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MOLECULAR SYSTEMATICS OF THE GENERA *MICROCYSTIS*, *ANABAENA*, AND *APHANIZOMENON* OF THE CYANOBACTERIA

Charles R. Williams

A dissertation submitted in partial fulfillment of the
requirements for the degree of

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Abstract

MOLECULAR SYSTEMATICS OF THE GENERA *MICROCYSTIS*, *ANABAENA*, AND *APHANIZOMENON* OF THE CYANOBACTERIA

Charles R. Williams

Chairperson of the Supervisory Committee:
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A phylogenetic analysis of the Genus *Microcystis*, Order Chroococales, and the Genera *Anabaena* and *Aphanizomenon*, Family Nostocaceae, Order Nostocales, within the Division Cyanophyta was conducted using DNA sequences from the gene subunits *cpc*B and *cpc*A with intergenic spacer, the *rpo*C1 gene subunit and the 16S-23S rRNA ITS region. Pacific Northwest lakes were sampled for the above genera to examine regional diversity and determine the position of regional strains.

Molecular data confirm that previously recognized species of *Microcystis* show little divergence and represent 3-4 distinct genotype clusters. Morphological characters appear too plastic to determine relatedness between two distinct Pacific Northwest, U.S.A. strains that can be present in the same lakes.

Phylogenetic analyses of the Family Nostocaceae suggest that major taxonomic revision of this family and perhaps the entire Order Nostocales is necessary. The genus

Anabaena was polyphyletic with members of the genera *Nostoc*, *Aphanizomenon*, *Anabaenopsis*, *Cylindrospermopsis* and *Fischerella* contained within what were previously thought to be good *Anabaena* groupings based on traditional morphological characterization.

At least two major lineages of *Anabaena* were found by *cpc*BA-IGS and *rpo*C1 analysis. One clade contained the planktonic strains, *A. flos-aquae* and *A. circinalis*, and included the genus *Aphanizomenon*. It is proposed under the rules of the International Code of Botanical Nomenclature that *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* be subsumed within *Anabaena flos-aquae*. The second major *Anabaena* clade contained soil and planktonic isolates and seven species as described under the International Code of Botanical Nomenclature. *Anabaena* strains UTEX(1444), UTEX(2557), UTEX(2558) and UTEX(1823) should be designated as *Nostoc*.

The distribution of planktonic *Anabaena* in eight lowland Puget Sound lakes appeared limited to 4 genotype clusters representing 6 morphotypes. One *Anabaena flos-aquae* genotype was found exclusively in the late fall/winter through early spring and was responsible for toxic blooms (anatoxin a). An identical morphotype from the same lake exhibited a significantly different genotype and life history (summer and early fall dominant). Trichome habit, cell, heterocyst and akinete shape and size were shown to be poor characters for distinguishing several genera and many strains but were useful features in distinguishing *Anabaena circinalis* from *Anabaena flos-aquae* in the Pacific Northwest.

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DEDICATION

To my mother, who patiently encouraged me to follow the dream, my father who expected me to answer the hard questions, Bill for significant help in pursuing my academic adventures and Deacon who almost got to see me finish.

INTRODUCTION

Molecular techniques are needed to conduct the studies necessary to reconstruct the phylogeny of the cyanobacteria and accurately describe their diversity and distribution (Castenholz, 1992; Komárek and Anagnostidis, 1999). Molecular data provide systematic characters that are independent of morphology. These independent characters provide a phylogenetic framework upon which the utility of diagnostic taxonomic characters (e.g., morphology) can be evaluated objectively. Establishing a phylogenetic framework for cyanobacteria has been difficult because the presence of consistent unifying traits is accompanied by morphological characters that are homoplastic (Castenholz, 1992; Castenholz, 2001). Reliance solely on morphological characters can lead to erroneous interpretations of phylogeny (Neilan et al., 1994; Palinska et al., 1996) and conversely, molecular data without a context can provide only limited utility as patterns of gene evolution may obscure the true phylogeny. In addition, cyanobacteria are treated under two different nomenclature codes. Botanists apply the International Code of Botanical Nomenclature (ICBN) (St. Louis Code) and bacteriologists apply the International Bacteriological Code of Nomenclature (ICNB) to cyanobacteria (Sneath, 1992).

Microcystis and *Anabaena* have also been responsible for serious public health risks from toxic blooms (Codd et al., 1999; Chorus and Bartram, 1999). Both genera are known for their taxonomic difficulties because of the morphological similarity among species, and morphological plasticity (Fahrenkrug et al., 1992; Fergusson and Saint,

2000). Misidentification of these genera can have serious public health implications. Efforts to identify toxic species or strains have been ongoing for over 40 years.

Microcystis's position within Section I of the ICNB or what might be called a reduced order Chroococales appears settled (Turner, 1997; Wilmotte and Herdman, 2001) and Komárek (1999) constructed the family Microcystaceae within the order Chroococcales which places *Eucapsa*, *Gloeocapsa*, *Chondrocystis* and *Microcystis* together, but the Microcystaceae remains untested phylogenetically. The strains and species within *Microcystis* have only very recently begun to be sorted out based on 16S rRNA and DNA-DNA reassociation (Otsuka et al., 2001). An exploration of the distribution of *Microcystis* strains within and between geographical regions is the next step in understanding *Microcystis* diversity. Research in regions such as the Pacific Northwest of the United States of America will be combined with the work of other regional investigators to generate a global picture of the diversity of *Microcystis* strains, elucidate the frequency and mechanisms of genetic exchange and identify the physiological adaptations of *Microcystis* strains to specific environmental conditions. Ultimately, researchers will be able to use this information to elucidate the environmental factors that favor toxic strains over non-toxic strains and reconstruct aquatic ecosystems damaged by anthropogenic impacts.

Evolutionary relationships within *Anabaena* and among related genera in the family Nostocaceae and the order Nostocales remain poorly understood (Komárek and Anagnostidis, 1989; Komárek et al., 1993; Lu et al., 1997; Wilmotte and Herdman,

2001). Members of the family Nostocaceae such as *Anabaena, Nostoc, Nodularia, Aphanizomenon, Cylindrospermopsis,* and *Anabaenopsis* are separated under the ICBN based on morphological characters such as trichome/filament habit, cell size and shape, heterocyst size, shape and placement and akinete size, shape and placement (Komárek and Anagnostidis, 1989). Several classification schemes have been proposed to remedy the confusion left in the wake of the historical ICBN designations. Disagreement among these classifications focuses on morphological characteristics such as trichome symmetry, akinete and heterocyst shape, size, frequency and placement as well as newer methods included in polyphasic taxonomical analyses such as fatty acid composition, DNA-DNA reassociation and the use of phylogenetic molecular markers (Baker, 1981; Barker et al., 2000; Caudales and Welles, 1992; Fergusson and Saint, 2000; Hindak, 2000; Hiroki et al., 1998; Li et al., 2000a; Li et al., 2000b; Li and Watanabe, 2001; Li et al., 1998; Lyra et al., 2001; Stulp and Stam, 1984a). Unfortunately, most of the studies examined a very limited number of taxa that are often not shared between studies, and key taxa are missing or misidentified such that resolution is not sufficient to distinguish the species and genera in this group.

This dissertation presents data regarding the distribution of *Anabaena* genotypes and morphotypes in lowland Pacific Northwest lakes as well as reconstructions of the phylogenetic relationships for Pacific Northwest strains of *Microcystis*, *Anabaena* and *Aphanizomenon* to other global strains based on sequence data from three different DNA regions. The *cpc*BA-IGS region was used to examine *Microcystis, Aphanizomenon* and

Anabaena directly from individual colonies or filaments collected in the field. The *rpo*C1 region was also used to confirm the position of *Anabaena* and *Aphanizomenon* within the Nostocaceae as inferred from the *cpc*BA-IGS analysis. Select *Microcystis* strains were examined using the 16-23S rRNA ITS region to further elucidate the relationships among strains of *Microcystis* as documented in the most complete survey to date in the literature (Otsuka et al., 1999b).

This study specifically addresses the following questions:

1. What are the phylogenetic relationships of Pacific Northwest strains to other members of the genus *Microcystis*?

2. Do the phylogenetic relationships from this *Microcystis* study support a monotypic genus?

3. Do multiple *Microcystis* strains co-exist in Pacific Northwest lakes spatially or temporally and, if so, can morphology be used to identify toxic vs. non-toxic genotypes?

4. Is *Anabaena* monophyletic?

5. Does *Aphanizomenon* belong in the genus *Anabaena*?

6. What level of diversity of *Anabaena* strains and species exists in lowland Pacific Northwest lakes?

7. Which strain of *Anabaena* is responsible for toxic blooms in American Lake, WA, and what is its relationship to other *Anabaena* strains and what is its distribution?

8. Is morphology useful in describing species of *Anabaena*?

9. Are the phylogenetic relationships among the genera within Nostocaceae testable using the *cpc*BA-IGS and *rpo*C1 markers?

10. Does the current classification within the Nostocaceae reflect phylogenetic relationships inferred from the molecular data? If not, what suggestion for revising the classification can be made?

The ultimate goal of this research is to provide an evolutionary framework for *Microcystis, Anabaena*, and *Aphanizomenon* in order to interpret key morphological character evolution, biogeographical history, and to provide the basis for a classification scheme that reflects phylogenetic relationships.

CHAPTER 1: MOLECULAR SYSTEMATICS AND DIVERSITY OF PACIFIC NORTHWEST *MICROCYSTIS* BASED ON *cpc*BA-IGS AND 16S rDNA-23S rDNA ITS

ABSTRACT

A phylogenetic analysis of *Microcystis* was constructed from Pacific Northwest direct PCR lake samples (single colonies) and culture isolates from around the world using DNA sequence from the *cpc*BA-IGS and 16-23 rRNA-ITS regions. Sequence from over seventy toxic and non-toxic strains representing a variety of morphotypes were examined using 585 bp of DNA from the *cpc*BA-IGS region and 623 bp from the 16-23S rRNA-ITS region. One Pacific Northwest *Microcystis* genotype cluster was consistently associated with toxic blooms. Two distinct Pacific Northwest genotype clusters were found; however, morphological characteristics did not prove useful for field delimitation due to the plasticity of colonial morphology. This study confirms recent proposals in the literature that *Microcystis* be designated a monotypic genus, *Microcystis aeruginosa.* Based on the taxa represented in our sample, *Microcystis aeruginosa* consists of at least 4 clusters/clades of strains that grade into each other morphologically and appear to have a cosmopolitan distribution with the possible exception of the toxic strain cluster from the Pacific Northwest.

INTRODUCTION

The genus *Microcystis* Kützing ex Lemmerman 1907 is a member of the family Microcystaceae (Elenkin 1933) in the Order Chroococcales (Komárek and Anagnostidis, 1999). *Microcystis* is colonial in habit and predominantly occurs in eutrophic lakes,

ponds and reservoirs (Paerl and Tucker, 1995). Like most cyanobacteria, *Microcystis* is quite sensitive to physical features of the aquatic environment and tends to dominate in marginally inhabitable (to other organisms) but environmentally stable habitats (Paerl, 1988). *Microcystis* has been implicated in numerous toxic bloom events as a producer of the hepatotoxin, microcystin, a serine/threonine protein phosphatase 1 and 2A inhibitor (Honkanen et al., 1990). Water management authorities are dependent on accurate identification of *Microcystis* and information regarding the distribution of toxin producing strains or face potential catastrophic consequences such as the recent death of hemodialysis patients in Brazil (Pouria et al., 1998), or the death of animals (Chorus and Bartram, 1999). Humans may also experience chronic effects (e.g., liver tumor promotion) that may be associated with drinking toxin-laden water or in consuming contaminated produce irrigated with water containing viable *Microcystis* that survives on plants such as lettuce (Codd, Metcalf and Beattie, 1999).

Cyanobacteria are currently treated systematically under both the International Code of Botanical Nomenclature (ICBN) and the International Bacteriological Code of Nomenclature (ICNB) (Castenholz, 1992). While there are differences in these systems, both have moved slowly toward polyphasic taxonomy in an attempt to more accurately describe the phylogenetic relationships among cyanobacteria (Castenholz, 2001; Waterbury and Rippka, 1989; Wilmotte, 1994). Under the Botanical Code, unicellular and colonial cyanoprokaryotes, including *Microcystis,* are primarily classified according to morphological characters that are considered constant and genetically stable. Families within the Order Chroococcales *sensu* Komárek are organized according to the following

morphological characteristics: 1) type of cell division; 2) simple and more complex families are delineated by the presence or absence of polarity of cells and colonies; 3) unique combinations of several cell division types are regarded as unique family characters; and 4) the form and structure of a colony, including mucilaginous strands (the layers and the position of cells in a colony are particularly important) may be regarded as a species or genus character (Komárek and Anagnostidis, 1999).

The number of species in the genus *Microcystis* has varied historically from the revision of the genus by Geitler describing 32 species (Geitler, 1932) based on cell morphology and colonial habit, to two species in Drouet's compression of cyanobacteria taxa based largely on herbarium and live samples (Drouet, 1981). Colony formation such as clathrate, lenticular, lobed, etc. in field samples was regarded as the primary character to distinguish species under the ICBN (Desikachary, 1959). The development of laboratory culturing of *Microcystis* resulted in a better understanding of the plasticity of morphology in the genus; however, many strains grew poorly under axenic conditions and some strains were more amenable to culture than others. Subsequent work with culture isolates excluded forms such as *Microcystis elebans* that do not produce gas vesicles and lack gas vesicle genes (Stanier et al., 1971; Waterbury and Rippka, 1989). Many of the *Microcystis* species identified in the Geitlerian system were maintained, however, because the problem of plasticity remained with respect to interpreting the taxonomic significance of colonial habit. Most strains of *Microcystis* placed in culture lost their colonial habit suggesting that habit is an unlikely character for accurately determining evolutionary relationships (Otsuka et al., 2000), and the cell dimensions of

one species often overlapped other species although significant effort was made using morphometrics to identify clusters designated as "species"(Kersner and Komárek, 2000).

The advent of ultrastructural analysis in the 1960s brought a profound change in blue-green algal systematic thinking when investigators discovered that blue-greens were not algae but bacteria (Stanier et al., 1971). Microbiologists began to apply the ICNB to cyanobacteria strains that were amenable to culturing and *Microcystis* was initially placed in Section I in the *Synechocystis* group based on the shared character of reproduction by binary fission (Rippka et al., 1979). The description in the 1989 edition of Bergey's Manual of Systematic Bacteriology defined *Microcystis* as having a coccoid cell shape, gas vesicles, a tendency to form aggregates or colonies and an amorphose mucilage or sheath (Waterbury and Rippka, 1989).

The recently revised 2001 Bergey's Manual, however, moved *Microcystis* out of the *Synechocystis* group into Subsection I of the Chroococcales based on 16S rRNA data and designated a form genus characterized by transverse binary fission in two or three planes, structured sheath absent, with spherical cells 3-4 μm in diameter, gas vesicles normally present and a G + C content around 42 mol% (Herdman et al., 2001). The proposed type strain of the type species, *Microcystis aeruginosa*, is represented by UTEX 2385 with a mol% of 42.5 and genome size of 3.1 Gdal (Herdman et al., 2001).

Watanabe et al. (2000) argue that the current classification system of the genus *Microcystis* is invalid as a result of numerous demonstrations that morphospecies have overlapping morphological characteristics. More troubling was the discovery that even one strain can have multiple colonial forms (Otsuka et al., 2000). Palinska et al. (1996)

also demonstrated phenotypic variability in identical genotypes in *Merismopedia*. These results combined with other phylogenetic studies demonstrated a clear need to apply a combined approach to cyanobacterial taxonomy (Waterbury and Rippka, 1989). The most recent proposal calls for *Microcystis* to be designated a monotypic genus based on 16S rRNA, DNA-DNA reassociation and fatty acid composition with a type strain NIES 843 (Otsuka et al., 2001). The unpublished DNA-DNA reassociation data of Rippka and Herdman combined with 16S rRNA gene sequences (Wilmotte and Herdman, 2001) also support a single species genus with type strain UTEX 2385 (Herdman et al., 2001) although their conclusion was based on a smaller dataset of strains than the Otsuka dataset. Numerous geographical regions such as the Pacific Northwest are unrepresented in both studies and need to be examined to verify thse conclusions.

The increased work in and understanding of cyanobacteria systematics has also allowed researchers to develop tools to describe the temporal and spatial diversity in cyanobacterial communities. Current research in phylogenetics suggests that a small number of different cyanoprokaryotic genotypes are present in regional waterbodies despite notable phenotypes present in these different environments. Only a small number of taxonomic units are in culture (Komárek and Anagnostidis, 1999), thus research into the diversity of cyanobacteria is in a very early stage and techniques for analyzing specimens *in situ* are clearly needed. Neilan (1995) first demonstrated genetic variation in *Microcystis* as measured by restriction length polymorphism of PCR amplified *cpc*B-*cpc*A intergenic spacer (*cpc*BA-IGS) and flanking coding regions. This preliminary investigation of diversity suggested that *Microcystis* might show significant diversity

within and between regional lakes and provided a mechanism to obtain PCR amplification of *Microcystis* DNA without concern for bacterial contamination as only cyanobacteria produce cyanophycocyanin or its subunits. Significant diversity in strain clusters has been demonstrated in other cyanobacteria genera by Palenik (Palenik, 1994) who employed the RNA Polymerase subunit, *rpo*C1, to identify isolates of *Synecchococcus* from the Sargasso Sea and by Ernst in showing the diversity of *Synechocystis* culture isolates from Lake Constance (Ernst et al., 1995).

