

Fig. 2. A neighbour-joining tree based on the 16S rRNA gene sequences (1386 bp), showing clustering of the *Anabaena*, *Aphanizomenon* and *Trichormus* strains studied (in bold). Bootstrap values over 70% are shown at the nodes. The numbers 1-6 refer to the cluster and the letters A-I to the subclusters, which are discussed in text. Accession numbers of the sequences retrieved from the database are shown in parentheses. Potential hepatotoxic strains by mcyE-PCR are indicated by bullet points. The tree was constructed with a PAUP v10b (Swofford 2003) with 1000 bootstrap replicates, and using an F84 substitution model. Outgroup sequences, *Scytonema* sp. U-3-3 (AY069954) and *Chroococcidiopsis thermalis* PCC7203 (Z82789), are not shown.

16S rRNA gene similarities between the subclusters were in many cases above 97.5% (Table 3 in II). Nevertheless, most of the phylogenetic subclusters of *Anabaena* and *Aphanizomenon* strains were distinct from one other in at least one morphological feature (II). These features were trichome width, and morphology of terminal cells as well as morphology and development of akinetes (II). Potential hepatotoxic strains formed cluster F (III). Dense coiling was a stable characteristic for *An. compacta* (subcluster C), whereas other subclusters were comprised of strains with both coiled and straight trichomes. All the strains in cluster 1 were separated from the benthic *Anabaena* and *Trichormus* strains by the lack of terminal heterocytes (II).

Only three of the nine subclusters were formed by strains assigned to the same species (Fig. 2; Fig. 4-6 in II): The three strains of *An. compacta* formed the only monophyletic subcluster (Fig. 2; Fig 4 in II). The *Aphanizomenon flos-aquae* strains isolated in this study were placed in subcluster B; however, a few other *Ap. flos-aquae* strains (PCC7095, NIES81) were found in subcluster C. Two *An. oscillarioides* strains formed subcluster H and were distantly related to other *An. oscillarioides* strains in cluster 3 (Fig. 2; Fig. 4 in II). These separately clustered *An. oscillarioides* strains differed from the others by the morphology of terminal cells and the location of heterocytes (II). The six benthic *Anabaena* and *Trichormus* strains formed five distantly related clusters outside cluster 1 (Fig. 2; Fig. 4-6 in II). The three *Trichormus* strains included in this study were only distantly related to each other and did not form a monophyletic cluster.

4.3 Phylogeny of *Snowella* and *Woronichinia* strains

The four *Snowella* strains from Italy and Finland formed a monophyletic cluster in the phylogenetic tree based on 16S rRNA gene sequences, and they shared a 16S rRNA gene similarity of >98.4% (Fig. 3; Fig. 3 in III). The *Snowella* strains were most closely related to *Woronichinia naegeliana* 0LE35s01, which was also isolated here (16S rRNA sequence similarity 95-95.4%) (Fig. 3; Fig. 3 in III). These strains of the Gomphosphaerioideae subfamily and the strains *Merismopedia glauca* OBB39S01 identified in this study formed a cluster with *M. glauca* B1448-1 (Palińska et al. 1996) and *Synechocystis* strains [cluster 2.1 in Herdman et al. (2001)] (Fig. 3), which all belong to the same family, by Komárek and Anagnostidis (1999).

4.4 Phylogeny of *Limnothrix redekei* strains

Limnothrix redekei strains 007a, 165a, and 165c from Lake Kastoria (Greece) clustered together with the strain FP1 from Italy (Fig. 3; Fig. 2 in I), which has possibly been misidentified as *Planktothrix*. These four strains had trichomes characteristic of *Limnothrix* (i.e., cell length exceeding cell width) (I). The strains formed a separate cluster, which was only distantly related (< 91%) to the previously cultivated *L. redekei* strains Meffert 6705 (type strain) and NIVA/CYA 277/1 or to any other cyanobacteria (Fig. 2; Fig. 2 in I).

4.5 RNA polymerase β subunit (*rpoB*) as a phylogenetic marker gene

Primers were designed to amplify approximately 600 bp-long fragment of the *rpoB* gene from cyanobacteria (II). Phylogenetic trees were constructed either using all DNA codon positions or only the first two codon positions and the translated

amino acid sequences. The constructed trees were all fairly similar, and the conflicting nodes had bootstrap values less than 65% (II). The *rpo*B and 16S rRNA gene trees were congruent with the exceptions of two *Anabaena* strains (Fig. 4 and 5 in II). A highly variable region of insertions or deletions (indel) (33-144 bp) was found among the amplified *rpoB* gene fragments. The lengths and sequences of the indel region agreed with the clustering of strains in the *rpoB* and 16S rRNA gene trees (II). Most of the substitutions in the *rpoB* fragment occurred at the third codon position, and only six bases in the third codon position were conserved within