A large number of genotypes appear to exist as stable populations as demonstrated by stable strains in culture. Komárek and Anagnostidis (1999) however caution phycologists that "Only in some cases should strains be grouped together in larger natural and delimited units that can be ascribed the rank of species." Research on cyanobacterial diversity has been hampered for years by the difficulty in isolating colonies or filaments into culture to obtain sufficient clonal axenic material for subsequent genetic analysis (Barker et al., 1999). Barker and Hayes (1997) pioneered a method of directly sequencing single *Nodularia* filaments using a two step PCR protocol. Direct PCR of single colonies or filaments from the field provides a new mechanism to test the hypotheses surrounding cyanbacterial genetic diversity and ecological adaptation in *Microcystis*.

This study pioneers direct colony PCR of *Microcystis* from field samples in one PCR reaction. *Microcystis* from Pacific Northwest lakes was compared with culture strains from across the globe using *cpc*BA-IGS and 16-23S rRNA-ITS as molecular markers. Both markers have been used to elucidate *Microcystis* diversity (Bolch et al.,

1996; Neilan et al., 1995; Otsuka et al., 1999b; Tillett et al., 2001) and represent the largest dataset for comparison of Pacific Northwest strains to other regional strains. The phycocyanin gene marker allows the use of direct PCR from field samples containing bacterial contaminants as no other prokaryotes contain this gene (Bolch et al., 1996). A systematic framework was constructed using parsimony and maximum likelihood analyses to better understand the diversity of *Microcystis* within the Pacific Northwest and the relationship of Pacific Northwest strains to strains from other regions.

MATERIALS AND METHODS

SAMPLING

Sampling was designed to include as many of the putative *Microcystis* species and geographic locations as were available from culture collections. Field collections focused on toxic blooms of *Microcystis* from 1991-2000 to determine whether the same strain was responsible for multiple toxic bloom events in the Pacific Northwest region. Local collections of *Microcystis* were made in response to reports of toxic water blooms ranging from the Central Coast of Oregon to northwestern Washington State. Additional samples were also obtained from blooms not associated with toxic events to determine whether genotype diversity existed in Pacific Northwest *Microcystis* populations that distinguished toxic from non-toxic strains. Toxicity was verified by mouse bioassay (Lambert et al., 1994), HPLC (Lawton et al., 1994) or ELIZA (Chu et al., 1990) by the following collaborators (Crayton, M., Pacific Lutheran University, unpublished data;

Beattie, K., University of Dundee, unpublished data; Jacoby, J., Seattle University, unpublished data).

Microcystis samples were obtained from 8 Pacific Northwest lakes to assess the diversity of *Microcystis* in the region and additional culture isolates were obtained from the University of Texas Culture Collection (UTEX) (Starr and Zeikus, 1993), the Culture Collection of Algae and Protozoa (CCAP), Pasteur Culture Collection of Cyanobacterial Strains (PCC) and the Institute of Applied Microbiology (IAM) to determine the placement of local *Microcystis* genotypes within the global phylogenetic hierarchy. All samples collected and sequenced are listed in Table 1 and include accession numbers from sequences obtained from Genbank as well as any known information on origin and toxicity.

Culture isolates were grown in either BG-11 Medium (Rippka et al., 1979) or MLA (Bolch et al., 1997). Field samples were frozen at –20°C for later DNA isolation or placed in growth chambers until used for direct colony PCR. All strains were maintained in a lighted incubator at 100 $\mu m \cdot^{-2} \cdot sec^{-1}$ on a 16:8 hour light/dark cycle at 20°C. The Portage Bay, Lake Union, WA strain was isolated and grown in MLA.

MICROSCOPY

Black and white photographs were obtained using a Zeiss compound microscope with a Nikon M-35 1S mounted camera using TMAX 400 film. Color microphotographs were obtained using a Zeiss compound microscope using Kodachrome 64 slide film.

DNA ISOLATION AND SEQUENCING

Two methods were employed in isolating cyanobacteria DNA for PCR: 1) MASS PCR- DNA from culture isolates was obtained by centrifuging approximately 0.5 ml log phase culture and processing the pelleted cells. Pellets were either immediately extracted, or frozen and extracted at a later date. DNA was isolated from fresh, frozen, lyophilized or formalin preserved (1%) material either according to a modified Instagene method intermediate to that of Bolch et al. (1996) and Neilan et al. (1995) (reagents and washes per Bolch but temperature, time and vortexing per Neilan) or using a xanthogenate-SDS extraction protocol detailed by Tillett and Neilan (2000) but incubating all samples for two hours at 60°C. 2) DIRECT PCR- Approximately 20 μl of field collected material were pipetted onto a depression slide and examined at 40x under a dissecting microscope. Individual *Microcystis* colonies were pipetted (Eppendorf ultramicropipetter) into a second depression slide containing 100 μl of BG-11 medium; this washing procedure was repeated 5 times. Single colonies were pipetted in a 1 μl liquid volume into 0.6 ml eppendorf tubes and frozen for later PCR or placed on ice until addition of PCR reaction mix. DNA from culture isolates was examined by gel analysis and quantified with approximately 5 ng used for each 25 μl PCR reaction.

PCR reaction conditions were optimized for each molecular marker primer set and for each type of DNA amplified (whole cell direct colony PCR vs. extracted DNA from culture isolates). Double stranded PCR reaction mix for *cpc*BA-IGS and 16-23S rRNA-ITS of extracted DNA consisted of 10 mM Tris-HCl, pH. 9.2, 25 mM KCl, 3.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 µg BSA, 0.0125 units of Taq polymerase, 0.1 mM of

each primer, and 1 μl total DNA template either undiluted or diluted up to 1:100 in a 25 μl reaction volume. PCR reaction mix for direct colony PCR consisted of 10 mM Tris-HCl, pH 8.8, 25mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 μg BSA, 0.0125 units Taq polymerase, 0.4 mM each primer in a total volume of 25 μl. 3.75 μl glycerol was substituted in some reactions for BSA in direct colony PCR and concentration of primers was doubled in some reactions to remedy DNA derivitization problems. PCR reaction mix was added to the 1 μl colony mix and immediately frozen or amplified. A PTC-100 Programmable Thermal Controller (MJ Research, Inc.) was used for PCR amplification.

Primers used for *cpc*BA-IGS amplification and sequencing are described in (Bolch et al., 1996) (forward *cpc*βF: 5'-GGCTGCTTGTTTACGCGACA-3' and reverse *cpc*αR: 5'-CCAGTACCACCAGCAACTAA-3'). This primer set amplified a 610 bp fragment that included the 3' end of the β subunit of phycocyanin, an approximate 100 bp ITS region, and the 5' end of α subunit of phycocyanin. Primers used for 16-23S rRNA-ITS PCR amplification are described in (Otsuka et al., 1999b) (forward MSR-S2f: 5'-TCAGGTTGCTTAACGACCTA-3' and reverse 242r: 5'-(G/T)TTCGCTCGCC(A/G)CTAC-3') and yielded a 623 bp fragment spanning the ITS and flanking regions that included 74 bp of tRNA-Ile.

The reaction profile for *cpc*BA-IGS amplification of *Microcystis* colonies was: 1) an initial denaturation at 96° C for 4 minutes followed by; 2) 94°C for one minute; 48°C for one minute; 72°C for two minutes for 38 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for *cpc*BA-IGS amplification of extracted DNA was: 1) an initial denaturation at 96°C for 4 minutes followed by; 2) 94°C for one

minute; 52°C for one minute; 72°C for two minutes for 35 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for the 16-23S rRNA-ITS region amplification was the following: 1) an initial denaturation at 94°C for three minutes followed by; 2) 94°C for one minute; 55°C for one minute; 72°C for two minutes for 30 cycles followed by; 3) final extension at 72°C for 5 minutes.

Double stranded PCR products were cleaned using Qiaquick PCR Purification Kit (Qiagen Inc., Chatworth, CA) or electrophoresed in a 1.5% agarose gel (SeaKem, FMC), stained with ethidium bromide and the appropriate sized bands cut and cleaned using Quiquick Gel Purification or Quiex II (Quigen Inc., Chatworth. CA) and quantified by spectrophotometry. All 16-23S rRNA-ITS PCR products and PCR product from samples that provided poor data from direct sequencing or samples suspected to contain more than one strain were purified and ligated into the cloning vector pGEM-T Easy (Promega). Transformation was performed using the *E.coli* JM109 strain. The recombinant plasmids were verified by restriction digestion followed by agarose electrophoresis. A minimum of 4 clones from each sample was sequenced using the T7 and SP6 primers provided by Promega. Direct PCR sequencing was accomplished with the same primers as used in the PCR reaction. DNA was sequenced using the cycle sequencing method for both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Foster City, CA) and analyzed on an ABI 377 automated sequencer. DNA sequences were assembled using Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI).

DATA ANALYSIS

Sequence alignment for *cpc*BA-IGS and 16-23S rRNA-ITS was peformed using ClustalX (Thompson et al., 1997) and subsequently adjusted manually to correct errors and to align difficult portions of the intergenic/intron spacer regions. Due to the number of insertions and deletions in the intergenic/intron region and the difficulties in aligning large data matrices, sequence alignment was performed in several steps. First, taxa within *Microcystis* and those from outside the genus *Microcystis* were aligned separately, and those alignments were adjusted manually. Second, selected taxa from both inside and outside the genus *Microcystis* were aligned with ClustalX, and this alignment was adjusted manually. *Synechococcus* PCC 7902 was selected as the most closely related taxon for the purposes of aligning the intergenic spacer for *cpc*BA. *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 were selected as outgroups for 16-23S rRNA-ITS based on prior studies. Final alignments were constructed by placing these alignments together and represent the best estimate of the positional homology.

tRASA was performed on the aligned sequences to determine phylogenetic signal (Lyons-Weiler, 2001). Outgroups were identified and tested for suitability in the *cpc*BA-IGS and 16-23S rRNA-ITS analyses. Previous studies (Lu et al., 1997; Neilan, Jacobs and Goodman, 1995; Neilan et al., 1997b; Otsuka et al., 1999b) using the *cpc*BA-IGS or 16-23S rRNA-ITS markers rooted trees in *Synechococcus* and/or *Synechocystis* strains but did not test their outgroup taxa for phylogenetic signal or variance ratio. Recent work by Lyons-Weiler has demonstrated that inappropriate outgroup usage can degrade phylogenetic signal and result in inaccurate tree topology (Lyons-Weiler and Hoelzer,

1997). A negative tRASA is one of the symptoms of long branch attraction (Lyons-Weiler et al., 1996) and declining power curves and high taxon variance ratios are also evidence of problematic taxa (Lyons-Weiler and Hoelzer, 1997). Taxa violating these criteria were removed from the alignments.

*cpc*BA-IGS and 16-23S rRNA-ITS data were analyzed using PAUP* (version 4.0b8)(Swofford, 1999). Both parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981) analyses were conducted. Regions of ambiguous alignment were excluded from analyses. Short regions of sequence at the beginning and the end of both *cpc*BA-IGS and the 16-23S rRNA-ITS regions were excluded from analyses to account for some sequencing reactions that did not yield full-length fragments. Gaps were introduced in the alignment in order to optimize positional homology (submitted to EMBL).

One thousand heuristic searches with random sequence addition were conducted with all characters weighted equally with TBR swapping and MULPARS in effect for parsimony analyses. Five hundred bootstrap replications were conducted to assess relative group support (Felsenstein, 1985), and each bootstrap replicate used ten heuristic searches with random sequence addition.

For maximum likelihood analyses, a substitution model where all base transformations are equally likely and a discrete gamma distribution (Yang 1994) with three rate classes to account for rate heterogeneity were used. Ten heuristic searches with random sequence addition were conducted with MULPARS turned off (DeBry and Olmstead, 2000). The tree with the maximum likelihood was retained to represent the

best estimate of phylogenetic patterns. One hundred bootstrap replications were conducted under maximum likelihood criteria to assess relative group support.

RESULTS

Previously used outgroups in prior *cpc*BA-IGS and 16-23S rRNA-ITS studies (Lu et al., 1997; Otsuka et al., 1999b; Tillett, Parker and Neilan, 2001) produced negative tRASA values both in our dataset and in recreations of the prior studies indicating serious degradation of phylogenetic signal and likely long branch attraction (Felsenstein, 1981). Datasets were carefully examined for phylogenetic signal using the tRASA test and for degradation of signal using the variance ratio described by Lyons-Weiler (Lyons-Weiler, Hoelzer and Tausch, 1996).

Review of the alignment and choice of outgroup in this study and the16-23S rRNA-ITS study by Otsuka (2000) uncovered serious errors in Otsuka's analysis. The ITS region was alignable between *Microcystis* genotypes, but an exhaustive search of suitable outgroups, including PCC6803 (Neilan et al., 1997a) and NIBB10667 as used by Otsuka (2001) failed to provide a suitable taxon that could be aligned in the ITS region without significant loss (negative values) in phylogenetic signal as measured by tRASA. In view of this problem, the phylogenetic analysis in this study was conducted both unrooted and rooted with *Microcystis wesenbergii* (NIES 111). The 46 taxa when unrooted yielded a tRASA of –5.26 indicating poor phylogenetic signal and possible long branch attraction amongst taxa. Rooting with NIES 111 raised tRASA to 18.7828. After a preliminary analysis, removal of outlier taxa according to the variance plot and reducing the number of duplicative representatives in well-represented clades to facilitate

computer analysis, the total taxa were reduced to 22. The unrooted tRASA of the final dataset was –2.322 and 4.2328 when rooted with NIES 111. The highest tRASA was obtained by rooting with strain CC2 (15.877); however, Lyons-Weiler cautions that use of an ingroup to root a tree can result in an incorrect topology when only maximum tRASA is considered. As NIES 111 was the most distant genotype represented in the remaining taxa, it represented the most stable taxon for rooting and was consistent with other studies.

The *cpc*BA-IGS phylogenetic analysis initially contained 50 *Microcystis* taxa. The coding regions of *cpc*BA were aligned with the entire set of cyanobacteria sequences available from Genbank, and a preliminary alignment and phylogenetic analysis indicated that *Synechococcus* PCC 7902 was the most closely related sister taxon. Alignment of PCC 7902 and the *Microcystis* taxa in the IGS region was suspect, and phylogenetic signal was unacceptably low (tRASA = -5.492). Removal of *Synechoccocus* PCC 7902 and several other problematic taxa increased phylogenetic signal of the unrooted dataset containing 41 taxa to –1.7 tRASA. Rooting with *Microcystis wesenbergii* NIES 111 gave an acceptable tRASA of 18.750372 with 699 degrees of freedom and was consistent with the rooting of the only other *cpc*BA-IGS study available (C. S. Bolch, IVth International Toxic Cyanobacteria Symposium, 1997).

Sequences from 41 taxa included in the *cpc*BA-IGS study and 22 taxa in the 16-23S rRNA-ITS study (Table 1) provided a total aligned length of 585 bp for *cpc*BA-IGS and 623 bp for 16-23S rRNA-ITS (Submitted to EMBL). Nine gaps with one gap of two bases were introduced in the *cpc*BA-IGS alignment to account for unique sequences

found in both the coding and IGS region. Five parsimony informative gaps were included in the *cpc*BA-IGS analysis as two-state characters. Five gaps, ranging from one to 5 bases long, were introduced into the 16-23S rRNA-ITS alignment. Five parsimony informative gaps were included in the 16-23S rRNA-ITS analysis as two-state characters.

In the *cpc*BA-IGS dataset, 71 sites were variable of which 62 sites were parsimony informative. Parsimony analyses resulted in 14 most parsimonious trees of 130 steps (CI=0.638, RI=0.895, HI=0.362 for informative characters). The strict consensus of the 14 trees is shown in Fig. 1. Maximum likelihood analyses yielded one tree of maximum likelihood (-lnL= 1690.82702). The overall topology of the maximum likelihood tree is similar to that of the parsimony strict consensus tree (Figs. 1 & 2). Differences between the maximum likelihood tree and the parsimony strict consensus tree are restricted to resolution of the relationship of Cluster II to other major lineages and in areas with weak bootstrap support and short branch lengths. Tree 4 from the parsimony analysis is characteristic of the placement of Cluster II in the majority of parsimony trees (Fig. 3) and is similar to that shown in the maximum likelihood tree (Fig. 2). Strains 127a and c EAWAG form a separate clade in the maximum parsimony analysis that has been included in Cluster II because of the short distance and low bootstrap support separating the strains from other Cluster II taxa. The position of these strains is part of an unresolved polytomy in the maximum likelihood analysis that has also been designated Cluster II.

In the 16-23S rRNA-ITS dataset, 39 sites were variable of which 35 sites were parsimony informative. Parsimony analyses resulted in 18 most parsimonious trees of 93

steps (CI=0.527, RI=0.762, HI=0.473 for informative characters). The strict consensus of the 18 trees is shown in Fig. 4. Maximum likelihood analyses yielded one tree of maximum likelihood (-lnL= 1483.84065). The overall topology and bootstrap support of the maximum likelihood tree (Fig. 5) is similar to that of the parsimony strict consensus tree (Fig. 4). Tree 4 from the parsimony analysis (Fig. 6) is nearly identical to the maximum likelihood tree (Fig. 5). Differences between the maximum likelihood tree and the parsimony strict consensus tree are restricted to areas with weak bootstrap support and short branch lengths.