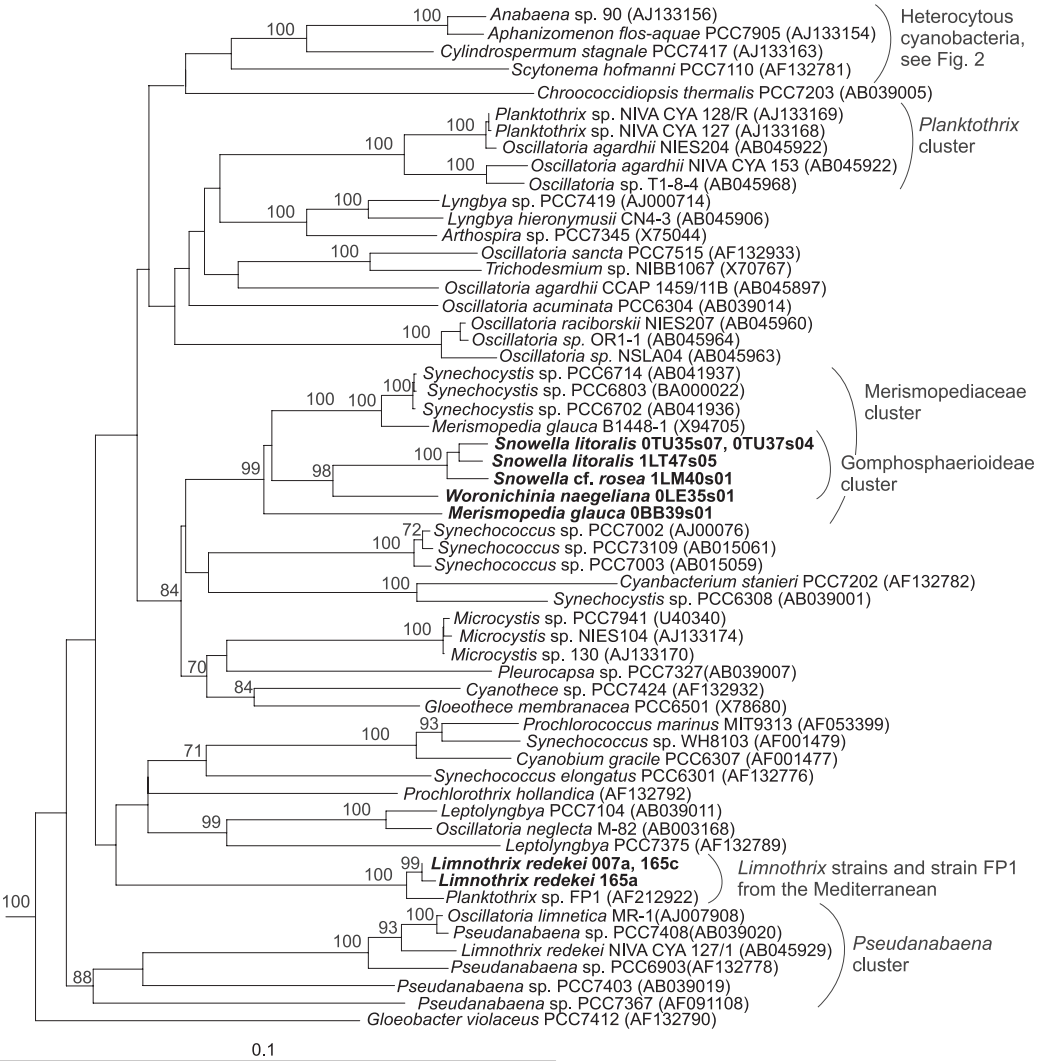

Fig. 3. A neighbour-joining tree based on 16S rRNA gene sequences (1386 bp) showing clustering of the *Limnothrix*, *Snowella*, *Woronichinia*, and *Merismopedia* strains studied (in bold). Bootstrap values over 70% are shown at the nodes. The tree was constructed with a PAUP v10b (Swofford 2003) with 1000 bootstrap replicates using an F84 substitution model. Accession numbers of sequences retrieved from the database are shown in parentheses. Outgroup sequences, *Bacillus subtilis* NCD0769 (X60646) and *E. coli* K12 (U00096), are not shown.

600 bp-long alignment of all heterocytous cyanobacterial, *Planktothrix*, and *Microcystis* sequences (data not shown).

4.6 Environmental factors related to cyanobacterial genotypes and morphotypes in Finnish lakes

The cyanobacterial community composition in Lake Tuusulanjärvi was studied by microscopy, strain isolation, as well as 16S rRNA gene-based methods including DGGE, cloning and sequencing of clones and DGGE bands (IV). In addition, the occurrences of the genera *Snowella*, *Woronichinia*, and *Merismopedia* were investigated in 56 Finnish lakes by microscopic counting (III).

4.6.1 Cyanobacterial community composition in Lake Tuusulanjärvi

During the two-year monitoring period, the cyanobacterial community in Lake Tuusulanjärvi was formed mainly of *Microcystis*, heterocytous cyanobacteria (*Anabaena/Aphanizomenon*), and *Synechococcus* (IV). The biomass levels of *Microcystis* and heterocytous cyanobacteria were at their highest in mid and late summer respectively (IV). Members of Chroococcales other than *Microcystis* (picocyanobacteria) contributed little to the total cyanobacterial biomass (Fig. 2 in IV). However, picocyanobacteria were abundant based on the cell numbers and the sequencing of clones (III). Based on the cloning results, *Synechococcus* was the most common picocyanobacterial genus, and generally more common than *Microcystis*. Unlike the cloning results, *Synechococcus* morphotypes always accounted for less than 1% of the total cyanobacterial biomass, and other picocyanobacterial morphotypes (*Aphanocapsa* and *Chroococcus*) were more common than *Synechococcus* in microscopic counting (IV). By microscopy, 32 Chroococcales and 11 *Anabaena/Aphanizomenon* species were recognised during the two-year monitoring period. By molecular methods, *Anabaena/Aphanizomenon* and *Synechococcus* populations in Lake Tuusulanjärvi contained 8 and 10 genotypes respectively. These results indicate that diverse heterocytous cyanobacterial and picocyanobacterial populations were present in Lake Tuusulanjärvi (IV).

4.6.2 Cyanobacterial community composition in relation to environmental conditions

The occurrence and the biomass of the main cyanobacterial groups (heterocytous cyanobacteria, *Microcystis*, and *Synechocystis*) were related to different seasons and environmental conditions by canonical correspondence analysis (CCA) (IV). Colonial and buoyant *Microcystis* populations dominated in mid-summer but were replaced by nitrogen-fixing and buoyant *Anabaena* and *Aphanizomenon* population in late summer (Fig. 1 and 6 in IV). Picocyanobacteria contributed to the biomass mostly in early summer, although another biomass maximum peaked in late summer. In CCA the environmental factors that significantly ($p<0.05$) contributed to the model based on the DGGE data were global radiation, TP, DIN:DIP, and water temperature. In CCA, based on microscopic data, global radiation, TP, and DIP were the most significant factors.

According to CCA based on the microscopic data, high temperatures (>17°C), global radiation (>110 MJ m^{-2}), and concentrations of TP (>90 µg l^{-1}) and DIP (>30 µg l^{-1}) were related to *Microcystis* dominance (except *M. ichthyoblabe*), whereas low phosphorus concentrations (TP <85 µg l^{-1}; DIP <30

μg l^{-1}) and temperatures between 13-17°C were related to the success of heterocytous cyanobacteria (except *Aphanizomenon* sp.) in Lake Tuusulanjärvi (Fig. 6 in IV). Both *Microcystis* and heterocytous cyanobacteria dominated and reached high biomass levels in low DIN concentration (<100 μg l^{-1}) and DIN:DIP (<5 w:w) (Fig. 6 in IV). The occurrence of picocyanobacteria was related to intermediate global radiation and DIP concentrations (Fig. 6 in IV).

Cyanobacterial genotypes found in DGGE were not as clearly related to specific environmental conditions as were the morphotypes. The reason was probably the use of less informative presence/absence results instead of band intensities in DGGE. The nested PCR protocol used did not allow the use of DGGE band intensities as estimates of cyanobacterial abundances. *Microcystis* was present in almost all samples and therefore could not be related to any specific environmental condition (Fig. 6 in IV). In contrast, different genotypes of heterocytous cyanobacteria were related to different environmental conditions (Fig. 6 in IV). *Aphanizomenon flos-aquae*-related bands were associated with high DIN:DIP in early summer, whereas a few bands representing potentially hepatotoxic *Anabaena* were found in late summer (Fig. 6 in IV). The potentially hepatotoxic *Anabaena* genotypes were also abundant, but not dominant in late summer samples, when studied by cloning. In the samples, these genotypes co-occurred with non-hepatotoxic *Anabaena* genotypes (IV). Different non-toxic *Anabaena/Aphanizomenon*-related bands were found during all seasons and unidentified Chroococcales bands in early and late summer (Fig. 6 in IV).

4.6.3 Occurrence of *Snowella* and *Woronichinia*

A survey of 56 Finnish lakes showed that the genera *Snowella* and *Woronichinia* were commonly detected in lakes and occasionally dominated at least in July, before cyanobacteria reached its maximum (III). *Snowella* was most commonly detected and reached the highest biomass levels in oligo-mesotrophic (<35 μg P l^{-1}) lakes, while *Woronichinia* was found in mesotrophic (10-35 μg P l^{-1}) conditions (Fig. 4 in III). On the other hand, *Woronichinia* commonly formed blooms in eutrophic Czech reservoirs (III).

Snowella and *Woronichinia* had low biomass (<0.05 μg f.w. l^{-1}) in Lake Tuusulanjärvi throughout the two-year study period (IV). In addition, their abundance was generally low when analysed by molecular methods except in July 2000, when *Snowella* accounted for 16% of the cyanobacterial clones.

4.7 Cyanobacterial community composition by DGGE, cloning of the 16S rRNA gene, and microscopic counting

Three method – microscopic counting of cyanobacterial morphotypes, DGGE with cyanobacterial specific primers, and cloning of 16S rRNA gene – as well as sequencing of DGGE bands and clones were used to study cyanobacterial community composition in Lake Tuusulanjärvi. Generally, all three methods detected the major cyanobacterial groups, i.e., heterocytous cyanobacteria, *Microcystis*, and unicellular picocyanobacteria (IV). However, their relative abundance in the cyanobacterial community differed, depending on the method (IV). Cloning seemed to give higher estimates for proportions of heterocytous genera (*Anabaena*/*Aphanizomenon*)

and unicellular picocyanobacteria (*Synechococcus*) than did microscopic counting (IV). Those genotypes of heterocytous cyanobacteria that were most abundant when measured by cloning were also detected by DGGE in all samples (IV). *Microcystis* was detected in the same samples by DGGE and cloning (Fig. 4 in IV). *Synechococcus* was rarely detected by DGGE. In two out of eight samples in which it was detected by both DGGE and cloning, a single *Synechococcus* genotype made up a large proportion of the total cyanobacterial population (IV). In the other cloned samples, several *Synechococcus* genotypes were present, and the most abundant *Synechococcus* genotype accounted for less than 13% of the total abundance of cyanobacteria by cloning (IV).

5 DISCUSSION

5.1 Phylogenetic relationships of heterocytous cyanobacteria

The re-evaluation of the classification of cyanobacteria has been complicated, owing to the misidentification of strains, the lack of isolates and genetic information for many morphotypes, as well as inadequate morphological data on many genetically characterised strains (Komárek and Anagnostidis 1989; Wilmotte and Herdman 2001). In addition, the phenotype of heterocytous cyanobacteria is known to change occasionally during prolonged laboratory cultivation (Rippka et al. 2001b; Lehtimäki et al. 2000; Gugger et al. 2002a), which makes their identification at the species level difficult. Therefore, in this study morphology and phylogenetic relationships were characterised from freshly isolated *Anabaena* and *Aphanizomenon* strains. The genera *Anabaena* and *Aphanizomenon* were found to be intermixed (II), a finding consistent with previous studies based on sequences of the 16S rRNA gene (Lyra et al. 2001; Gugger et al. 2002a; Iteman et al. 2002), the *rbcLX* and ITS regions (Gugger et al. 2002a), and cellular fatty acids (Gugger et al. 2002b).

Although the strains belonging to the genera *Anabaena* and *Aphanizomenon* have complex morphology with differentiated cells in comparison to many other cyanobacteria, defining the *Anabaena* and *Aphanizomenon* species has been problematic due to a continuous variation of morphological features (Komárek and Kováčik 1989). The *Anabaena* and *Aphanizomenon* strains studied here formed several stable clusters and subclusters in the 16S rRNA, *rpoB*, and *rbcLX* gene trees (Fig. 2). However, most of the *Anabaena*/*Aphanizomenon* subclusters contained several "botanical species". Similar results have been found by others in studies based on the 16S rRNA gene (Gugger et al. 2002a; Iteman et al. 2002), the *rbcLX* and ITS region (Gugger et al. 2002a), whole-cell protein patterns (Lyra et al. 1997), whole-genome fingerprinting (Lyra et al. 2001), and fatty-acid profiles (Li and Watanabe 2001; Gugger et al. 2002b). Only the *An. compacta* strains were monophyletic (II). Most of the *Ap. flos-aquae* strains were clustered together in phylogenetic trees (cluster B in Fig. 2), but few strains were placed outside the cluster. However, the identification of these *Ap. flos-aquae* strains was problematic: the morphology of PCC7905 no longer corresponds to the description of this species (Rippka et al. 2001b), and morphology NIES81 is not described. As Wilmotte and Herdman (2001) have pointed out, misidentification as well as lack of morphological description of cyanobacterial strains complicates the interpretation of phylogenetic trees. Nevertheless, the genetic relationships of the *Anabaena* and *Aphanizomenon* species do not follow their current classification by Komárek and Anagnostidis (1989) or Boone and Castenholz (2001).