Two distinct genotype clusters were present in Pacific Northwest lakes and well supported in both the Parsimony and Maximum Likelihood analyses in the *cpc*BA-IGS analysis (Figs 1-3). The Pacific Northwest strains were found in Clusters I & III of the four *Microcystis* strain clusters identified and showed a 3% difference across the *cpc*BA-IGS sequence. Pacific Northwest strains found in Cluster I were not associated with any toxic bloom events although many of the other strains within Cluster I are toxic. The cluster of Pacific Northwest strains found in Cluster III were all toxic except there are no positive data supporting toxicity of a strain that uncharacteristically bloomed in the spring in Lake Union (*Microcystis* typically blooms in the Pacific Northwest in the late summer to early fall) and was associated with a fish kill. No microcystin was detected in an ELISA test conducted toward the end of the Lake Union bloom (Jean Jacoby, Seattle University, personal communication). Both the Cluster I and Cluster III genotypes were found to occur in several Pacific Northwest lakes (Lake Sammamish, Lake Terrell and Lake Union) and were coincident in bloom samples as well (Lake Terrell and Lake

Union). No quantifiable difference in morphology was noted on examination of colonial habit or cell size. Figure 7a shows the colonial habit of Pacific Northwest *Microcystis* from Cluster I and Figures 7b, 7c and 7d illustrate the habit of Pacific Northwest Cluster III *Microcystis*. All show the characteristic clathrate habit most often associated with *Microcystis aeruginosa* and *M. wesenbergii*.

The *cpc*BA-IGS analysis strongly supports at least four major clades/clusters of *Microcystis aeruginosa* strains (Figs. 1-3) although additional taxa may reduce the certainty with which these divisions are drawn or represent additional clades. Strains from Cluster IV were used to root the tree because no suitable sister groups were identified, NIES 111 and M-178 were most distant (6% from Cluster I) from all other *Microcystis* strains in the analysis and prior unpublished data supported this topology (C. S. Bolch, IVth International Toxic Cyanobacteria Symposium, 1997). Cluster IV may not represent the basal lineage of *Microcystis*; however, the topology of the tree did not vary from that of an unrooted analysis in either parsimony or maximum likelihood analysis. The two strains in Cluster IV are known as *M. wesenbergii* (NIES 111) and *M. flos-aquae* (M-178). M-178 shows a distinct spherical colonial habit even in culture (Figure 7d) whereas NIES 111 demonstrates a clathrate, reticulate habit. Suprisingly, despite their disparate morphology, M-178 and NIES111 share identical *cpc*BA-IGS and 16-23S rRNA-ITS sequence (submitted to Genbank).

The toxic cluster of Pacific Northwest strains formed a unique clade in the *cpc*BA-IGS tree (Cluster III) that included strains from Green Bay, Wisconsin that are not known to be toxic (Figs. 1-3). The source of the 110 EAWAG strain also in Cluster

III is unknown. The morphology of these Pacific Northwest strains was most often highly reticulate but also showed other concurrent morphologies characteristic of the plasticity of *Microcystis*. *cpc*BA-IGS Cluster II is the least well supported of the clades and contains strains from North America, South Africa and Australia as well as several strains of unknown origin. Both maximum likelihood analysis and the maximum parsimony strict consensus showed weak support for the inclusion of the strains EAWAG127a and c in Cluster II with bootstrap values of 52% supporting the two strains as a separate clade. As with the other clusters, both toxic and non-toxic strains are represented in Cluster II. Additional taxa may resolve any ambiguity in retaining a separate clade for the two EAWAG strains or placing them within either Cluster I or Cluster II.

Cluster I was highly supported and contains both of the proposed type strains for *Microcystis aeruginosa*. The percentage difference between Cluster I and Cluster IV is approximately 6% and strain representatives from Europe, North America, Japan and South Africa were tightly grouped together. Cluster II appears to be distinct from Cluster I based both on the distance between strains within the clusters and the high bootstrap support separating the clusters in maximum likelihood and maximum parsimony analysis despite the polytomy leaving the Cluster II taxa unresolved. EAWAG 127a and c appear responsible for much of the uncertainty in this Cluster as indicated by the low bootstrap support for these strains in Fig. 3.

The 16-23S rRNA-ITS analysis shared few strains with the *cpc*BA-IGS analysis but also supports a minimum of four strain clusters with weak to moderate support (Figs.

4 & 5). Cluster III and Cluster IV were highly supported as distinct lineages within *Microcystis* under both Maximum Likelihood and Maximum Parsimony analysis. Cluster IV contained the same strains as Cluster IV of the *cpc*BA-IGS tree with the addition of a *M. wesenbergii* strain from Japan (TAC57). Cluster III also mirrored that of the *cpc*BA-IGS tree with the Pacific Northwest representative as the single taxon (Lake Union strain). Cluster I contained one representative (CCAP1450/10) known to be a member of either Cluster I or II in the *cpc*BA-IGS tree (not shown). Cluster II, however, is weakly supported and possibly includes several clusters given the relative base differences between strains and the possibility that TAC20 is not a member of Cluster I (Figs. 4-6). Additional available taxa did not clarify these relationships due to the loss in phylogenetic signal when included in the analysis. Cluster II also does not include any representatives of the *cpc*BA-IGS analysis so that comparison of these clusters between data sets is not possible. The percentage difference between Clusters I and IV is 5.3% with major cluster delineation appearing to occur at approximate 1.1% intervals. Resolution of 16-23S rRNA-ITS is less than *cpc*BA-IGS (1.3% vs. 2.9% difference between Clusters III & IV), and the weaker bootstrap support between these markers supports this finding.

DISCUSSION

Two phylogenetic reconstructions of *Microcystis* containing strains from the Pacific Northwest using DNA sequence from the molecular markers, *cpc*BA-IGS and 16-23S rRNA-ITS, are presented. The strains sequenced and supplemented with Genbank

data represent much of the known morphological and geographical diversity of this genus. Because patterns of phylogenetic relationships may change with additional sampling, some of the systematic conclusions may be modified as more data become available, particularly if new clades are discovered. Numerous *Microcystis* studies have been conducted, but few taxa overlap in these studies due to proprietary practices in a field of research dependent on the isolation of strains. The development of direct PCR techniques such as those used in this study promises to expand the availability of data such that the diversity and biogeography of cyanobacteria will be better understood.

Otsuka et al. (2000) examined the morphological variability of five *Microcystis* morphospecies (*M. aeruginosa, M. ichthyoblabe, M. novacekii, M. viridis* and *M. wesenbergii*) in culture and concluded that the current classification of the genus *Microcystis* was no longer valid due to the overlapping morphological variability between strains. Numerous attempts to validate the morphological taxonomy by morphometrics (Komárek and Anagnostidis, 1999; Otsuka et al., 2000), pigment content (Otsuka et al., 1998), allozymes (Tatsumi et al., 1991) and other characters failed to describe the evolutionary relationship of this genus accurately, and the most recent proposals based on the ICNB recommend reshaping *Microcystis* as a genus with a single species, *M. aeruginosa* (Otsuka et al., 2001; Wilmotte and Herdman, 2001). Our study is consistent with these recommendations as distinctly different genotypes from the Pacific Northwest are present in the same lakes yet mature colonies are indistinguishable using light microscopy and the molecular differences between these genotypes are insufficient to merit splitting into two species.

The phylogenetic analyses in this study reveal that *Microcystis* strains from disparate sources and with significant morphological differences show limited genotypic divergence in both *cpc*BA-IGS and 16-23S rRNA-ITS sequence, a finding consistent with other recent taxonomic analyses of *Microcystis* based on morphology (Otsuka et al., 2000; Otsuka et al., 1999a) and on DNA sequence data from 16S rRNA (Herdman et al., 2001; Neilan et al., 1997a; Otsuka et al., 1998). Most of the morphological characters from the Geitlerian system are not phylogenetically informative; however, features such as the presence of gas vesicles have been validated as clearly separating *Microcystis* from other unicellular cyanobacteria formerly included in *Microcystis* (i.e., *M. elebans* and *M. holsatica*) (Herdman et al., 2001). Lack of gas vesicles in *M. elebans* and *M. holsatica* is due to the absence of gas vesicle forming genes rather than mutation(s) blocking vesicle formation which is known to occur frequently in lab isolates but is unlikely to be found in lakes (Lyra et al., 2001). 16S rRNA and GC content studies have also conclusively shown *M. elebans* and *M. holsatica* to belong to other Chrococcalean families (Herdman et al., 2001).

RAPD analysis of *Microcystis* strains (Neilan, 1995) suggested the current morphospecies designations were incorrect; however, subsequent studies suggest that Neilan's analysis and assignments based on Dollo parsimony were flawed (Wilmotte and Herdman, 2001). Studies using 16S rRNA and *rbc*L and X clearly distinguished morphospecies designated as *Microcystis holsatica*, *M. elebans* and *M. botrys* as distinct genotypes outside the genus *Microcystis* and more closely related to *Synechococcus* PCC 6301 (Neilan et al., 1997a; Rudi et al., 1998). Rudi's analysis also concluded that at least

with respect to the *rbc*L and X markers, gene transfer between phyletically closely related cyanobacteria in neutrally evolving genes likely occurs based on differences between the topology of the 16S rRNA tree and the *rbc*L and X tree (Rudi, Skulberg and Jakobsen, 1998). Additional work on Group I introns in $tRNA^{LEU}$ (UAA) confirmed the polyphyletic origin of introns in cyanobacteria and that genetic mobility is possible in cyanobacteria based on either a homing endonuclease mechanism or reverse splicing and reverse transcription (Rudi and Jakobsen, 1999). These findings placed earlier *Microcystis* phylogenetic conclusions in doubt but do not appear to be a concern here where phylogenies based on molecular markers are consistent with 16S rRNA, DNA-DNA reassociation, and other markers. The results of this study were consistent with the above factors and no evidence of gene transfer for *cpc*BA-IGS or 16-23S rRNA-ITS was detectable within the sequences of *Microcystis* strains examined.

Partial and full sequence of the 16S rRNA fail to delineate *Microcystis* species and strains due to the high similarity in sequence (exceeds 97%) across the entire *Microcystis* cluster (Neilan et al., 1997a; Otsuka et al., 1998; Rudi, Skulberg and Jakobsen, 1998). DNA-DNA reassociation of 9 Japanese strains of *Microcystis* representing 4 of the recognized morphospecies found a difference of only 29.9% between the most disparate strains in the study (Kondo et al., 2000). The phylogenetic definition of a bacterial species typically includes strains that share 70% or greater DNA-DNA relatededness (Stackebrandt and Goebel, 1994; Wayne et al., 1987). Our *cpc*BA-IGS study shared two of the strains (NIES 111 and NIES 298) from the Kondo study with

a DNA-DNA reassociation value of 79.5% that translates to a difference in *cpc*BA-IGS sequence of 6.5% between our most distant Clusters (I & IV).

DNA-DNA reassociation data from a more complete study by Otsuka (2001) also places the similarity between the most disparate of *Microcystis* strains above 70% and when combined with high 16S rRNA similarity and the *cpc*BA-IGS similarity ~ 7% from this study, strongly support the contention that the genus *Microcystis* contains a single species. The relationship of *Microcystis* strains to each other and their geographic distribution, however, remains at issue. Several molecular markers have identified distinct strain clusters/clades within *M. aeruginosa* (Otsuka et al., 1999b; Tillett, Parker and Neilan, 2001), and this study confirms these studies in part and expands their results showing that stable clusters of strains exist and that most of the clades/clusters of strains have a cosmopolitan representation. The *cpc*BA-IGS data from this study strongly support at least four clades/clusters of *Microcystis aeruginosa* while the 16-23S rRNA-ITS data are less conclusive but consistent with that of *cpc*BA-IGS. The approximate two fold increase in resolving power present in the *cpc*BA-IGS data is likely responsible for differences in topology, and this is also borne out in previous data sets when comparing the two markers (Otsuka et al., 1999b; Tillett, Parker and Neilan, 2001). Otsuka's 16-23S rRNA-ITS data set is entirely Asian in origin and the DNA-DNA reassociation data set (Otsuka et al., 2001) is also Asian with the exception of one strain from the United Kingdom. An even wider selection of strains would facilitate further our understanding of the diversity within *Microcystis* and the distribution of that diversity.

Bolch has suggested that Australia may have a unique cluster of *Microcystis* strains (C. S. Bolch, IVth International Toxic Cyanobacteria Symposium, 1997); however, the *cpc*BA-IGS data supporting this assertion remains unpublished and the toxic Pacific Northwest strains in this study have not been compared with the Australian taxa. The one representative of the toxic Pacific Northwest cluster sequenced for both *cpc*BA-IGS and 16-23S rRNA-ITS appears in approximately the same topological region of the 16-23 rRNA-IGS and *cpc*BA-IGS trees with moderate support. No other strain outside of North America included in this analysis or available in Genbank was found in Cluster III except for EAWAG110. Depending on the origin of EAWAG110 (unknown origin), Cluster III may represent a North American genotype not shared with other regions. Additional taxa from Australian samples and from other under-represented regions should be sequenced to determine whether this clade or any other purported clade is truly regional in distribution.

The diversity of clusters found in the Pacific Northwest appeared similar to that of other regions. Asian and Australian strains were represented in three of the four clusters (absent from Cluster III). European strains were present in two and possibly three clusters (Clusters I and II with Cluster III depending on the origin of EAWAG110). North American strains were present in Clusters I-III while the two Pacific Northwest genotypes were only found in Clusters I and III. Further study of the ecological requirements of *Microcystis* clusters may shed light on succession within *Microcystis* communities and the diversity of strains in lakes and estuaries with distinct environmental conditions. Isolation and PCR amplification of single *Microcystis*

colonies via the techniques described in this study allow for quantitative studies of *Microcystis* populations over space and time. Studies of this type for the genus *Nodularia* already have provided significant information on the dynamics of cyanobacterial communities in the Baltic Sea (Barker et al., 1999). Linking these techniques with extensive environmental data should provide a mechanism at last to track succession within microbial freshwater communities at depth, across waterbodies and over time.

Of the 40-100 genes that are useful phylogenetically (for bacteria), *cpc*BA-IGS and 16-23S rRNA-ITS appear well suited for cyanobacterial phylogenetic analysis at the strain cluster/clade level. The sequence divergence among *Microcystis* strains for *cpc*BA-IGS and 16-23S rRNA differed with *cpc*BA-IGS providing approximately twice the phylogenetic information (11% vs. 5.6%) of 16-23S rRNA-ITS. The first *cpc*BA-IGS RFLP analysis (Neilan, 1995) identified four clades/clusters of *Microcystis* strains in contrast to the three clusters found in the first 16-23S rRNA ITS study (Otsuka et al., 1999b). This study supplements the data of Otsuka (1999) with a new cluster (Cluster III) and adds strains from additional regions. A recent *cpc*BA-IGS study partitions *Microcystis* into 3 clusters (IA, IB & II) based on neighbor joining analysis (Tillett, Parker and Neilan, 2001). Tillet's analysis includes no members of Cluster IV from our analysis. Cluster IA strains are represented in our Cluster III and Cluster IB strains correspond to our Cluster II. Tillett's Cluster II, however, is broader than our equivalent, Cluster I. Strains EAWAG 127 a and c, and UWOCC- MRC & D are placed in Tillett's Cluster II but fall into our Cluster II which corresponds to Tillet's IB. This disparity is

not surprising given the weak to moderate bootstrap support in both Tillett's and our analyses and the polytomy in our *cpc*BA-IGS maximum likelihood tree reinforces the fact that the exact placement of these strains is uncertain. Both analyses do, however, place these strains in the same relative branch of the phylogeny regardless of cluster assignment and are therefore consistent with the general description of the relationship of the strains.

The cyanobacteria are an ancient lineage and the phylogeny of the group based on 16S rRNA analysis is deeply branched with a fan-like topology with little resolution that indicates a likely explosive radiation of this group after the acquisition of oxygenic photosynthesis (Wilmotte and Herdman, 2001). Given the current uncertainty regarding phylogeny, few suggested phylogenetic groupings were included in the 2001 Bergey's Manual at a higher resolution than the Subsection level and form genera are described as temporary vehicles for various species, ecotypes and strains. *Microcystis* is placed in Subsection I with other unicells that reproduce by binary fision. Hypotheses describing the mechanisms that have resulted in the level of cyanobacteria morphological plasticity and the stability of morphological forms over time vary widely. Several studies suggest that the apparent stability of cyanobacterial morphological characters is the result of the exchange of genetic material for neutrally evolving genes (Rudi, Skulberg and Jakobsen, 1998). As additional genomes of cyanobacteria taxa are sequenced, a clearer picture will emerge as to whether the exchange of genetic material is the primary force in shaping morphology while other selective pressures alter physiology or whether morphology is simply constrained by the limited number of successful ecological strategies available.

Neilan (1997) has commented "it appears that the morphological, functional and geographical adaptation of cyanobacteria is not mirrored by the molecular evolution of these organisms." The tremendous convergence in form, function and exploitation of environmental niches by cyanobacteria represent a significant challenge to our understanding of their evolutionary placement.

For example, the phylogeny of *Microcystis* based on microcystin synthetase sequence (*mcy*C) does not correspond to the *cpc*BA-IGS, 16S rRNA, or 16-23S rRNA-ITS based phylogenies. Interestingly, analysis of *mcy*C chromosomal location across strains clearly shows that this subunit is found at the same location in all strains and it is therefore unlikely that either intra- or intergenomic mobility (lateral gene transfer or other genomic reaarangement) is occurring (Tillett, Parker and Neilan, 2001). The more likely hypothesis then is that *Microcystis* was originally capable of toxin production and non-toxic strains are defective mutants. This does not explain the discovery of microcystin within other cyanobacterial lineages (i.e., *Anabaena* and *Oscillatoria*) and the alternative hypothesis that site specific recombination can occur frequently for genes such as *mcy*C remains untested. A combination of genome sequencing of key taxa and additional phylogenetic studies at the population level will be needed to fully understand this remarkable organism.