The cluster of planktic *Anabaena*/*Aphanizomenon* strains has been separated from benthic *Anabaena* strains in analyses of the 16S rRNA gene (Gugger et al. 2002a; Iteman et al. 2002) and the *rbcLX* and ITS regions (Gugger et al. 2002a). In this study, five benthic *Anabaena* strains (i.e. no visible gas vacuoles under light microscopy) were intermixed with planktic *Anabaena* strains (III). Three of these five benthic strains were isolated from sediment and surfaces of plants on

the shores of the Baltic Sea (strains BECID 22, BECID33, and XP6B), whereas two strains (Itu34s7, 277), which did not have visible gas vacuoles and were not buoyant in culture, originated from lake plankton samples (II). However, it is not known whether these benthic strains are capable of forming gas vacuoles in different culturing conditions or whether they have the *gvp* genes. The loss of ability to produce gas vacuoles has been reported in several cyanobacterial strains, e.g., in *Aphanizomenon* sp. PCC7905 (Rippka and Herman 1992), and in *Limnothrix redekei* Meffert (Meffert 1987). Moreover, spontaneous mutations in gas vesicle genes of *Planktothrix* (Beard et al. 2002) and *Microcystis aeruginosa* (Mlouka et al. 2004) strains have been observed in laboratory cultures (Beard et al. 2002). The rearrangements of *gvp* genes of these strains led to the loss of gas vesicles or the production of less and weaker gas vesicles, which could not provide buoyancy (Beard et al. 2002; Mlouka et al. 2004). Nevertheless, both benthic and planktic strains in cluster 1 were distinguished from other benthic *Anabaena* and *Trichormus* clusters by the absence of terminal heterocytes (III). *Trichormus* strains, which were morphologically separated from *Anabaeana* on the basis of akinete development (Komárek and Anagnostidis 1989), did not form a monophyletic cluster. More strains need to be studied before phylogenetic relationships of these distantly related benthic strains can be accurately inferred.

Morphological features were found to separate most of the phylogenetic subclusters (II). Those features included akinete parameters, trichome width, and morphology of terminal cells. The same morphological features were found to be stable in several *Anabaena* strains under different light and temperature conditions (Stulp and Stam 1982; 1985). However, the 16S rRNA gene similarities between the subclusters were in most cases over 97.5% (II) - the suggested cut-off point for species definition (Stackebrandt and Goebel 1994). Thus, the strains of subclusters might belong to the same or to different species. Zeigler (2003) found that phylogenies based on one to three carefully selected genes could reliably predict the genomic relatedness of organisms. Unfortunately, it is not known which genes might be reliable phylogenetic marker genes for cyanobacteria. Recently, Sánchez-Baracaldo et al. (2005) tested congruence between cyanobacterial tree topologies based on rRNA genes and 34 genes coding for metabolic, cellular processing, and information processing proteins. Of these gene trees, only three metabolic protein gene-based trees showed significant incongruence (Sánchez-Baracaldo et al. 2005). In this study, *rpoB* and *rbcLX* gene tree topologies supported subclustering of the strains in the 16S rRNA gene tree (II).

The DNA:DNA reassociation studies performed for a few *Anabaena* strains (Lachance 1981; Stam 1980; Stulp and Stam 1984, 1985) have shown that their DNA: DNA relatedness is well in accordance with their distances in the 16S rRNA gene trees (Wilmotte and Herdman 2001). The DNA:DNA reassociation studies also verified high genetic heterogeneity among *Anabaena* strains (Lachance 1981; Stam 1980; Stulp and Stam 1984; 1985). Stulp and Stam (1984) found that morphology is well in accordance with DNA:DNA reassociation values for *Anabaena* strains. However, this study was based on a rather small set of strains (Stulp and Stam 1984; Rippka et al. 2001a).

Intragenomic heterogeneity in 16S rRNA gene copies could effect phylo-

genetic relationships of heterocytous cyanobacteria. The *Anabaena/ Aphanizomenon* strains included in this study had several different copies (up to six) of the 16S rRNA gene according to 16S rRNA-DGGE (II). Information on intragenomic divergence of the 16S rRNA genes was available for four heterocytous cyanobacterial strains (*Nostoc* PCC7120, *N. punctiforme* PCC73102, *Anabaena* sp. PCC9302, and *An. variabilis* ATCC29413) and varied from 0 to 1.3% (Table 2). Generally, the intragenomic divergence of the 16S rRNA genes among bacteria seems to be low (less than 1%), whereas exceptionally high divergence (up to 11.6%) seemed to be related to thermophilic bacteria (Acinas et al. 2004). Among cyanobacteria, the highest divergence of the 16S rRNA gene copies (1.3% in *Anabaena* sp. PCC9302) was enough to make the copies appear in two different taxonomic units within the same cluster (Iteman et al. 2002). Thus, the small distances within *Anabaena/Aphanizomenon* subclusters might be affected by heterogeneities in their 16S rRNA gene copies, but probably would not change the subclustering of the *Anabaena/Aphanizomenon* strains. The classification of these planktic *Anabaena/ Aphanizomenon* strains remains to be confirmed by the DNA:DNA reassociation method, by genome sequencing, or by a novel technique such as the random genome fragment hybridisation on a DNA microarray (Cho and Tiedje 2001).

5.2 Phylogeny of *Snowella*, *Woronichinia*, and *Limnothrix* strains

Many cyanobacterial species, or even genera or subfamilies described under the botanical code, have not been studied genetically, and thus their phylogenetical positions are not known. The *Snowella* and *Woronichinia* strains isolated in this study are the only strains of the entire Gomphosphaerioideae subfamily that have been studied phylogenetically. The *Snowella* strains formed a monophyletic cluster that, based on the 16S rRNA gene analysis, was a sister cluster to the *Woronichinia naegeliana* strain (III). The results showed that the morphology of these cyanobacterial genera was congruent with their phylogeny (III). Thus, phylogeny supports their classification by Komárek and Anagnostidis (1999) (see Table 1). Probably due to the lack of isolates and genetic information, these genera have not been included in Bergey's Manual of Systematic Bacteriology (Boone and Castenholz 2001).

During the publication of paper III, two short (316 and 645 bp) 16S rRNA gene sequences, which originated from a DGGE band and a clone of environmental sample and were related to *Snowella* and *Woronichinia*, were published (Boutte et al. 2005). The affiliation of these two sequences was based on unpublished sequences of the *Snowella* 1ES42-s2 and *Woronichinia* 1ES42-s1 strains (Boutte et al. 2005). The *Snowella*-related DGGE sequence had high similarity (97.8-99.4%) and clustered with sequences of *Snowella* strains isolated in this study. In addition, the *Woronichinia*-related clone sequence shared high 16S rRNA sequence similarity (99.8%) with the *Woronichinia* 0LE35s01 strain (data not shown). These findings further support the relationships of *Woronichinia* and *Snowella* strains.

The classification of filamentous, non-heterocytous cyanobacteria is currently confusing; the 16S rRNA gene as well as the DNA:DNA reassociation studies have revealed that the classification is not congruent with the genetic relationships (Wilmotte and Golubić 1991; Turner 1997; Suda et al. 2002). Revision of the

classification was proposed by Suda et al. (2002). According to the phylogenetic analysis of 16S rRNA gene sequences, *Limnothrix redekei* strains NIVA CYA 277/1 and Meffert 6705 clustered with *Pseudanabaena* strains (Wilmotte and Golubić 1991; Turner 1997; Suda et al. 2002). The Greek strains isolated in this study formed a cluster with the *Planktothrix* sp. FP1 strain (Pomati et al. 2000) and were separated from the *Pseudanabaena* clusters according to 16S rRNA gene analysis (Fig. 3; Fig. 2 in I). These results indicate a high diversity among these thin, filamentous, morphologically similar cyanobacterial strains.

The classification of thin, filamentous cyanobacteria on the basis of morphology is difficult (Komárek 2000). The *Limnothrix redekei* strains in this study and the *Planktothrix* sp. FP1 strain shared the trichome morphology characteristic of *Limnothix/Pseudanabaeana* (I). Thus, the *Planktothrix* sp. FP1 strain was probably misidentified. In addition, the *L. redekei* strains had the typical polar gas vacuoles at the beginning of the isolation process (I). Furthermore, electron microscopy (EM) showed that the thylakoids of *Limnothrix* strains were arranged parallel to cell surface (I), which separates the thin, filamentous genera *Pseudanabanena*, *Geitlerinema*, and *Jaaginema* from the large filamentous "Oscillatoriales" (*Planktothrix*, *Oscillatoriales*, *Phormidium*, and *Lyngbya*) (Komárek and Kaštovský 2003). The presence of gas vesicles was not verified in the EM, but the loss of gas vacuoles during laboratory cultivation of *L. redekei* has been pointed out previously by Meffert (1987).

The phylogenetic relationships of *Snowella*, *Woronichinia* and *Limnothrix* strains were based on the 16S rRNA gene analysis. The 16S rRNA copy number of these strains is not known. However, the genetic distances between these strains and their closest relatives were large, and thus, the low intragenomic divergence generally found probably did not play an important role in the inferred phylogenetic relationships. Moreover, based on the small set of cyanobacterial genome sequences, it appeared that Chroococcales strains had one or two identical copies of the 16S rRNA genes (Table 2).

5.3 *rpoB* as an alternative phylogenetic marker gene

The *rpoB* gene, which encodes the RNA polymerase subunit β, has been applied as a marker gene for bacterial (Morse et al. 2002; Mollet et al. 1997) and archaeal phylogeny (Klenk and Zillig 1994; Walsh et al. 2004) as well as for species identification (Kim et al. 1999; Renesto et al. 2001; Lee et al. 2000, 2003). Recently, *rpoB* gene has also been applied to the study of cyanobacteria (II; Lyra et al. 2005). The *rpoB* gene has many characteristics needed for a good marker gene; it is universally distributed, has a conserved function in DNA transcription, contains both conserved as well as variable regions, and is over one thousand amino acids in length (Palenik 1992; Ludwig and Klenk 2001). In addition, based on 175 bacterial genome sequences, Konstantinidis and Tiedje (2005a) found a strong correlation (r=0.78) between *rpoB* gene similarity and the AAI of genomes, indicating the usefulness of *rpoB* for phylogenetic analysis.

The present study found a high degree of congruence between the cyanobacterial *rpoB* and 16S rRNA gene tree topologies (II). Moreover, the length and sequence of the indel region in the *rpoB* gene supported the clustering and could be used as a signature sequence for the clusters. Similar

results were obtained with *Nodularia* strains (Lyra et al. 2005). The 451 bp fragment of the *rpoB* gene had a resolution more or less equal to the 1393 bp of the 16S rRNA gene. Sequencing of a longer fragment of *rpoB* of cyanobacterial strains would allow higher resolution than does the 16S rRNA gene, as has been reported for several bacterial genera (Mollet et al. 1997; Renesto et al. 2001; Taillardat-Bisch et al. 2003). The present work's results showed that in addition to the other RNA polymerase gene *rpoC* applied previously (Palenik and Haselkorn 1992; Palenik and Swift 1996), *rpoB* is a good alternative marker gene for cyanobacteria.

The attributes of *rpoB* and the fact that it is found in only one copy in bacterial genomes (Santos and Ochman 2004) also make *rpoB* an attractive marker gene for ecological applications such as DGGE and quantitative PCR. Actually, the *rpoB* gene has been applied in DGGE for marine bacterial isolates (Dahllöf et al. 2000), for soil microbial populations (Peixoto et al. 2002), and in the detection of *Bacillus anthracis* by real-time PCR (Qi et al. 2001). However, the great number of substitutions at the third codon position of *rpoB* gene caused problems in designing general primers for cyanobacteria; only six bases at the third codon position within a 600 bp fragment sequenced were constant in all cyanobacteria studied (data not shown). By analysing 132 complete genomes, Santos and Ochman (2004) found that 39 of the protein-coding genes had conserved regions useful for primer design within 143 single-copy genes present in 95% of sequenced bacteria (Santos and Ochman 2004). Primers with degenerate base(s), commonly used to recover all the target organisms, generate double bands in DGGE (Kowalchuk et al. 1997; Dahllöf et al. 2000) and should therefore be avoided.