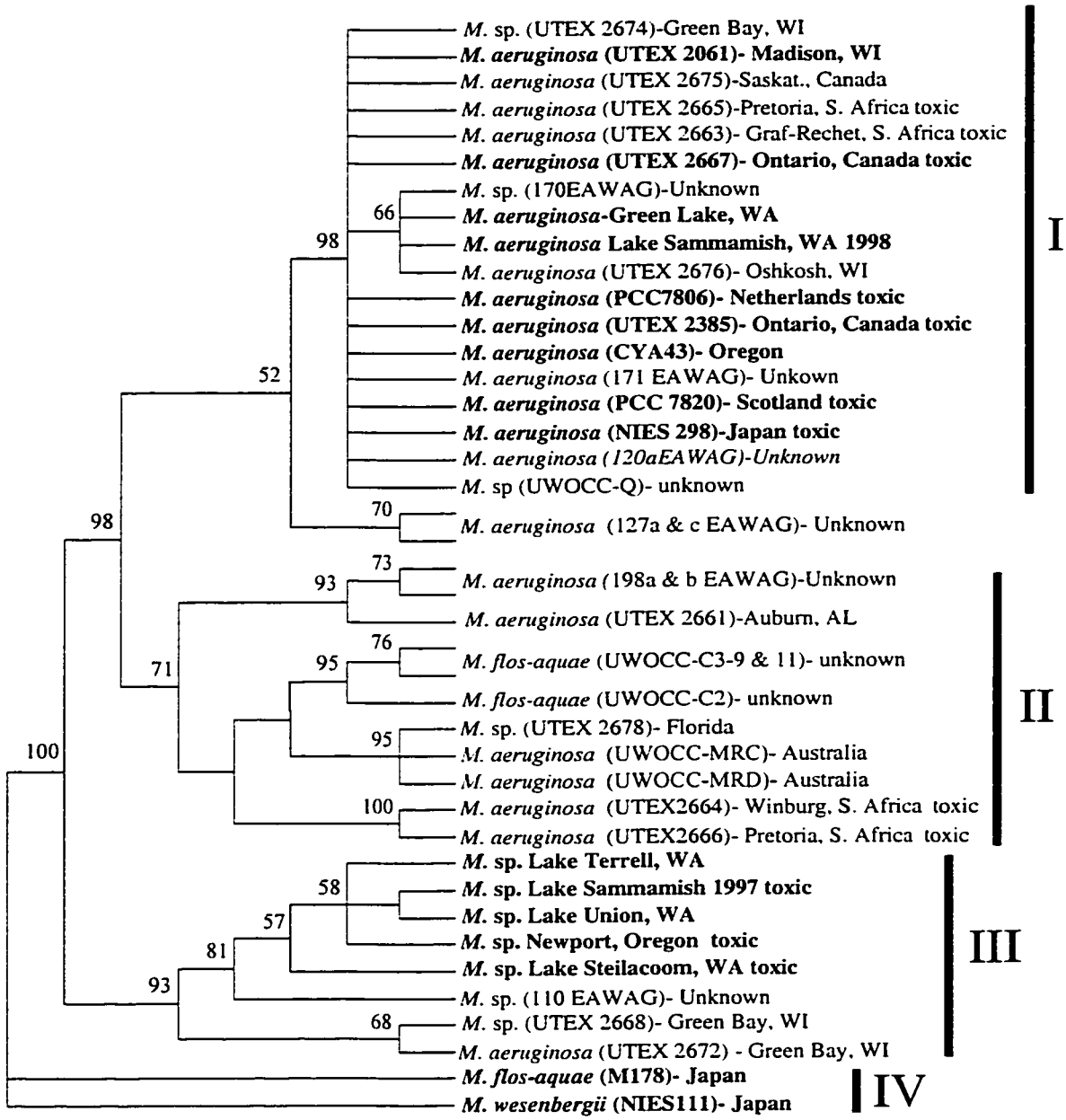

Figure 1. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *Microcystis cpc*BA-IGS sequences. Note that four distinct strain clusters are supported with Pacific Northwest strains found in Clusters I and III. No correlation appears with respect to toxicity. Cluster III may be strictly North American. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Nodes with bootstrap values of less than 50% are not labeled. Bold type indicates strains sequenced in this study.

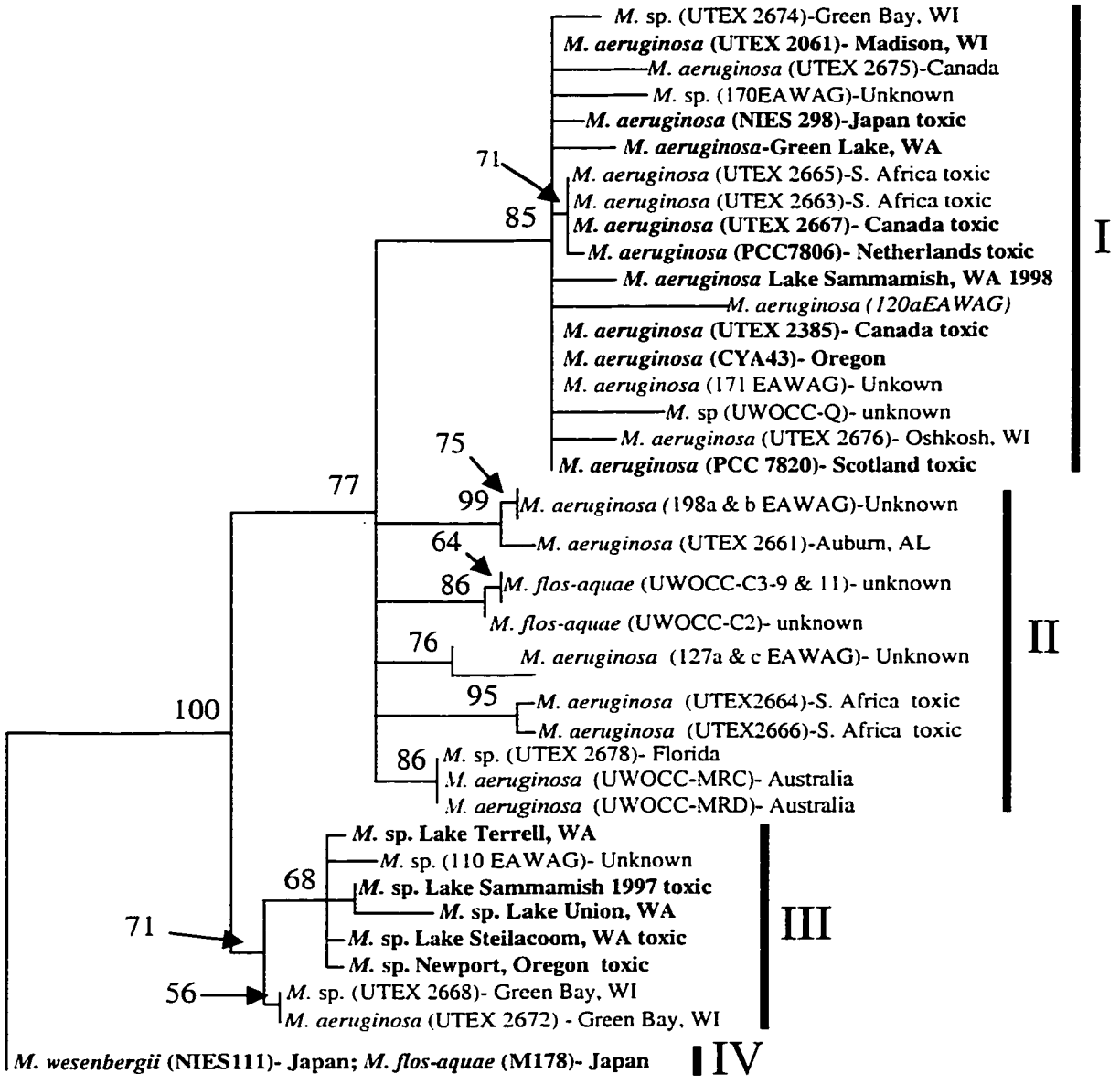

Figure 2. Phylogenetic tree inferred from a heuristic search using maximum likelihood from *Microcystis cpc*BA-IGS sequence data (-ln = 1690.82702). Note that four distinct strain clusters are supported with Pacific Northwest strains found in Clusters I and III. No correlation appears with respect to toxicity. Cluster III may be strictly North American. NIES 111 and M178 in Cluster IV share identical sequence. Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples) and nodes with bootstrap values less than 50% are not labeled. Bold type indicates strains sequenced in this study.

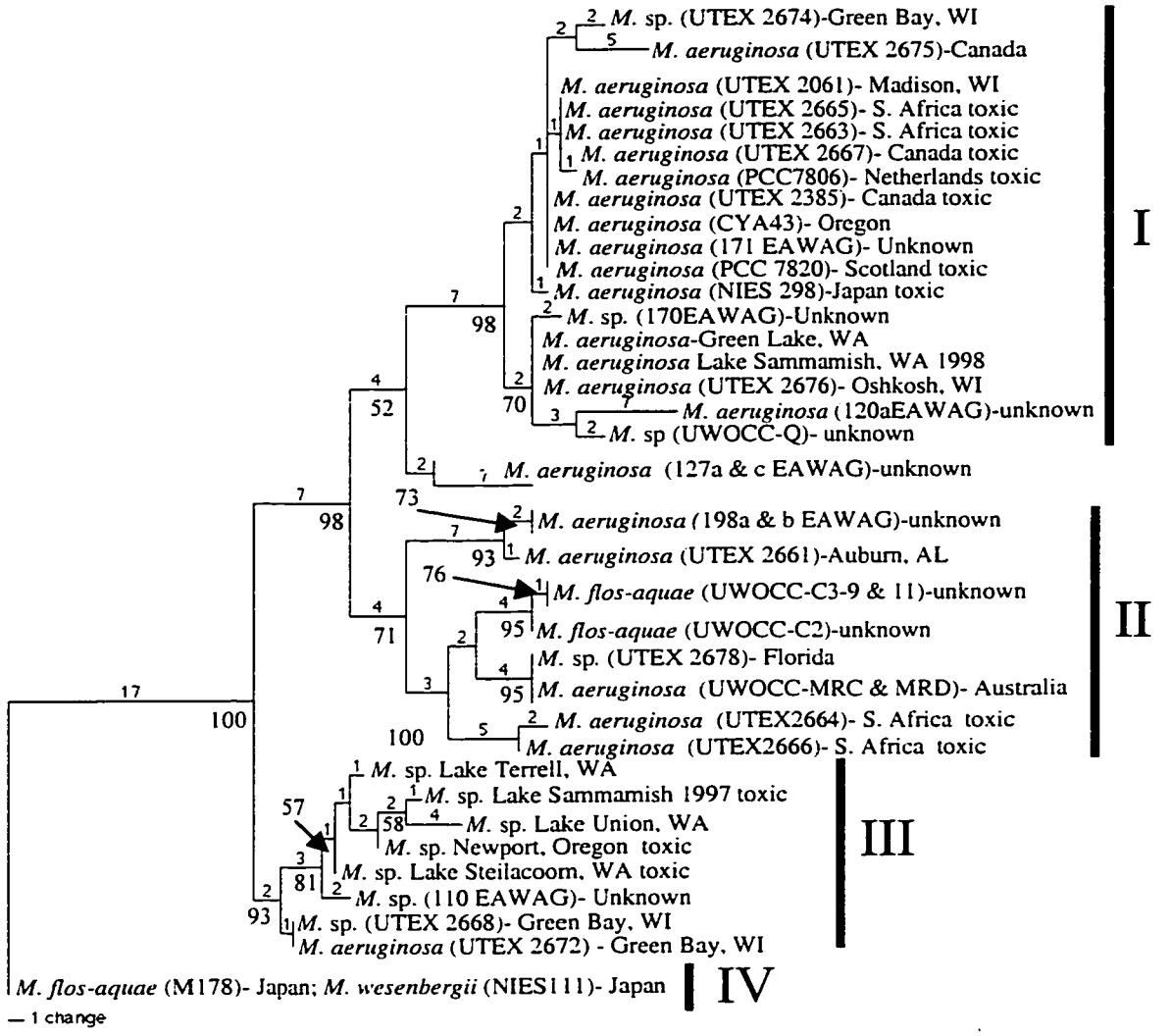

Figure 3. Phylogram based on maximum parsimony analysis of *Microcystis cpc*BA-IGS sequences. Tree shown is one of 14 most parsimonious trees recovered in a heuristic search. All trees supported four distinct strain clusters. Pacific Northwest strains are found in Clusters I and III. No correlation appears with respect to toxicity and geography. Cluster III may be strictly North American. Numbers above the nodes represent the number of base changes between nodes. Numbers below the nodes are labeled with bootstrap values for parsimony (1000 replicate samples) and nodes with less than 50% bootstrap values are not labeled. Tree length = 130 steps; CI = 0.638; RI = 0.895; RC = 0.74; HI = 0.362. Scale bar for 1 nucleotide change is shown at bottom.

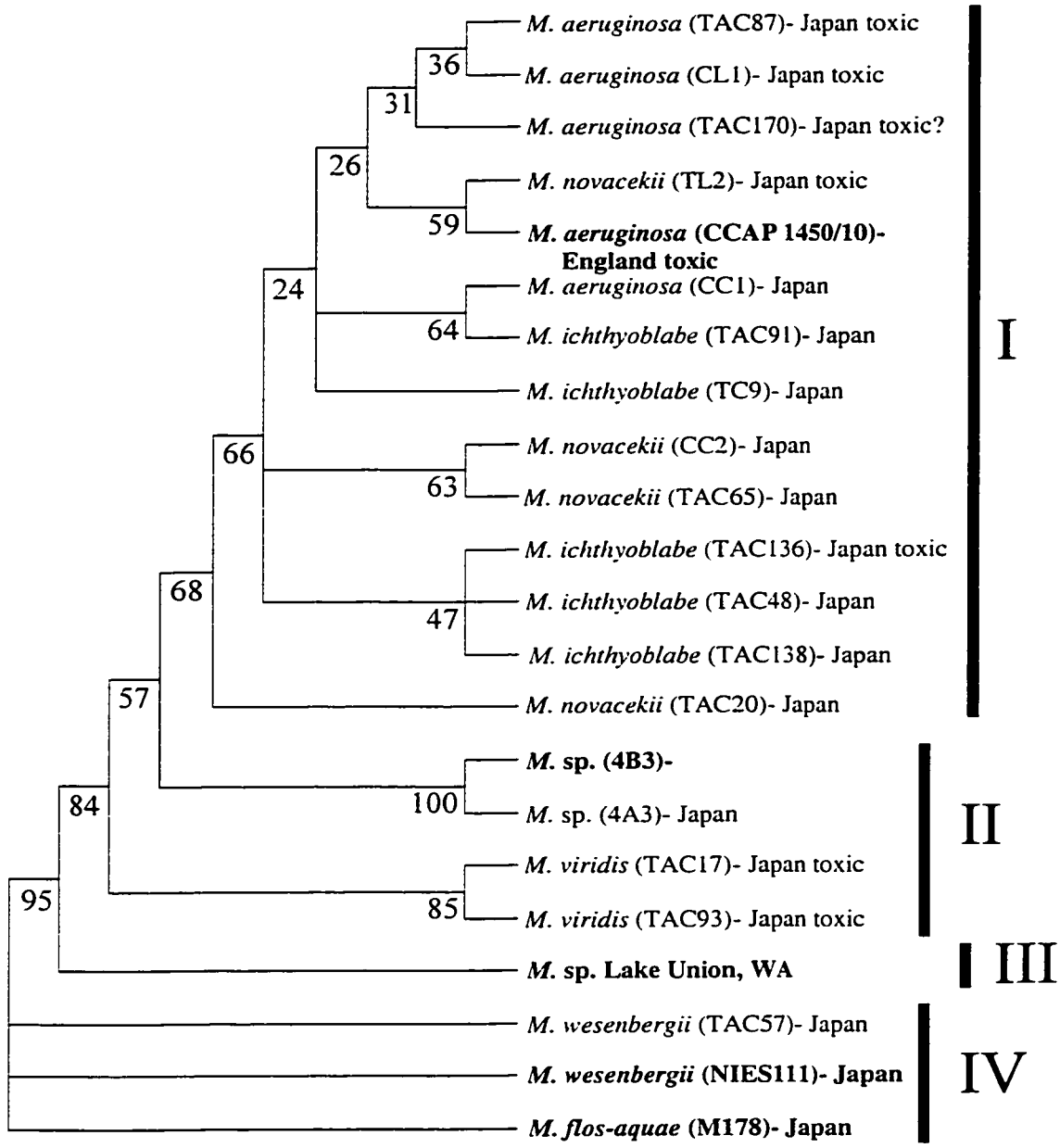

Figure 4. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *Microcystis* 16-23S rRNA-ITS sequences. Four clusters of *Microcystis* strains can be distinguished with Pacific Northwest strains found in Clusters I and III. Clusters I, III and IV contain the same strains as in the *cpc*BA-IGS analysis. Toxicity does not appear restricted to any geographic region or strain cluster. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Bold type indicates strains sequenced in this study.