5.4 The importance of isolation of planktic cyanobacterial strains

The identification of freshly isolated cyanobacterial cultures was underlined in this study. Many strains of this study changed their morphology during laboratory cultivation. Some heterocytous cyanobacterial strains no longer produced akinetes (II), and *Limnothrix redekei* showed no polar gas vacuoles (I). *Snowella* and *Ap. flos-aquae* strains lost their colony structure and grew unicellular and as a single filaments respectively (III, II). These morphological features are critical for the morphological identification of these genera. Changes in phenotype during laboratory cultivation seem to be common among cyanobacteria [*Microcystis* (Doers and Parker 1988; Mlouka et al. 2004), *Aphanizomenon* (Rippka et al. 2001b; Gugger et al. 2002a), *Nodularia* (Lehtimäki et al. 2000), *Merismopedia* (Palińska et al. 1996), and *Planktothrix* (Beard et al. 2002)]. The altered phenotype can make correct microscopic identification of the strains difficult or impossible: the *Anabaena* strains without akinetes cannot be identified at the species level (II), *L. redekei* without polar gas vacuoles could not be separated from the other thin filamentous cyanobacteria (I), and *Snowella* strains, which have lost their colony structure, could easily be misidentified as *Synechocystis* (III). Amended growth conditions could allow the re-appearance of these features for the strains; however, these conditions have not yet been determined. For example, Ernst et al. (2005) showed that the difficulty in culturing *Synechococcus* from mesotrophic lake was caused by the high nitrate and phosphate content of standard cultivation media. Lowering the phosphorus and nitrogen concentrations to 30% and below 10%, respectively, of

the original BG-11 medium enhanced the growth of *Synechococcus* (Ernst et al. 2005).

In this study, isolation was successful for all the main genotypes detected by DGGE and cloning in Lake Tuusulanjärvi (IV). However, some cyanobacteria cannot easily be isolated: the dominant *Aphanizomenon* genotype in the Baltic Sea, which frequently forms blooms, has not been isolated, despite several attempts (Laamanen et al. 2002). Knowledge of metabolic capabilities retrieved from a metagenomic study led to the design of successful isolation and cultivation strategy for *Leptospirillium* (Tyson et al. 2004).

5.5 Application of molecular biological tools for planktic cyanobacteria

In contrast to most other bacteria, cyanobacteria can be identified in most cases at the species level under microscopy. This makes it possible to compare molecular methods, and microscopic enumeration, and evaluate the usefulness of these methods in describing the cyanobacterial community composition (IV). Similar to previous studies combining molecular biological methods and microscopy (Table 6), the major cyanobacterial groups were generally found by both molecular biological methods (DGGE and cloning) and microscopic counting (IV).

However, cloning gave higher estimates for the proportion of heterocytous cyanobacteria than microscopy. Heterocytous cyanobacteria have been shown to contain up to five 16S rRNA gene copies, whereas Chroococcales have generally only one or two copies (Table 2). The high copy number of the 16S rRNA gene among heterocytous cyanobacteria has led to their overestimation in 16S rRNA gene-based methods. Crosby and Criddle (2003) compared the 16S rRNA gene/ITS based on community fingerprinting techniques (LH-PCR, DGGE, RFLP, and ARISA) by *in silico* analysis of hypothetical bacterial community constructed from genome database and observed that all the techniques skewed the diversity towards organisms with high rRNA gene copy number. Acinas et al. (2004) estimated that molecular methods based on rRNA genes caused overestimation of bacterial diversity by 2.5 to 3-fold. This finding was in agreement with the results of the present study, where the proportion of heterocytous cyanobacteria was on average 2.4 times higher by cloning of the 16S rRNA gene than by microscopic counting (IV).

Synechococcus was very rarely detected by microscopy or by DGGE, although *Synechococcus* was a major cyanobacterial group when measured by cloning. The heterogeneity of *Synechococcus* genotypes (ten clusters) in Lake Tuusulanjärvi probably caused their flawed detection in DGGE. In DGGE a single base difference in sequences cause the migration to different gel positions (Nübel et al. 1996) and consequently, their detection as different genotypes. Because many of the DGGE bands could not be sequenced, the presence of *Synechococcus* genotypes among numerous Chroococcales/Oscillatoriales related bands in gel is possible (Fig. 3 in IV). Moreover, *Synechococcus* was detected by DGGE when a single genotype made up a major fraction of the total *Synechococcus* population (IV). Similarly to the present study, Ferris and Ward (1997) also found that the detection of cyanobacteria in DGGE seems to be limited to major populations.

Incongruence between classification of cyanobacteria and their 16S rRNA gene phylogeny (Wilmotte and Herdman 2001)

Table 6. Studies of cyanobacterial community composition by polyphasic approach (DGGE, cloning, microscopy (MC), and/or strain isolation).

Samples	Methods*	Congruence of methods	Reference
Planktic cyanobacteria			
Lake Vilar and Lake Cisó, Spain	MC, DGGE[1]	Two major cyanobacterial populations in lakes were observed by both DGGE and MC. The abundance of morphotypes was reflected in the signal intensity of the bands.	Casamayor et al. 2000
Water reservoirs in Czech Republic	MC, DGGE[1] and cloning[3]	Major cyanobacterial groups were detected by all methods. Differences in detection of minor groups. Molecular methods were more sensitive in detecting picocyanobacteria than MC.	Boutte et al. 2005
Benthic cyanobacteria			
Microbial mat in evaporation ponds of saltern and in salt marsh, Mexico	MC, DGGE[1], carotenoids	General congruence in diversity indeces based on morphotypes, 16S rRNA gene sequences,and carotenoids.	Nübel et al. 1999
Endolithic cyanobacteria in dolomite, Switzerland	MC, isolation, DGGE[1], cloning	MC, DGGE, and cloning generally congruent, higher number of genotypes were detected with DGGE than morphotypes in MC. *Calothrix* not detected by MC. Enrichment isolation with BG-11 underestimated the diversity.	Sigler et al. 2003
Microbial mat, hot springs, Thailand, Philippines and China	MC, DGGE[1]	Higher number of genotypes (3-10) was detected in DGGE than morphotypes in MC (1-4). Novel sequence clusters were found.	Jing et al. 2005
Soil desert crusts, Colorado Plateau, USA	MC, DGGE[1], sequencing of picked filaments, strain isolation	Microscopy underestimated diversity of thin filamentous cyanobacteria and DGGE failed to detect heterocytous cyanobacteria with thick sheets. Isolation successful for 5 of 6 sequence clusters. A novel sequence clusters were found.	Garcia-Pichel et al. 2001
Microbialites, Lagoon of Pacific atoll, French Polynesia	MC, DGGE[1], strain isolation	MC and DGGE results were generally congruent. Isolation with ASN media biased towards filamentous cyanobacteria with narrow trichomes. Novel clusters of sequences and strains were found.	Abed et al. 2003
Solar Lake mat, Sinai, Egypt	MC, DGGE[1], strain isolation	General congruence between the diversity of molecular methods and MC. Novel sequence clusters detected. Isolation successful for one of the found cluster.	Abed and Garcia-Pichel 2001
Microbial mats, McMurdo Dry Valleys, Antarctica	MC, DGGE[1], cloning[2]	Althought a higher number of genotypes were detected by molecular methods than morphotypes by MC, some cyanobacteria were detected only in MC. Detection of some major groups failed in DGGE. Novel sequence clusters were found.	Taton et al. 2003
Microbial mat in evaporation ponds of saltern, Mexico	MC, DGGE[1], strain isolation	Majority of DGGE sequences were related to cultivated strains. The sequences not related to cultivated strains represented a minor population. Congruence between microscopy counts and abundances based on DGGE band intensities.	Nübel et al. 2000

* with cyanobacteria –specific primers. [1]Nübel et al. 1997; [2]primers 16S27f (=pA) (Edwards et al. 1989) -23S30R (Taton et al. 2003)

complicates the comparison of results of molecular biological methods and microscopy. Morphological identification of the simple unicellular Chroococcales species is difficult (Komárek et al. 2004). In this study, Chroococcales, which were not identified at the species level, accounted as many as 17% of the total cyanobacterial biomass in some samples (IV). Nevertheless, the proportion of all unicellular Chroococcales was higher when measured by cloning than by microscopy. However, unicellular Chroococcales dominated when cell numbers were used instead of biomass in microscopy enumeration. The large *Microcystis* colonies might have also masked picocyanobacteria in microscopic enumeration.

Molecular ecology tools have been applied together with microscopy mainly in studies of cyanobacterial mats in extreme environments such as hot springs and ponds of salterns (Table 6). In these environments a small number (1-10) of cyanobacterial morphotypes and genotypes was usually found, whereas up to 30 morphotypes in one sample were detected in Lake Tuusulanjärvi. Generally, in the studies of cyanobacterial mats, DGGE have revealed higher diversity (or species richness) than microscopy (Table 6.). In this study, the highest number of different species was detected by microscopy (IV). However, most of these species were present in low biomass levels, a factor that could explain why lower number of species were detected by DGGE or cloning. Recently, Boutte et al. (2005) compared cloning and DGGE methods in reservoirs in the Czech Republic and obtained similar results. Based on the polyphasic studies, it can be concluded that the simultaneous use of several methods gives a more complete picture of cyanobacterial community composition. However, in some cases a significant population can be underestimated (*Synechococcus* in this study) or even undetected if only a single method is used (e.g., microscopy or DGGE) (Garcia-Pichel et al. 2001; Taton et al. 2003).

The nested PCR protocol used in this study was necessary to obtain high enough sensitivity, and thus DGGE band intensities could not be used as estimates for genotype abundances (IV). DGGE band intensities have been successfully used to quantify cyanobacterial genotypes (Casamayor et al. 2000; Kolmonen et al. 2004), although PCR-based biases have been shown to affect the results of DGGE and cloning (see von Wintzingeroda et al. 1997; Wilson 1997). The differences in community composition between the methods found in this study seem to be more related to the 16S rRNA copy number and the identification difficulties among picocyanobacteria. FISH, which do not need DNA extraction and PCR, might give more quantitative results than PCR-based methods (Zwirglmaier 2005). FISH combined with flow cytometry makes it possible to analyse a large number of samples (Zwirglmaier 2005; Sekar et al. 2004). Microarrays also have potential as a rapid detection method for numerous bacterial genera by single hybridisation reaction and have been applied to planktic cyanobacteria (Rudi et al 2000; Castiglioni et al. 2004).

5.6 Environmental factors affecting the cyanobacterial community composition in Lake Tuusulanjärvi

The cyanobacterial biomass has been shown to increase with increasing nutrient concentration, especially to total phosphorus (TP) (Schindler 1977; Watson et al. 1997). However, the effect

of phosphorus concentration on the success of different cyanobacterial groups is contradictory (Jensen et al. 1994; Varis 1993; Schreurs 1992). According to our data, *Microcystis* was favoured by high TP and DIP, and heterocytous cyanobacteria by low TP and DIP concentrations (IV). In comparison to *Anabaena*, *Microcystis* has a low affinity to phosphorus (Visser et al. 2005), and thus could not compete with *Anabaena* in low phosphorus concentrations.