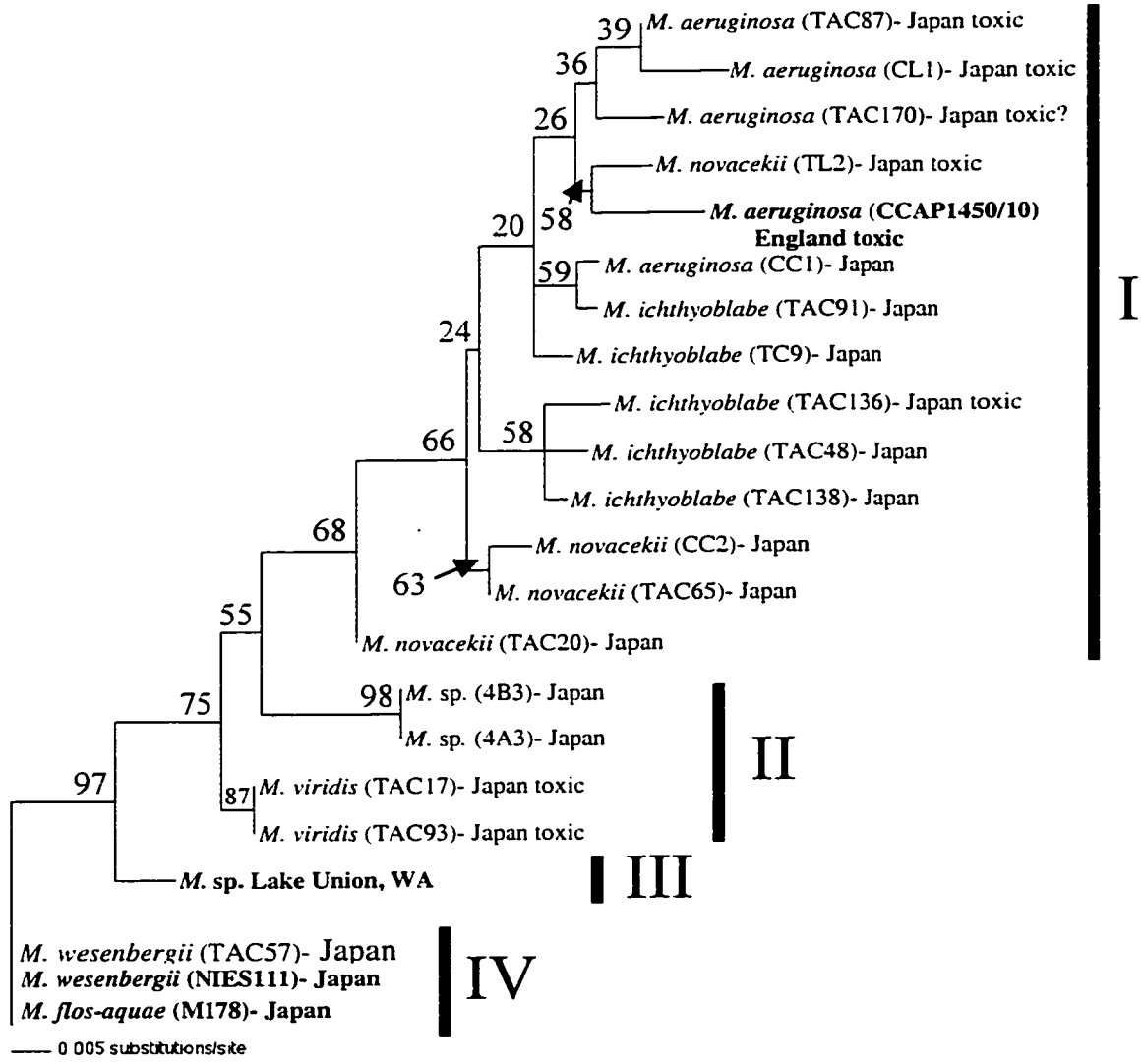

Figure 5. Phylogenetic tree inferred from a heuristic search using maximum likelihood from *Microcystis* 16-23S rRNA-ITS sequence data (-ln = 1483.84065). Four strain clusters are distinguished with Pacific Northwest *Microcystis* strains found in Clusters I and III. Topology mirrors that of the maximum parsimony tree. Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). Bold type indicates strains sequenced in this study.

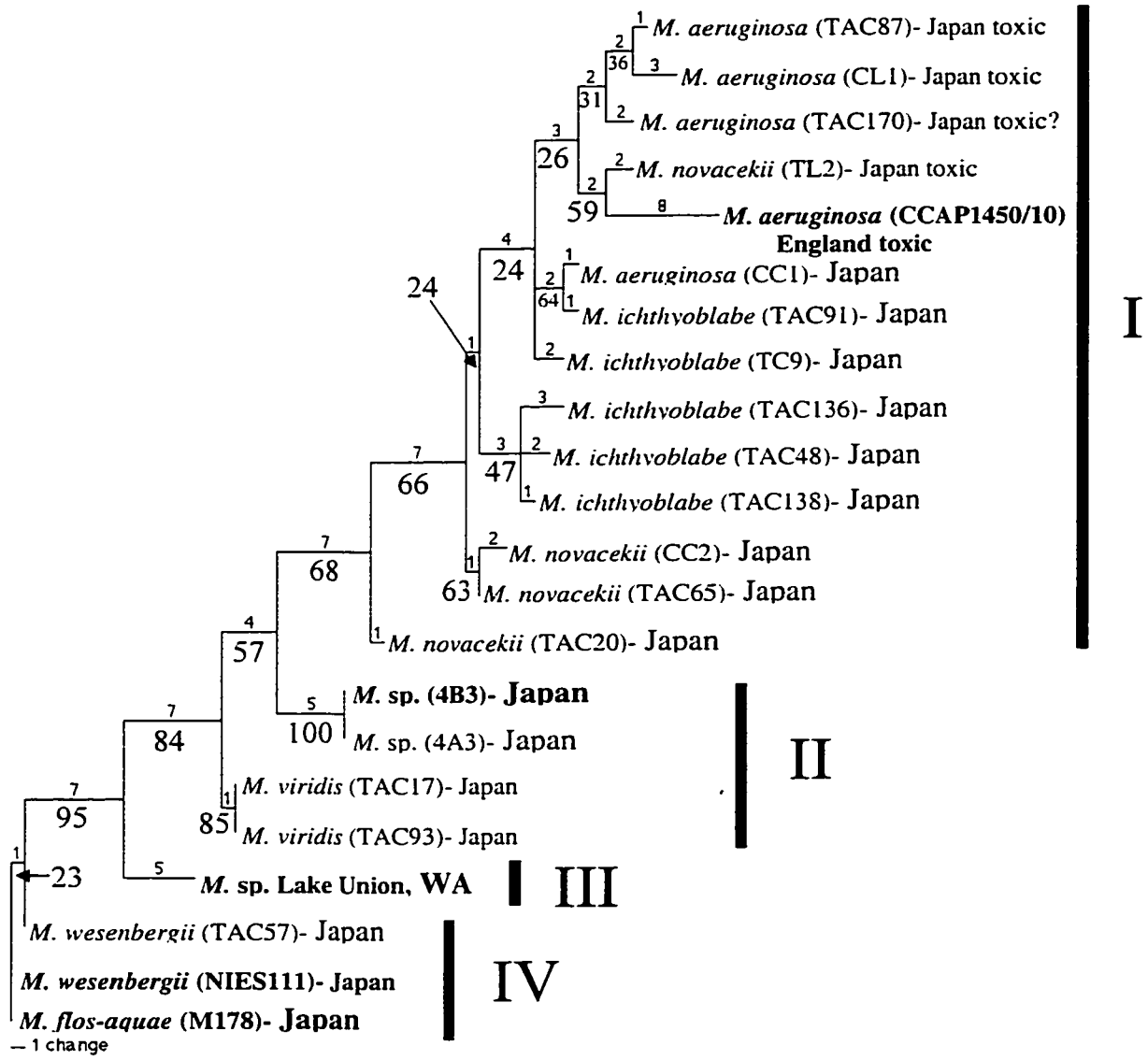

Figure 6. Phylogram based on maximum parsimony analysis of *Microcystis* 16-23S rRNA-ITS sequences. Tree shown is one of 18 most parsimonious trees recovered in a heuristic search. All of the trees shared similar topology with Pacific Northwest *Microcystis* strains found in strain Clusters I and III. Numbers above the nodes represent the number of base changes between nodes. Numbers below the nodes are labeled with bootstrap values for parsimony (1000 replicate samples). Tree length = 93 steps; CI = 0.527; RI = 0.762; RC = 0.402; HI = 0.473. Scale bar for 1 nucleotide change is shown at bottom. Bold type indicates strains sequenced in this study.

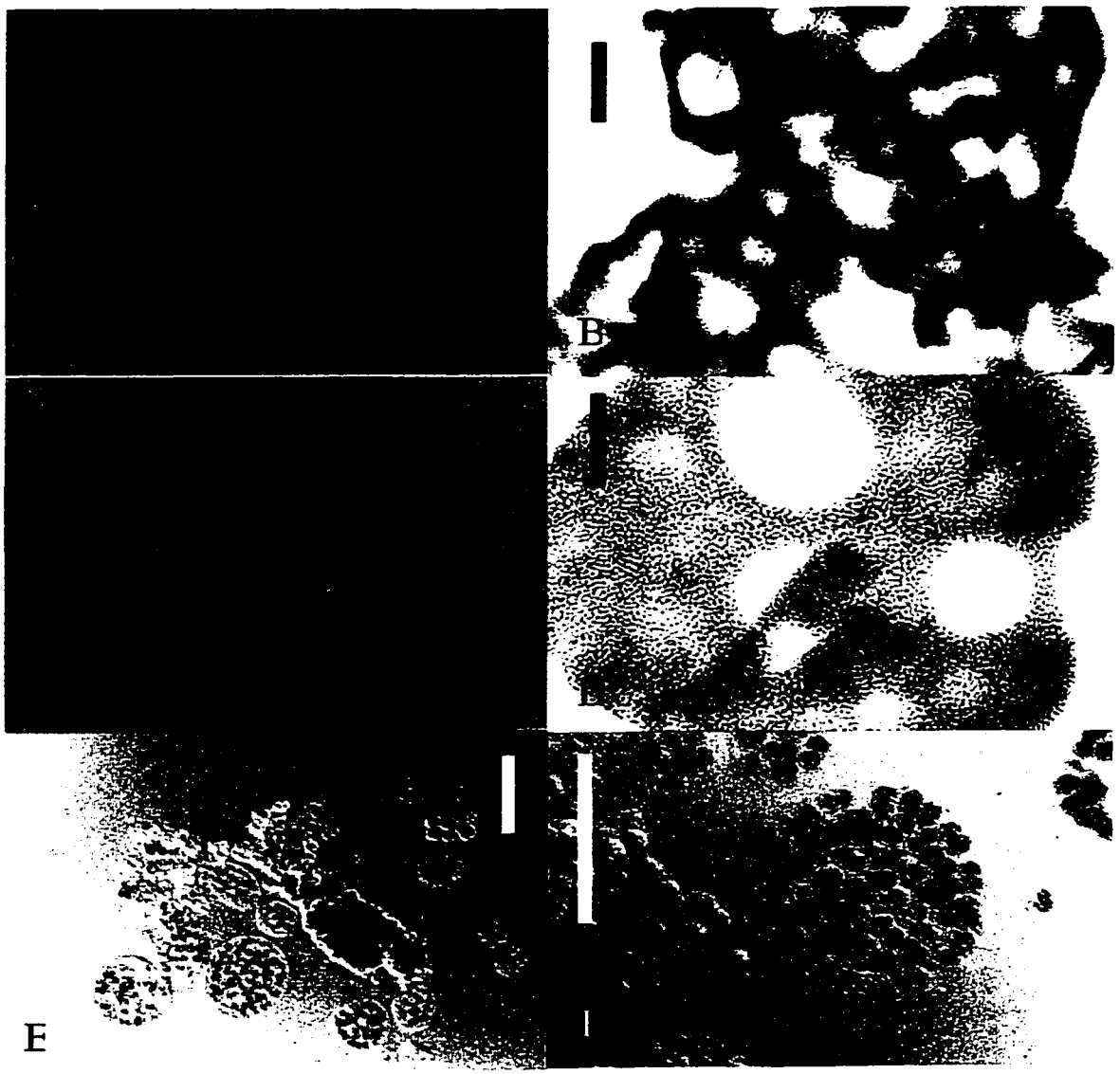

Figure 7. *Microcystis* habit as seen by light microscopy. Note the difference in clathration, colony shape and size in contrast to the very small differences in genetic makeup. (a) Cluster I *Microcystis* from Green Lake, WA; (b) Cluster III *Microcystis* from Lake Union, WA; (c) Cluster III *Microcystis* from Lake Terrell; (d) Cluster III *Microcystis* from Lake Steilacoom; (e) Cluster IV *Microcystis flos-aquae* (M-178); and (f) Cluster IV M-178. Bar = 30 μm.

Table 1. Cyanobacteria strains included in the *Microcystis* study.

Taxon	Strain	Toxicity	Source	Accession Number
Microcystis				
aeruginosa Kützing	PCC7806	Yes	Braakman Reservoir, Netherlands	NA
aeruginosa Kützing	PCC7820	Yes	Lake Balgavies, Scotland	NA
aeruginosa Kütz. em. Elenkin	CYA43	No	Oregon	NA
aeruginosa Kützing	NIES298	Yes	Japan	NA
aeruginosa Kütz. em. Elenkin	UTEX 2061	No	Madison, Wisconsin	NA
aeruginosa Kütz. em. Elenkin	UTEX 2675	No	Saskatchewan, Canada	AF195175
aeruginosa Kütz. em. Elenkin	UTEX 2665	Yes	Pretoria, South Africa	AF195170
aeruginosa Kütz. em. Elenkin	UTEX 2663	Yes	Graff-Reinet, South Africa	AF195169
aeruginosa Kütz. em. Elenkin	UTEX 2667	Yes	Ontario, Canada	AF195165
aeruginosa Kütz. em. Elenkin	UTEX 2676	No	Oshkosh, Wisconsin	AF195171
aeruginosa Kütz. em. Elenkin	UTEX 2385	Yes	Ontario, Canada	NA
aeruginosa Kütz. em. Elenkin	UTEX 2661	No	Auburn Univ, AL	AF195166
aeruginosa Kütz. em. Elenkin	UTEX 2664	Yes	Winburg, South Africa	AF195167
aeruginosa Kütz. em. Elenkin	UTEX 2666	Yes	Pretoria, South Africa	AF195168
aeruginosa Kütz. em. Wesenberg-Lund	UTEX 2672	No	Green Bay, WI	AF195159
aeruginosa Kützing	TAC87	Yes	Shimane, Japan	AB015364
aeruginosa Kützing	TAC170	Unknown	Tokyo, Japan	AB015365
aeruginosa Kützing	CL1	Yes	Hubei, P. R. China	AB015381
aeruginosa Kützing	CC1	No	Neinengo, P. R., China	AB015383
aeruginosa Kütz. em. Elenkin	CCAP 1450/10	Yes	England	NA
aeruginosa Kützing	EAWAG170	Unknown	Switzerland	AJ003178
aeruginosa Kützing	EAWAG120a	Unknown	Switzerland	AJ224711
aeruginosa Kützing	EAWAG171	Unknown	Switzerland	AJ003179
aeruginosa Kützing	EAWAG198a	Unknown	Switzerland	AJ003182
aeruginosa Kützing	ESWAG198b	Unknown	Switzerland	AJ003183
aeruginosa Kützing	EAWAG127a	Unknown	Switzerland	AJ003174
aeruginosa Kützing	EAWAG127c	Unknown	Switzerland	AJ003175
flos-aquae (Wittr.) Kirshn.	*M178*	No	Japan	NA
flos-aquae (Wittr.) Kirshn. em. Wesenberg-Lund	*UWOCC-C2*	Unknown	Unknown	AF195162
flos-aqaue (Wittr.) Kirshn. em. Wesenberg-Lund	*UWOCC-C3-9*	Unknown	Unknown	AF195163
flos-aqaue (Wittr.) Kirshn. em. Wesenberg-Lund	UWOCC-C3-11	Unknown	Unknown	AF195164
ichthyoblabe Kützing	TC9	No	Bangkok, Thailand	AB015373
ichthyoblabe Kützing	TAC91	No	Hokkaido, Japan	AB015367
ichthyoblabe Kützing	TAC136	No	Fukuoka, Japan	AB015369
ichthyoblabe Kützing	TAC48	No	Nagano, Japan	AB015366
ichthyoblabe Kützing	TAC138	No	Fukuoka, Japan	AB015370
novacekii (Komárek) Compére	TL2	Yes	Chon Buri, Thailand	AB015380
novacekii (Komárek) Compére	CC2	No	Hubei, P. R., China	AB015378

Table 1. Continued

Taxon	Strain	Toxicity	Source	Accession Number
Microcystis				
novacekii (Komárek) Compére	TAC65	No	Nagano, Japan	AB015375
novacekii (Komárek) Compére	TAC20	No	Ibaraki, Japan	AB015374
sp.	4A3	No	Hubei, P. R. China	AB015357
sp.	4B3	No	Hubei, P. R. China	AB015358
sp.	Union	Unknown	Portage Bay, Lake Union, Seattle, WA (spring bloom)	NA
sp.	Terrell	Unknown	Lake Terrell, Cherry Point, WA	NA
sp.	Steilacoom	Yes	Lake Steilacoom, Steilacoom, WA	NA
sp.	Green Lake	Unknown	Green Lake, Seattle, WA	NA
sp.	Sammamish 1997	Yes	Lake Sammamish, Issaquah, WA	NA
sp.	Sammamish 1998	No	Lake Sammamish, WA	NA
sp.	Newport	Yes	Big Creek Reservoir #2, Newport, Oregon	NA
sp.	Spanaway	Yes	Lake Spanaway, Spanaway, WA	NA
sp.	Pendleton	Yes	Pendleton Pond, Silvana, WA	NA
sp.	110 EAWAG	Unknown	Switzerland	AJ003172
sp.	Black Lake	Unknown	Olympia, WA	NA
sp.	UWOCC-Q	Unknown	Unknown	AF195179
sp.	UTEX 2668	No	Green Bay, WI	AF195160
sp.	UTEX 2674	No	Green Bay, WI	AF195178
sp.	UTEX 2678	No	Florida	AF139344
viridis (A. Brown) Lemmermann	TAC17	Yes	Ibaraki, Japan	AB015398
viridis (A. Brown) Lemmermann	TAC93	Yes	Hokkaido, Japan	AB015403
wesenbergii (Komárek) Komárek in Kondratieva	NIES111	No	Ibaraki, Japan	AB015388
wesenbergii (Komárek) Komárek in Kondratieva	TAC57	No	Nagano, Japan	AB015391

CHAPTER 2: PHYLOGENETIC AND BIOGEOGRAPHIC ANALYSIS OF PACIFIC NORTHWEST *ANABAENA* BASED ON *cpc*BA-IGS AND *rpo*C1 DNA

ABSTRACT

A phylogenetic analysis of *Anabaena* was constructed from Pacific Northwest, U.S.A. direct PCR lake samples (single filaments) and culture isolates from around the world using DNA sequence from the *cpc*BA-IGS and *rpo*C1 regions. Sequences from over 15 species, including several toxin producing strains, representing a variety of morphotypes were examined using 680 bp of DNA from the *cpc*BA-IGS region and 585 bp from the *rpo*C1 region. One Pacific Northwest *Anabaena flos-aquae* strain (AL89) was identified as the only toxic strain sampled and blooms appeared restricted to late fall through early spring. *Anabaena* blooms in lowland Puget Sound lakes were limited to the two common species, *Anabaena flos-aquae* and *Anabaena circinalis*, that exhibited five morphotypes. Morphological characteristics proved useful in delimiting the two species from each other but not in distinguishing toxic and non-toxic *A. flos-aquae* strains.

This study indicates that at least two major clades of *Anabaena* exist: a cluster including *Anabaena flos-aquae* (Group IIA) and *Anabaena circinalis* (Group IIB) and a cluster including numerous other *Anabaena* spp. (Groups IA and IB). Based on the taxa represented in our sample, *Anabaena* appeared paraphyletic in both the *cpc*BA-IGS and *rpo*C1 phylogenies with several of the *Anabaena* culture isolates distributed in a clade including the genera *Nostoc* and *Nodularia*. These results suggest that many of the

"good" morphological characters used to distinguish genera within the Nostocaceae are not valid.

INTRODUCTION

The genus *Anabaena* Kützing ex Lemmerman 1907 is a member of the family Nostocaceae (Dumort. 1829) in the order Nostocales (Komárek and Anagnostidis, 1989). *Anabaena* is a non-branching trichome with nitrogen-fixing heterocysts and akinetes and is typically associated by the public with blooms in eutrophic lakes, ponds and reservoirs (Paerl and Tucker, 1995). *Anabaena* is widely distributed throughout the world not only in lakes and estuarine conditions but is also found in soils as an important member of cryptogamic crusts and is also found as a symbiont in several plants (Lee, 1999). Like most cyanobacteria, *Anabaena* thrives in limited ecological conditions where it tends to dominate seasonally to the exclusion of other organisms, particularly in environmentally stable habitats (Paerl, 1988). Planktonic *Anabaena* are scum-forming ecostrategists that use gas vesicles in combination with carbohydrate ballast to control access to light and nutrients by moving up and down in the water column (Chorus and Bartram, 1999)

Anabaena has been implicated in numerous toxic bloom events in lakes and reservoirs as a producer of the hepatotoxin, microcystin, a serine/threonine protein phosphatase 1 and 2A inhibitor (Honkanen et al., 1990), saxitoxin, an alkaloid nerve cell sodium channel blocker, anatoxin a, an acetyl choline mimic, and anatoxin a (S), an anticholinesterase (Chorus and Bartram, 1999). The breadth of toxins produced by

Anabaena strains has puzzled ecologists and taxonomists as no correlation between phylogenetic relationships and toxin production has been identified (Tillett, Parker and Neilan, 2001). Regardless of their evolutionary origins, toxins can be traced to particular strains and this information is critical in understanding the ecological conditions and distribution of strains that pose risks to the public's health.

Li (2000) discusses the paucity of morphological and genetic information available for identification of planktonic species of *Anabaena* at a time when water management authorities need information regarding the distribution of toxic producing strains. We face potential catastrophic consequences such as the recent death of hemodialysis patients in Brazil (Pouria et al., 1998), death of livestock (Chorus and Bartram, 1999) or chronic effects on fish, invertebrates and other organisms using water resources where *Anabaena* blooms if we ignore the taxonomic questions that remain unresolved in this genus. Construction of a classification system that provides an accurate description of the natural relationship between organisms and a useful identification tool on a day-to-day basis is difficult, as the concept of a "species" in the cyanobacteria has no distinct boundaries. Phylogenetic approaches provide an objective framework that will allow us to understand key ecological relationships from the exchange of genetic information to the ecophysiological parameters that govern succession in waterbodies.

Cyanobacteria are currently treated systematically both under the International Code of Botanical Nomenclature (ICBN) (Greuter et al., 2000) and the International

Code of Nomenclature of Bacteria (ICNB) (Sneath, 1992) with most researchers in agreement that as prokaryotes, cyanobacteria are fit more appropriately under the ICNB (Castenholz, 1992). While there are differences between these systems, both have moved slowly toward polyphasic taxonomy in an attempt to describe more accurately the phylogenetic relationships among cyanobacteria (Castenholz, 2001; Waterbury and Rippka, 1989; Wilmotte, 1994). It is important to note, however, that the priority of species names rests with the ICBN, and it is therefore necessary for studies at the species level to integrate this information with the polyphasic taxonomy approach of the ICNB (Vandamme et al., 1996). Under the ICBN, filamentous cyanobacteria with heterocysts, including *Anabaena*, are primarily classified according to morphological characters that are considered constant and genetically stable (Komárek et al., 1993).

Taxa within the ICBN order Nostocales *sensu* Komarárek conform to the following morphological characteristics: 1) hormogonal structure of filaments (uniserial row of cells with cross walls); 2) perpendicular cell division; 3) the ability to produce heterocysts; 4) absence of true branching; and 5) facultative false branching and production of akinetes (Komárek and Anagnostidis, 1989). The Nostocales includes the families Rivulariaceae, Scytonemaceae, and Nostocaceae. Komárek defines the family Nostocaceae (Dumort., 1829) as a group of taxa with non-branching isopolar trichomes composed of terminal cells that may be rounded or conical, sometimes slightly narrowed or attenuated but rarely elongated and pointed, and trichomes containing akinetes (resting spores) and heterocysts (nitrogen-fixing cells). Members of the Nostocaceae do not have

meristematic zones and reproduction may proceed by motile hormogonia or immotile hormocytes. The first valid publication of the Nostocaceae under the ICBN is Bornet and Flahault's 1886 monograph (Greuter et al., 2000).

Genera within the Nostocaceae are distinguished by a number of characters which include: 1) the position of heterocysts as either terminal (*Cylindrospermum, Cylindrospermopsis, Anabaenopsis*) or intercalary (*Anabaena, Nostoc, Nodularia, Aphanizomenon*); 2) the symmetry of filaments based on the position of heterocysts/akinetes that can be either symmetric, subsymmetric or metameric (i.e., if heterocysts form terminally, complete symmetry results (*Cylindrospermum*), if heterocysts form intercalary within the trichome and are more or less equidistant, then metameric symmetry results (*Anabaena, Nostoc, Nodularia*), and if several intercalary heterocysts are in irregular connection with akinetes, a subsymmetric pattern results such as in *Aphanizomenon*); and 3) characterization of akinetes as either paraheterocystic (*Anabaena, Aphanizomenon, Anabaenopsis, Cylindrospermopsis*) or apoheterocystic (*Nostoc, Nodularia*). There are disagreements in the literature regarding the importance and consistency of these characters and the classification of taxa with exceptions that are not fully explained by this classification system (Komárek and Anagnostidis, 1989). While these characters may be fixed and stable, they may not represent the true phylogenetic relationship of a cyanobacterial lineage (Nadeau et al., 2001), and no rationale exists in the literature to suggest that any particular character state represents an ancestral state within the Nostocales or Nostocaceae.

The number of species in the genus *Anabaena* under the ICBN has varied historically from the revision of the genus by Geitler describing 40 species (Geitler, 1932) based on cell morphology and habit, to 2 species in Drouet's compression of cyanobacteria taxa based largely on herbarium and live samples (Drouet, 1981). Trichome symmetry in field samples (cell shape and size across the trichome including heterocyst and akinete development) was regarded as the primary character useful in distinguishing *Anabaena* species under the ICBN (Desikachary, 1959). The development of laboratory culturing of *Anabaena* has reduced the reliance of taxonomy on characters demonstrating a high degree of plasticity in response to environmental conditions, but these data have been limited to those strains that are amenable to culturing and production of heterocysts and akinetes (Hiroki et al., 1998). A more accurate description of *Anabaena* is difficult to obtain based on morphology given the shortage of taxa presently in culture (Li, Watanabe and Watanabe, 2000b), and not all cultures are available for comparison.

The ICBN type species, *Anabaena oscillarioides* Bory ex Born. et Flah. 1886, is a representative of one of two proposed subgenera in Komárek's description of the genus, and it is characterized by "non-gas vacuolate filaments that are unified into mats on a substrate, floating or living in soils." The proposed representative species for the planktonic *Anabaena* subgenus is *A. flos-aquae* Breb. ex Born. et Flah. 1886. *A. flos-aquae* can be a solitary trichome or may occur in clusters and may be either straight, helical or irregularly coiled and is gas vacuolate. Komárek (1989) also distinguishes two

groups within the planktonic *Anabaena* subgenus. One group has differentiated trichome parts, and the other does not (all vegetative cells are identical). Komárek splits off a genus from *Anabaena* named *Trichormus*, which has apoheterocystic akinetes rather than paraheterocystic akinetes. This leads to some confusion intergenerically because apoheterocystic akinetes are also present in *Nostoc* and *Nodularia*.

Criticism of Komárek's description has focused on the phylogenetic dilemma that characters in separate evolutionary lines can develop at different rates or multiple times and thus have different or no taxonomic value between and within taxonomic groups (Castenholz, 2001). This is particularly problematic in the Nostocales where several genera such as *Anabaenopsis* and *Aphanizomenon* have been accepted based on features not applied to other genera (Komárek and Anagnostidis, 1989). Several studies have also criticized the designation of *Anabaenopsis, Cylindrospermum* and *Cylindrospermopsis* based primarily on terminal heterocyst formation (Rippka et al., 2001). Given the lack of linkage between traditional morphological characters and the emerging phylogeny of the Nostocales, the traditional taxonomy used to distinguish Nostocalean taxa has proven inconsistent in elucidating their evolutionary history and these morphological characters need to be reexamined for their consistency with recent phylogenetic studies that include a suitable breadth of taxa to evaluate their utility.

This dissatisfaction with morphology based taxonomy and the advent of ultrastructural analysis in the 1960s, brought a profound rethinking in blue-green algal systematics when investigators confirmed that blue-greens clearly were eubacteria

(Stanier et al., 1971). Microbiologists then began to apply the ICNB to cyanobacteria strains that were amenable to culturing, and *Anabaena* was initially placed in Subsection IV based on its filamentous structure and exclusive division by binary fission in one plane (Rippka et al., 1979). *Anabaena* was distinguished from *Nostoc* under the ICNB by its reproduction by random trichome breakage as opposed to *Nostoc's* formation of hormogonia, which are distinguishable from mature trichomes by 1) rapid gliding motility, 2) smaller cell size or 3) gas vacuolation when grown in culture (Kantz and Bold, 1969).

While the inclusion of life history based studies was an improvement, the bacteriology community has not yet defined new characters to replace or supplement the traditional morphological characters used to distinguish genera and species within the Nostocales. Genera such as *Cylindrospermum* continue to be distinguished from *Anabaena* based on heterocyst and akinete placement (terminal and adjacent respectively) and *Nodularia* is distinguished from *Anabaena* solely by cell shape (isodiametric to cylindrical in *Nodularia* versus spherical, ovoid or cylindrical in *Anabaena*) (Rippka et al., 1979). The use of cell shape to distinguish *Anabaena* from *Nodularia* is entirely subjective as both genera include strains that have cylindrical cells that create a character overlap between the genera. Other techniques such as DNA-DNA reassociation (Stam, 1980), fatty acid profiles (Caudales and Welles, 1992), and salt tolerance (Stulp and Stam, 1984b) have been used in the developing arsenal of the polyphasic taxonomical approach, but these studies suffered from the very limited availability of axenic strains

and the apparent misidentification of some (50%) culture strains (Komárek and Anagnostidis, 1999).

The recently revised 2001 Bergey's Manual of Systematic Bacteriology (Castenholz, 2001), however, recognizes the current uncertainty in Nostocales taxonomy and indicates that the division between Subsection IV and Subsection V is unjustified and must be reevaluated (Rippka, Castenholz and Herdman, 2001). Rippka (2001) designates a temporary form genus, *Anabaena*, characterized by motile or immotile trichomes composed of barrel-shaped or cylindrical cells with conical end cells (gliding species) or undifferentiated end cells (planktonic members) and heterocysts that are exclusively intercalary or both intercalary and terminal. Akinetes are adjacent to or remote from heterocysts and some members contain gas vesicles (Rippka, Castenholz and Herdman, 2001).

Three reference strains are proposed for three clusters representing the proposed major lineages of *Anabaena*. Cluster I is represented by *Anabaena cylindrica* (PCC 7122) with a molecular % G+C of 43.9% and a genome size of 3.2 Gdal. Trichomes are composed of cylindrical cells 4-5 μm in diameter with conical terminal cells, both intercalary and terminal heterocysts, and cylindrical akinetes either singly or in pairs on one or both sides of heterocysts (Rippka, Castenholz and Herdman, 2001). Cluster II is represented by *Anabaena* sp. (PCC 7108), an intertidal isolate, with trichome characteristics similar to Cluster I, but with ellipsoidal akinetes found in short chains distant from heterocysts, a molecular % G+C of 42.3% and a genome size of 3.7 Gdal.

Rippka (2001) suggests that PCC 7108 could be *Anabaena variabilis* but recommends confirmation by DNA-DNA reassociation studies. Cluster III is represented by an immotile planktonic strain, *Anabaena flos-aquae* (PCC 9332), with cells 4-5 μm in diameter, heterocysts predominantly intercalary, no data on akinetes, a molecular % G+C of 39.1% and genome size of 3.3 Gdal. This study examines several species meeting the description of two of Rippka's three proposed clusters and includes strains of Pacific Northwest planktonic *Anabaena* to assess regional diversity.

The increased work in and understanding of cyanobacteria systematics has also allowed researchers to develop tools to describe the diversity in cyanobacterial communities both temporally and spatially. Komárek (1999) identifies one of the key problems in determining cyanobacteria diversity as the difficulty in assessing the relatedness of physiologically or morphologically diverse strains (i.e., pigment, width, habit) within established populations that represent ecological adaptations within what would be defined as a "species" based on ecological and morphological criteria. Komárek admits that the evaluation of morphological features is subjective, and current research in phylogenetics suggests that a small number of different cyanoprokaryotic genotypes may be present in regional waterbodies despite notable phenotypes present in the environment (Barker et al., 1999). Palinska et al. (1996) demonstrated phenotypic variability in identical genotypes in *Merismopedia*, and subsequent studies have confirmed this trend (Barker et al., 1999; Moore et al., 1998). These results combined

with new relationships found in other phylogenetic studies demonstrate a clear need to apply a combined approach to cyanobacterial taxonomy (Waterbury and Rippka, 1989).

Only a small number of taxonomic units are in culture (Komárek and Anagnostidis, 1999) thus research into the diversity of cyanobacteria is in a very early stage and techniques for analyzing specimens *in situ* have never been more important. Stulp (1984) first demonstrated genetic variation in *Anabaena* by DNA-DNA reassociation by using the limited number of axenic cultures available. Neilan (1995) followed by demonstrating genetic variation in *Anabaena* as measured by restriction length polymorphism of PCR amplified *cpc*B-*cpc*A intergenic spacer (*cpc*BA-IGS) and flanking coding regions. Neilan's preliminary investigation of diversity suggested that *Anabaena* might show significant diversity within and between regional lakes and provided a mechanism to obtain PCR amplification of *Anabaena* DNA without concern for bacterial contamination as no other bacteria produce cyanophycocyanin or its subunits. Significant diversity in strain clusters has also been demonstrated in other cyanobacteria genera by Palenik (1994) who employed the RNA Polymerase subunit, *rpo*C1, to identify isolates of *Synechococcus* from the Sargasso Sea and by Ernst in showing the diversity of *Synechocystis* culture isolates from Lake Constance (Ernst, Paul and Postius, 1995)

A large number of genotypes appear to exist as stable populations as demonstrated by strains in culture. (Komárek and Anagnostidis, 1999), however, cautions phycologists that "Only in some cases should strains be grouped together in

larger natural and delimited units that can be ascribed the rank of species." Research on cyanobacterial diversity has been hampered for years by the difficulty in isolating colonies or filaments into culture to obtain sufficient clonal axenic material for subsequent genetic analysis (Barker et al., 1999). Barker and Hayes (1997) pioneered a method of directly sequencing single *Nodularia* filaments using a two step PCR protocol, and this direct PCR of filaments from the field provided a new mechanism to test the hypotheses surrounding genetic diversity and ecological adaptation in cyanobacteria.