The nitrogen deficiency or low DIN: DIP levels have suggested favouring heterocytous cyanobacteria over the non-nitrogen-fixing cyanobacterial genera (Schindler 1977; Hyenstrand et al. 1998; Levine and Schindler 1999). However, in Lake Tuusulanjärvi, both *Microcystis* and heterocytous cyanobacteria dominated and reached high biomass levels in low DIN: DIP (<5 w:w) (Fig. 1 and 2 in IV). Similarly, Jensen et al. (1994) did not find a correlation between low DIN:DIP levels and the biomass of heterocytous cyanobacteria in Danish lakes. In Lake Tuusulanjärvi, DIN concentration was high at the beginning of *Microcystis* dominance, and although DIN concentrations decreased to the level (<100 mg m^{-3}), which has been found to induce nitrogen fixation and thus to favour heterocytous cyanobacteria (Horne and Commins 1987), *Microcystis* dominance continued for weeks before being replaced by heterocytous cyanobacteria. During this period of nitrogen deficiency, *Microcystis* may have used the cyanophycin and phycobilin nitrogen reserves and/or alternatively, ammonium sources in deeper water layers (hypolimnion). Blomqvist et al. (1994) and Hyenstrand et al. (1998) have suggested that the low DIN in the water column favours heterocytous cyanobacteria over the non-nitrogen fixing buoyant genera such as *Microcystis* only when ammonium sources in hypolimnion are also depleted. Actually, ammonium concentrations in Lake Tuusulanjärvi were higher in deeper water layers (6-8 m) than in the surface water layer (0-2 m) and decreased to the surface layer level only a week before the end of *Microcystis* dominance in both 2000 and 2001 (IV).

The start of a cyanobacterial bloom has commonly been connected to an increase in temperature, which correlates with the amount of light and radiation (Robarts and Zohary 1987). In Lake Tuusulanjärvi, the *Microcystis* dominance was related to the higher global radiation and temperature (>19°C), while the dominance of heterocytous cyanobacteria was associated with lower temperature and global radiation. *Microcystis* has been found to tolerate high surface irradiances (Paerl et al. 1985) and seems to require and be better adapted to high irradiances than *Anabaena* (Oliver and Ganf 2000; Huisman and Hulot 2005). Similar to this study, Hammer (1964), Reynolds (1984), and Schreurs (1992) found that *Microcystis* dominated in higher temperatures than did *Anabaena*.

During the two-year monitoring period of eutrophic Lake Tuusulanjärvi, biomass levels of genera *Snowella* and *Woronichinia* (<0.05 mg f.w. l^{-1}) were low (IV). However, *Snowella* and *Woronichinia* species were commonly present and could occasionally constitute a major part of the total cyanobacterial biomass in many oligo-mesotrophic and mesotrophic Finnish lakes, at least before cyanobacterial maxima later in the summer (III). Generally, picocyanobacteria, to which *Snowella* belongs, have a large surface-to-volume ratio, and nutrients are acquired more effectively than in large cells (Raven et al. 1998). As a consequence, picocyanobacteria are favoured by low-

nutrient conditions, when they contribute to the total biomass more than in nutrient-rich conditions (Raven et al. 1998; IV). The ecology of picocyanobacteria is poorly known (Stockner et al. 2000). The previous lack of sequence information about the genera *Snowella* and *Woronichinia* has hindered their detection by molecular biological methods.

6 CONCLUSIONS

At present, species description and classification of a large number of cyanobacterial strains are based solely on morphology. In many cases, the classification and phylogeny of cyanobacteria are not congruent. The only way to solve the current problematic situation of cyanobacterial classification is to study both morphology and genetic relationships of cyanobacterial strains. In this study, a number of cyanobacterial strains belonging to the genera *Anabaena, Aphanizomenon, Snowella, Woronichinia, Merismopedia* and *Limnothrix* were isolated and their morphology and phylogenetic relationships were examined.

The phylogenetic analysis revealed that the planktic and benthic *Anabaena, Aphanizomenon* and *Trichormus* strains were heterogeneous and intermixed. The heterogeneous *Anabaena* strains could be divided into more than one genera. Despite the complex relationships between their morphology and phylogeny, some morphological features (akinetes, morphology of terminal cells, and trichome width), that were related to the phylogenetic clusters, and that could be used in the identification of heterocytous cyanobacterial clusters, were found. This study also showed that the *rpoB* gene is a good marker gene for cyanobacterial phylogeny.

Snowella strains formed a monophyletic cluster, which was a sister cluster to a *Woronichinia* strain. The phylogeny of *Snowella* and *Woronichinia* strains, common members of planktic cyanobacteria in lakes, has not been studied before this study. The phylogenetic relationships of these strains reflected their morphology and followed the current classification by Komárek and Anagnostidis (1999). In addition, this study revealed a new cluster of thin, filamentous cyanobacteria, formed by Mediterranean *Limnothrix redekei* strains. This cluster was not closely related to any other known cyanobacteria, which indicates the high genetic diversity among the thin, filamentous cyanobacteria.

Microcystis as well as the heterogeneous *Anabaena*/*Aphanizomenon* and *Synechococcus* populations were the major groups forming the cyanobacterial community in Lake Tuusulanjärvi. These groups showed seasonal succession and were favoured by different environmental conditions. Picocyanobacteria were common in early summer. *Microcystis* dominance in midsummer was followed by dominance of heterocytous cyanobacteria in late summer. The high phosphorus concentrations, temperatures, and global radiation were associated with *Microcystis* dominance. The success of heterocytous cyanobacteria in low nutrient concentrations was explained by the higher phosphorus uptake affinity, and nitrogen fixation. Non-nitrogen fixing *Microcystis* could not compete with heterocytous cyanobacteria during the long period of nitrogen deficiency in both surface and deeper water layers and decreasing temperature. *Snowella* seems to be common in oligo-mesotrophic and *Woronichinia* in mesotrophic Finnish lakes.

Molecular biological methods (DGGE, cloning of the 16S rRNA gene, and sequencing of DGGE bands and clones), microscopic counting, and strain isolations were used to study cyanobacterial community composition in Lake Tuusulanjärvi. Although all methods detected the major groups

of the cyanobacterial community, the study revealed differences in detection of the minor groups and in proportions of the major groups. The heterocytous cyanobacteria were overestimated by the molecular biological methods based on the 16S rRNA gene, whereas the identification and detection of unicellular simple cyanobacteria caused problems in microscopy.

This study combined microscopic (morphological) and molecular biological methods in studying cyanobacterial strains and populations. The results showed that utilisation of both microscopic and molecular methods gave a more complete picture of the cyanobacterial community composition in nature. The morphological and phylogenetic evaluation of freshly isolated cyanobacterial strains revealed the phylogeny of *Snowella* and *Woronichinia,* and showed high diversity among filamentous cyanobacterial genera, *Anabaena*, *Trichormus,* and *Limnothrix.* The results shed light on the phylogeny of cyanobacteria and on the needed revision of their classification.

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