The present study pioneered direct filament PCR of *Anabaena* from field samples in one PCR reaction. *Anabaena* collections from Pacific Northwest lakes were compared with culture strains from across the globe using *cpc*BA-IGS and *rpo*C1 as molecular markers. Both markers have been used to elucidate *Anabaena* diversity (Bolch et al., 1996; Fergusson and Saint, 2000; Neilan, Jacobs and Goodman, 1995) and represent the largest dataset for comparison of Pacific Northwest strains to other regional strains by direct filament PCR. The phycocyanin and *rpo*C1 gene markers allow the use of direct PCR from field samples containing bacterial contaminants as no other prokaryotes are known to contain either gene (Bolch et al., 1996; Palenik, 1992). A systematic framework was constructed using parsimony and maximum likelihood analysis to better understand the diversity of *Anabaena* within the Pacific Northwest and the relationship of Pacific Northwest strains to strains from other regions.

MATERIALS AND METHODS

SAMPLING

Sampling was designed to include a broad representation of *Anabaena* species from a range of geographic locations available from culture collections and planktonic morphotypes from the Pacific Northwest. Representatives of soil (benthic) strains were also included to assess their similarity to planktonic strains. Field collections initially focused on toxic blooms of *Anabaena* to determine the phylogenetic relationship and distribution of the strain(s) responsible for multiple toxic events in the Pacific Northwest region. Local collections of *Anabaena* were made in response to reports of toxic water blooms ranging from Black Lake, Idaho to American Lake, WA and isolates (AL89, AL89 N39, AL89 55D & AL89 61L) were obtained from Professor Michele Crayton at Pacific Lutheran University. A comprehensive sampling (monthly) of 8 Puget Sound lowland lakes (Fig. 8) was conducted from May-November of 1999 and additional samples were obtained from May-October of 2000 to confirm and supplement collections from the previous summer. *Anabaena flos-aquae* at American Lake was also sampled monthly throughout 1999 to assess whether *Anabaena flos-aquae* populations in the lake were primarily toxic or non-toxic strains. Toxicity was verified by mouse bioassay (Lambert, Holmes and Hrudey, 1994) and HPLC (Lawton, Edwards and Codd, 1994) by the following collaborators (Crayton, M., Pacific Lutheran University, unpublished data; Beattie, K., University of Dundee, unpublished data).

Culture isolates were obtained from the University of Texas Culture Collection (UTEX) (Starr and Zeikus, 1993), the Culture Collection of Algae and Protozoa (CCAP),

and Professor Michele Crayton at Pacific Lutheran University and were selected to assist in the placement of local *Anabaena* genotypes within the global phylogenetic hierarchy. All samples collected and sequenced are listed in Table 2 and include accession numbers for sequences obtained from Genbank as well as any known information on origin and toxicity. Table 2 also includes the sister taxa and outgroup taxa used in this study.

Culture isolates were grown in either BG-11 or BG-11_0 Medium (Rippka et al., 1979). Field samples were frozen at –20°C for later DNA isolation or placed in growth chambers until used for direct filament PCR. All strains were maintained at 100 micromoles photons·m^{-2}·sec^{-1} on a 16L:8D hour light/dark cycle at 20°C. The AL93 strain from American Lake, WA was isolated and grown on 1.5% agarose plates containing BG-11 Medium.

MICROSCOPY

Black and white photomicrographs were obtained using a Zeiss compound microscope with a Nikon M-35 1S photomicrographic camera using TMAX 400 film. Color microphotographs were obtained using a Zeiss Photomicroscope II compound microscope and Kodachrome 64 slide film.

DNA ISOLATION AND SEQUENCING

Two methods were employed in isolating cyanobacteria DNA for PCR: 1) DNA from culture isolates was obtained by centrifuging approximately 0.5 ml log phase culture and processing the pelleted cells. Pellets were either immediately extracted or frozen and extracted at a later date. DNA was isolated from fresh or frozen material

either according to a modified Instagene method intermediate to that of Bolch et al. (1996) and Neilan et al. (1995) (reagents and washes per Bolch but temperature, time and vortexing per Neilan) or using a xanthogenate-SDS extraction protocol detailed by (Tillett and Neilan, 2000) but incubating all samples for two hours at 60°C. 2) Approximately 20 microliters of field-collected material were pipetted onto a depression slide and examined at 40x under a dissecting microscope. The longest intact individual *Anabaena* filaments representing one phenotype were pipetted (Eppendorf ultramicropipetter) into a second depression slide containing 100 μl of BG-11 medium and this washing procedure was repeated 5 times. Single filaments were pipetted in a 1 μl liquid volume into 0.6 ml eppendorf tubes and frozen for later PCR or placed on ice until addition of PCR reaction mix. DNA from culture isolates was examined by gel analysis and quantitated with approximately 5 ng used for each 25 μl PCR reaction.

PCR reaction conditions were optimized for each molecular marker primer set and for each type of DNA amplified (whole cell direct filament PCR vs. extracted DNA from culture isolates). Double stranded PCR reaction mix for *cpc*BA-IGS and *rpo*C1 of extracted DNA consisted of 10 mM Tris-HCl, pH. 9.2, 25 mM KCl, 3.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 μg BSA, 0.0125 units of Taq polymerase, 0.1 mM of each primer, and 1 μl total DNA template either undiluted or diluted up to 1:100 in a 25 μl reaction volume. PCR reaction mix for direct filament PCR consisted of 10 mM Tris-HCl, pH 8.8, 25mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 μg BSA, 0.0125 units Taq polymerase, 0.4 mM each primer. 3.75 μl glycerol was substituted in some reactions for BSA in direct

filament PCR and concentration of primers was doubled in some reactions to remedy DNA derivitization problems. PCR reaction mix was added to the 1 µl filament mix and immediately frozen or amplified. A PTC-100 Programmable Thermal Controller (MJ Research, Inc.) was used for PCR amplification.

Primers used for *cpc*BA-IGS amplification and sequencing are described in (Bolch et al., 1996) (forward *cpc*βF: 5'-GGCTGCTTGTTTACGCGACA-3' and reverse *cpc*αR: 5'-CCAGTACCACCAGCAACTAA-3'). This primer set amplified a ~610 bp fragment that included the 3' end of the β subunit of phycocyanin, an approximate 100 bp ITS region, and the 5' end of α subunit of phycocyanin. Primers used for *rpo*C1 PCR amplification are described in (Palenik, 1994) (forward C: 5'-GAGCTCYAWNACCATCCAYTCNGG -3' and reverse N: 5'-GGTACCNAAYGGNSARRTNGTTGG –3') and yielded a ~623 bp fragment.

The reaction profile for *cpc*BA-IGS amplification of *Anabaena* filaments was: 1) an initial denaturation at 96°C for 4 minutes followed by; 2) 94°C for one minute; 48°C for one minute; 72°C for two minutes for 38 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for *cpc*BA-IGS amplification of extracted DNA was: 1) an initial denaturation at 96°C for 4 minutes followed by; 2) 94°C for one minute; 52°C for one minute; 72°C for two minutes for 35 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for the *rpo*C1 region amplification was the following: 1) an initial denaturation at 96°C for three minutes

followed by; 2) 94°C for one minute; 50°C for one minute; 72°C for two minutes for 35 cycles followed by; 3) final extension at 72°C for 5 minutes.

Double stranded PCR products were cleaned using Qiaquick PCR Purification Kit (Qiagen Inc., Chatworth, CA) or electrophoresed in a 1.5% agarose gel (SeaKem, FMC), stained with ethidium bromide and the appropriate sized bands were cut and cleaned using Quiquick Gel Purification or Quiex II (Quigen Inc., Chatworth. CA) and quantified by spectrophotometry. All *rpo*C1 PCR products and PCR products from samples that provided poor data from direct sequencing or samples suspected to contain more than one strain were purified and ligated into the cloning vector pGEM-T Easy (Promega). Transformation was performed using the *E. coli* JM109 strain. The recombinant plasmids were verified by restriction digestion followed by agarose electrophoresis. A minimum of 4 clones from each sample was sequenced using the T7 and SP6 primers provided by Promega. Direct PCR sequencing was accomplished with the same primers as used in the PCR reaction. DNA was sequenced using the cycle sequencing method for both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Foster City, CA) and analyzed on an ABI 377 automated sequencer. DNA sequences were assembled using Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI).

DATA ANALYSIS

Sequence alignment for *cpc*BA-IGS and *rpo*C1 was performed using ClustalX (Thompson et al., 1997) and subsequently adjusted manually to correct errors and to align

difficult portions of the intergenic spacer region in *cpc*BA-IGS. Due to the number of insertions and deletions in the intergenic region of *cpc*BA and the difficulties in aligning large data matrices, sequence alignment was performed in several steps. First, taxa within *Anabaena* and those from outside the genus were aligned separately, and those alignments were adjusted manually. Second, selected taxa from both inside and outside the genus *Anabaena* were aligned with ClustalX, and this alignment was adjusted manually. Regions of the intergenic spacer in *cpc*BA-IGS contained numerous insertions and deletions that made confident alignment with sister and outgroup impossible and these IGS regions were excluded from all analyses that included these taxa (base pairs 320-386). Final alignments were constructed by placing these alignments together and represent the best estimate of the positional homology.

tRASA was performed on the aligned sequences to determine phylogenetic signal (Lyons-Weiler, 2001). Outgroups were identified and tested for suitability in the *cpc*BA-IGS and *rpo*C1 analyses. Recent work by Lyons-Weiler has demonstrated that inappropriate outgroup usage can degrade phylogenetic signal and result in inaccurate tree topology (Lyons-Weiler and Hoelzer, 1997). A negative tRASA is one of the symptoms of long branch attraction (Lyons-Weiler, Hoelzer and Tausch, 1996), and declining power curves and high taxon variance ratios are also evidence of problematic taxa (Lyons-Weiler and Hoelzer, 1997). Taxa violating these criteria were removed from the alignments.

The *cpc*BA-IGS and *rpo*C1 data were analyzed using PAUP* (version 4.0b8)(Swofford, 1999). Both parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981) analyses were conducted. Regions of ambiguous alignment were excluded from analyses. Gaps were introduced in the alignment in order to optimize positional homology (submitted to EMBL).

One thousand heuristic searches with random sequence addition were conducted with all characters weighted equally with TBR swapping and MULPARS in effect for parsimony analyses. Five hundred bootstrap replications were conducted to assess relative group support (Felsenstein, 1985), and each bootstrap replicate used ten heuristic searches with random sequence addition.

For maximum likelihood analyses, a substitution model where all base transformations are equally likely and a discrete gamma distribution (Yang 1994) with three rate classes to account for rate heterogeneity were used. Ten heuristic searches with random sequence addition were conducted with MULPARS turned off (DeBry and Olmstead, 2000). The tree with the maximum likelihood was retained to represent the best estimate of phylogenetic patterns. One hundred bootstrap replications were conducted under maximum likelihood criteria to assess relative group support. Transition/transversion rate was also examined by varying from 2:1 to 1:1 to test whether differences in substitution changed the resulting trees.

RESULTS

Several dominant morphotypes of planktonic *Anabaena* were found in Puget Sound lowland lakes. All of the lakes contained a small-celled morphotype that could often be seen by the naked eye in blooms composed of snarled filaments (Fig. 9a), a larger-celled morphotype with spherical cells and helical filaments (Fig. 9b), and some lakes harbored long straight/linear filaments with cells very similar to the helical morphotype (Fig. 9c). The small-celled morphotype bloomed periodically throughout the summer months and persisted into the late fall (in American Lake winter blooms have occurred) while the large-celled morphotype typically came to prominence later in the growing season when lake temperatures warmed and disappeared by November.

Blooms of strains exhibiting the small-celled morphotype occurred at different times with the toxic strain in American Lake occurring in late fall through early spring after a documented turnover event providing phosphorus coupled with favorable light (sunny) and wind (light) conditions (Jacoby et al., 1994), and the non-toxic strain of the small-celled morphotype was found throughout the year but only achieved significant biomass from late spring through fall. The toxic strain was not detected in 1999 when extensive plankton tow sampling and sequencing of the small-celled morphotype was conducted, and no significant bloom occurred during sampling that winter. It is possible that the toxic strain was resident in very low numbers and undetected while growing in the littoral zone deeper than the surface tows (typically 0.25-2 meters) or remained as akinetes at the lake bottom because the required environmental cues never occurred that year. A lightly pigmented version of the small-celled morphotype was also present in

some lakes (Green Lake and Lake Terrell) but failed to PCR despite several attempts. Filaments in older blooms of the small-celled morphotype were often subject to attachment by the ciliate *Vorticella* (Fig. 10a) as observed under microscopy from field collections. *Vorticella* attachment appeared to be specific to this morphotype stage of *Anabaena*.

Vegetative cells of the small celled *Anabaena* morphotype were gas vacuolate, ellipsoidal to barrel shaped, approximately 4.5-6 μm in width by 4.5-8.5 μm in length with ellipsoidal to spherical shaped heterocysts slightly larger than vegetative cells and ellipsoidal akinetes approximately 10-11 μm wide and 28-35 μm long representative of the common species *Anabaena flos-aquae* (Lyngbe) Brébisson. *A. flos-aquae* typically have coiled trichomes usually forming an entangled and twisted mass. The filaments also had colorless mucilage which was difficult to detect in young filaments but copious in older filaments (India ink test), which is consistent with an *A. flos-aquae* designation. The toxic isolates from American Lake, AL89 & AL93, produced anatoxin a (Beattie, unpublished) and were morphologically indistinguishable from the non-toxic isolate, AL97, both in vivo (Fig. 11a and b) and in culture (Fig 11c and d). Subclones of originally isolated toxic strain material (AL89 N39, AL89 55D, and AL89 61L) varied in pigmentation and growth rate and one strain lost toxicity (Crayton, personal communication) but were identical in *cpc*BA-IGS sequence suggesting that some strain variation exists, but at levels not measurable by *cpc*BA-IGS. Sequencing of 6 clones of the *cpc*BA-IGS PCR product from multiple filaments (> 200) of *A. flos-aquae* from

Green Lake yielded identical non-toxic strain sequence. Surprisingly, the only other lake found by this study to harbor the toxic *A. flos-aquae* strain was Black Lake in Idaho. Jacoby (1994) found toxic *A. flos-aquae* in Clear Lake near American Lake in 1990 but not in any other lakes in the area.

The second Puget Sound lowland lake planktonic *Anabaena* morphotype was a gas vacuolate coiled/helical filament with large spherical cells approximately 10-13 μm in diameter, spherical heterocysts 10-13 μm in diameter and cylindrical akinetes 17-18 μm wide and 25-30 μm long. Both heterocysts and akinetes were intercalary with akinetes often close to heterocysts and in pairs. Populations also existed that had larger cells (1.5x) but retained the shape and placement of heterocysts and akinetes (Fig. 12). These may have been stationary phase or overly-mature filaments but could also represent a separate but closely related strain. The morphology of this helical morphotype was consistent with a designation of *Anabaena circinalis* Rabenhorst, a common planktonic species. Two distinct genotypes of the helical morphotype were present in Puget Sound lowland lakes. The *cpc*BA-IGS sequences from a Green Lake and Lake Terrell strain blooming from July-August were distinct from the strain blooming in Green Lake from September-November.

The last major planktonic *Anabaena* morphotype was a gas vacuolate straight filament with spherical shaped cells 10-13 μm in diameter with spherical shaped heterocysts 10-13 μm in diameter and cylindrical akinetes 18 μm wide and 25-30 μm long (Fig. 13a). Akinetes and heterocysts were intercalary. A clear designation of this

morphotype by morphology alone was not possible although *Anabaena circinalis* has been reported in rare conditions to exist in a straight/linear form (Whitton et al., 2000). The straight form found in the Puget Sound lowland lakes was dominant in several lakes in late summer with significant blooms occurring in Black Lake, WA in September of 1999. The straight phenotype also appeared as a co-dominant in several cyanobacterial blooms in the other lowland lakes and was present concomitantly with the helical morphotype and a twisted form that produced a copious mucilage matrix in the older filaments (Fig. 13b). Sequence analysis confirmed that the straight morphotype was nearly identical in *cpc*BA-IGS sequence to the coiled/helical morphotype with the typical *Anabaena circinalis* habit and also the mucilaginous form and therefore was designated *Anabaena circinalis* Rabenhorst.

The morphology of the *Anabaena* strains from culture collections sequenced in this study appeared consistent with their description in the literature. Komárek (1999) warns that many culture collection isolates have been contaminated or misidentified. A number of the strains were noted by the collections to be in taxonomic flux and carried multiple synonyms, problems which indicate that caution is advised in assigning correct designations to these strains. Micrographs of the culture strains were taken (Figs. 14-16) for use in examining morphology; however, most of the cultures did not contain heterocysts or akinetes which are required for effective use of the traditional botanical *Anabaena* taxonomy, and it is also common for cyanobacteria to drastically alter their morphology from their field habit under culture conditions. Culture collections typically

do not describe the original characteristics of deposited strains other than by assigning a species name meant to confer certain morphological attributes, and this makes comparisons with field material difficult.

RASA was performed on both the *cpc*BA-IGS and *rpo*C1 alignments to confirm adequate phylogenetic signal (Lyons-Weiler, Hoelzer and Tausch, 1996). The tRASA for the unrooted *rpo*C1 data was 25.926801 with 626 degrees of freedom. The tRASA for *rpo*C1 using the 11 outgroup taxa believed to be outside of the Nostocales was 13.370319 with 296 degrees of freedom. While the rooted tRASA offered less phylogenetic signal than unrooted, the tRASA value was acceptable with sufficient phylogenetic signal for the analysis. The tRASA for the *cpc*BA-IGS alignment with only *Anabaena* strains was 23.104836 with 776 degrees of freedom. The tRASA for the alignment including sister and outgroups was 15.558668 with 402 degrees of freedom when rooted using *Calothrix* and *Spirulina* and 14.241532 with 347 degrees of freedom when unrooted. Rooting slightly improved phylogenetic signal, but all of the alignments contained sufficient phylogenetic signal under the tRASA test. *Anabaena cylindrica* (UTEX 1611) was removed from the analysis after variance plot analysis indicated a strong likelihood of long-branch attraction. UTEX 1611 may have included either chimaeric sequence or sequence of a contaminant.

Sequences from 51 taxa included in the *cpc*BA-IGS study and 37 taxa in the *rpo*C1 study (Table 2) provided a total aligned length of 680 bp for *cpc*BA-IGS and 585 bp for *rpo*C1 (Submitted to EMBL). Twenty four gaps ranging from 1 to 22 bases long

were introduced into the *cpc*BA-IGS *Anabaena* alignment to account for unique sequences found in the IGS region of the *Anabaena* alignment. Five parsimony informative gaps were included in the *cpc*BA-IGS analysis as two-state characters. Three gaps ranging from 1 to 3 base pairs long were introduced into the broader *Anabaena*, *Nodularia*, and outgroup *cpc*BA-IGS alignment that excluded 67 base pairs of the IGS region (base pairs 320-386). Five gaps, ranging from 1 to 4 bases long, were introduced into the *rpo*C1 alignment and treated as missing.

In the *cpc*BA-IGS data set, 312 sites were variable, of which, 225 sites were parsimony informative. Parsimony analyses resulted in 865 most parsimonious trees of 683 steps (CI=0.647, RI=0.849, HI=0.353 for informative characters). The Maximum Parsimony (MP) Strict Consensus of the 865 trees is shown in Fig. 17. Maximum likelihood analyses yielded one tree of Maximum Likelihood (-lnL= 4316.05322). The Maximum Likelihood (ML) analysis was examined under 3 different transition/transversion ratios (1:1, 1.5:1, and 2:1) with the resultant trees producing similar clades to each other. The overall topology of the Maximum Likelihood tree (Fig. 18) is similar to that of the Maximum Parsimony strict consensus tree. Differences between the Maximum Likelihood tree and the Maximum Parsimony strict consensus tree are restricted to resolution of taxa at the tips of the trees' topologies and in areas with weak bootstrap support and short branch lengths. Both trees show the outgroup, *Calothrix* and *Spirulina,* clearly separated from the ingroup that contains a clade composed of *Nodularia* and *Nostoc* strains (with several *Anabaena* strains as well) and a

clade of *Anabaena* divided into 2 major *Anabaena* lineages (Groups I and II) with subgroups in each lineage. *A. verrucosa, A. spiroides*, and *A. inaequalis* compose Group IA while *A. subcylindrica, A. catenula* and *A. variabilis* represent Group IB. Group IIA is composed of *A. flos-aquae* strains and Group IIB contains *A. circinalis* strains.

Tree 54 from the MP analysis is characteristic of the placement of *Anabaena sphaerica* (UTEX 1616) in the majority of parsimony trees (Fig. 19) and is similar to that shown in the Maximum Likelihood tree although the ML tree places UTEX 1616 either in Group II or intermediate to Groups I and II with weak bootstrap support while the MP strict consensus tree places UTEX 1616 in or sister to Group I. *Anabaena flos-aquae* (AL89 and BLID) are placed within a larger *A. flos-aquae* clade in the MP analysis (Group IIA) with high bootstrap support but are placed outside of a combined *A. flos-aquae/A. circinalis* clade in the ML analysis with weak bootstrap support. An analysis of the entire *cpc*BA-IGS region using only representatives of *Anabaena* Groups I and II was performed to try and remove discrepancies in the MP and ML analyses.

In the Groups I and II *Anabaena cpc*BA-IGS dataset, 214 sites were variable, of which, 162 sites were parsimony informative. Parsimony analyses resulted in 998 most parsimonious trees of 316 steps (CI=0.780, RI=0.918, HI=0.220 for informative characters). The MP strict consensus of the 998 trees is shown in Fig. 20. Maximum likelihood analyses yielded one tree of maximum likelihood (-lnL= 2637.14860). The overall topology of the ML tree (Fig. 21) is similar to that of the MP strict consensus tree. The difference between the ML tree and the MP strict consensus tree appeared to resolve

the relationship of *A. sphaerica* (UTEX 1616) and placed UTEX 1616 in a clade containing Group II with high bootstrap support. UTEX 1616 appears only distantly related to *A. flos-aquae* and *A. circinalis*, however, and additional taxa will be necessary to completely understand the true placement of this strain in the phylogeny. *A. flos-aquae* (AL89 & BLID) were found in Group IIA in both the MP and ML trees with weak bootstrap support. AL89's relationship to the other *A. flos-aquae* strains was only weakly supported in part because one *A. flos-aquae* strain (BL-Aug) showed a high level of sequence variation from the other Pacific Northwest *A. flos-aquae* strains and moved back and forth between branches of the Group IIA clade in the analysis.

In the *rpo*C1 dataset, 362 sites were variable, of which, 299 sites were parsimony informative. Parsimony analyses resulted in 17 most parsimonious trees of 2031 steps (CI=0.319, RI=0.554, HI=0.681 for informative characters). The MP strict consensus of the 17 trees is shown in Fig. 22. Maximum likelihood analyses yielded one tree of maximum likelihood (-lnL= 9738.81565). The overall topology and bootstrap support of the ML tree (Fig. 23) is similar to that of the MP strict consensus tree. Tree 11 from the parsimony analysis (Fig. 24) is nearly identical to the ML tree. Differences between the maximum likelihood tree and the parsimony strict consensus tree are restricted to areas with weak bootstrap support and short branch lengths.

Testing of the molecular clock in *cpc*BA-IGS for a restricted dataset of the major *Anabaena* groups was conducted in Phylip 3.52 using an F-test of the difference in sums of squares between the Fitch and Kitsch trees. The Fitch sum of squares was 0.69019 and

the Kitsch sum of squares was 3.705. F was determined to be 30.577 and the critical value at 105 degrees of freedom using an alpha of 0.05 was 1.97. The F value therefore exceeded the critical value indicating that the Kitsch sum of squares was significantly worse than the Fitch fit and that the molecular clock was violated by the dataset. It is difficult therefore to determine a meaningful percentage difference in *cpc*BA-IGS sequence that equates to the designation of a "species" given the possibility that the rate of change between distant branches in the phylogeny may be significantly different.

DISCUSSION

Two phylogenetic reconstructions of *Anabaena* containing strains from the Pacific Northwest using DNA sequence from the molecular markers, *cpc*BA-IGS and *rpo*C1, are presented. The strains sequenced and supplemented from Genbank data represent much of the known morphological and geographical diversity of this genus. Because patterns of phylogenetic relationships may change with additional sampling, some of the systematic conclusions may be modified as more data become available; particularly if new clades are discovered. Numerous *Anabaena* studies have been conducted, but the overlap in taxa is often sparse due to the difficulty in obtaining and maintaining axenic material. The development of direct PCR techniques such as those used in this study promises to expand the availability of data such that the diversity and biogeography of cyanobacteria will be better understood.

The heterocystous cyanobacteria are the only well supported clade in the 16S rRNA cyanobacteria phylogenies currently available (Wilmotte and Herdman, 2001). Subsections IV (Nostocales) and V (Stigonematales) divide the heterocystous cyanobacteria based on cell division criteria, but this separation no longer appears valid given recent phylogenetic evidence (Rippka, Castenholz and Herdman, 2001). Prior to recent 16S rRNA studies (Turner et al., 1999; Wilmotte and Herdman, 2001), Subsection IV (Nostocales) had been regarded as a relatively stable group of filamentous cyanobacteria that divide exclusively by binary fission in one plane. The Nostocales sensu Komárek is divided into three families, Nostocaceae, Scytonemataceae and Rivulariaceae. *Anabaena* was placed in the Nostocaceae along with a number of other genera such as *Nostoc, Nodularia,* and *Aphanizomenon* that were characterized by trichomes that never exhibit basal-apical polarity and contain vegetative cells of uniform size (Castenholz, 1989). The ICNB, largely followed the Nostocacean designations until the latest revision of Bergey's Manual of Systematic Bacteriology (Rippka, Castenholz and Herdman, 2001) elected to withhold formal designations at the subsection and family levels and discusses taxa as "form genera" until such time as the relationship between the heterocystous cyanobacteria is clarified using polyphasic taxonomical principles (Rippka, Castenholz and Herdman, 2001).

Here, we examine the traditional scheme of the ICBN to determine whether any of the traditional characters remain useful in classifying *Anabaena* and the recent revisions based on the ICNB from Bergey's Manual of Systematic Bacteriology in the

context of our phylogenetic analysis and that of other recent studies. Genera in the Nostocaceae are separated based on distinct developmental cycles (hormogonia in the genus *Nostoc*), false branching, and placement of heterocysts and akinetes on the trichome (intercalary, terminal, singly, in chains, etc.). Komárek and Anagnostidis (1989) subdivided the Nostocaceae into two subfamilies, Anabaenoideae and Nostocoidaeae, based on akinete formation, but this character has been shown to vary within species (Stulp and Stam, 1984a) based on DNA-DNA hybridization studies and is no longer considered a valid distinction (Rippka, Castenholz and Herdman, 2001).

Rippka et al. (2001) operating under the ICNB designate a form genus, *Anabaena* Bory de St. Vincent 1822 sensu Rippka, Deruelles, Waterbury, Herdman, and Stanier 1979, with trichomes straight or slightly sinuous with constrictions at the crosswalls and vegetative cells ranging from barrel shaped to spherical. Trichomes contain both heterocysts and akinetes with heterocysts typically intercalary and akinetes either single or in groups adjacent to the heterocysts or distant. Geitler (1932) described the cell size of *Anabaena* under the ICBN as ranging from 2-15 μm and this range was followed by Komárek (1989). The bacteriology community recognizes a cell size range for *Anabaena* in Bergey's Manual of Systematic Bacteriology from 3-6 μm in diameter (Rippka, Castenholz and Herdman, 2001) that results in the exclusion of several *Anabaena* species recognized under the ICBN, including *A. circinalis* which was found to be a significant component of Pacific Northwest blooms. The ICNB is clearly in error and needs to emend *Anabaena* to include taxa with larger cells.

As noted in the Introduction, Rippka's *Anabaena* Cluster 1 includes *Anabaena variabilis* (UTEX 377 = PCC 6309) which is also examined in this study although this strain and Cluster 1 are designated *Anabaena cylindrica* in Rippka's classification and UTEX 377 was originally deposited as *A. subcylindrica* and renamed *A. variabilis* by Kantz and Bold (1969). The *cpc*BA analysis strongly supports a large *Anabaena* clade designated Group I (Fig 17 and 18), that includes strains designated *A. cylindrica, A. verrucosa, A. spiroides, A. inaequalis, A. subcylindrica* and A. *catenula* by UTEX. This clade is consistent with the similarity in fatty acid content shown by UTEX 1611, UTEX 1617, UTEX 1619, UTEX 375 and UTEX 380 that indicate that these strains are closely related (Caudales and Welles, 1992). Most of the strains in Groups IA and IB have serious identification problems based on morphological characters and many were named as *A. cylindrica* or *A. inaequalis* prior to revisions (Kantz and Bold, 1969) that preceded the availability of other evidence (Anand, 1988). UTEX 377, as a representative of Rippka's Cluster 1, shows very little difference in sequence to the other Group IB taxa in our *cpc*BA study, including *A. subcylindrica* (UTEX 1617) and *A. catenula* (UTEX 375), and all three strains are therefore likely members of the same species.

Group IB is separated with strong bootstrap support from Group IA (Figs. 17-19), which includes two strains designated *A. cylindrica* and a variety of other species that were likely misidentified by the isolator or are no longer the strain deposited originally with UTEX. All the strains except *A. spiroides* (UTEX 1552) are within a few base pairs of each other in *cpc*BA-IGS sequence and are therefore almost certainly the same species.

The difference in *cpc*BA sequence between Groups IA & IB represents a ~4% divergence, and this difference may merit separate species designations for each cluster although additional evidence from DNA-DNA reassociation and 16S rRNA will be necessary to make any formal reassignments. UTEX 1552 is synonomous with PCC 6310, which the Pasteur culture collection designated as a strain of *Nostoc* in Cluster 1. Rippka notes that PCC 6310 is an atypical member of *Nostoc* Cluster 1 as it does not form distinct hormonia with gliding motility (Rippka, Castenholz and Herdman, 2001). This life history and morphology is consistent with our placing UTEX 1552 in Group IA as an *Anabaena* strain; however, a dendrogram based on the unpublished DNA-DNA reassociation findings of Lachance and Stanier shows PCC 6310 within a larger clade of *Nostoc* separated from Clusters 1 and 2 of *Anabaena* (Rippka, Castenholz and Herdman, 2001). Without confidence estimates such as bootstrap support or the inclusion of Cluster 3 *Anabaena* taxa in the Rippka dendrogram, it is impossible to assess whether the DNA-DNA reassociation results contradict our placing UTEX 1552 within the genus *Anabaena* in Group IA. It is important to note, however, that additional strains designated *A. spiroides* all fall into *Anabaena* in both our *cpc*BA-IGS and *rpo*C1 trees and our *cpc*BA analysis does include representatives of Rippka's Cluster 3 (our Group II).

Rippka's Cluster 2 includes a single representative, PCC 7108, from the intertidal zone on the California coast. PCC 7108's morphology is listed as similar to Cluster 1 but with ellipsoidal akinetes more characteristic of *A. variabilis* (Rippka, Castenholz and

Herdman, 2001). This study did not include PCC 7108 and therefore does not contain data to support or dispute the existence of Rippka's Cluster 2. PCC 7108 may be a unique species belonging to Cluster 1 based on Rippka's DNA-DNA reassociation dendrogram that includes two Cluster 1 strains and numerous *Nostoc* strains (Rippka, Castenholz and Herdman, 2001) or PCC 7108 may indeed represent a unique cluster within *Anabaena*. Lyra (2001) does include PCC 7108 in a 16S rRNA tree, RFLP 16S rRNA tree and a REP fingerprint tree with PCC 7108 landing in the *Nostoc/Nodularia* clade in the 16S rRNA and REP fingerprint trees but within a clade containing *A. cylindrica* in the 16S RNA tree. It is suggested by Lyra that this indicates that PCC 7108 is roughly intermediate between *Anabaena* and *Nostoc* (Lyra et al., 2001). DNA-DNA reassociation data will be necessary to clarify the disparate results between the different molecular markers.

Our *cpc*BA-IGS analysis clearly shows a large difference in *cpc*BA-IGS sequence between our *Anabaena* Groups I and II that could include an additional *Anabaena* cluster and the difficulty in assigning *A. sphaerica* (UTEX 1616) to Groups I or II could indicate that a third cluster/group would emerge if additional taxa such as PCC 7108 were included. UTEX 1616 has been the subject of taxonomic debate as it was originally designated as *A. oscillariodes* (the type species under the ICBN), was reassigned to *A. ambigua* and *A. inaequalis*, and is currently designated *A. sphaerica* by UTEX. The fatty acid profile for UTEX 1616 clearly places it within *Anabaena* (Caudales and Welles, 1992) as does the high bootstrap support in our *cpc*BA-IGS tree, but neither study

definitively places UTEX 1616 within Group I or Group II. Additional taxa, DNA-DNA reassociation and 16S rRNA data will likely be needed to resolve the phylogenetic position of UTEX 1616 as either a Group I or Group II member or a representative of a new distinct Group III that may or may not overlap with Rippka's Cluster 2.

Cluster 3 in Rippka's classification includes PCC 9302, PCC 9332, and PCC 9349, all of which are planktonic isolates, 4-5 μm in diameter, immotile, heterocysts predominantly intercalary and two of the strains produce toxins (anatoxin a (s) and anatoxin (c)). Rippka's Cluster 3 conforms to our Group IIA containing *A. flos-aquae* strains. The reference strain proposed for Cluster 3 in Rippka's system is *Anabaena flos-aquae* (PCC 9332=CCAP 1403/13f) which is a reisolated strain of CCAP 1403/13B isolated from a lake in Cumbria, U.K. Our *rpo*C1 study includes CCAP 1403/13B, and it is nearly identical in sequence to the predominant spring-fall *A. flos-aquae* (AL97) strain from the Pacific Northwest and the closest relative to both of these strains is the toxic Pacfic Northwest *A. flos-aquae* (AL89) (Figs. 11b and d). Li (1998) also includes CCAP 1403/13B in a chemotaxonomy study of planktonic cyanobacteria that distinguishes an *Anabaena flos-aquae* clade equivalent to our Group IIA and a second clade likely equivalent to our *cpc*BA Group I although the two studies share only one species (*A. solitaria*) between them.

PCC 9349 is also placed in Cluster 3 by Rippka and as the only Canadian isolate reported in the literature to produce anatoxin (c), this strain is very likely identical to *A. flos-aquae* (UTEX 2391 = CCAP 1403/23) isolated by W. Carmichael. UTEX 2391

varies by only a few base pairs in *cpc*BA-IGS sequence from CCAP 1403/13D, an *A. flos-aquae* freshwater isolate from Wales, and is intermediate in sequence to the two *A. flos-aquae* strains from the Pacific Northwest (AL89 and AL97) (Fig. 18). UTEX 2391 is reported to have lost toxin production in culture, thus it would be interesting to test CCAP 1403/13D for anatoxin production, any genes associated with production and perform DNA-DNA reassociation on the two strains to determine how closely related these distant geographic strains are genetically.

The three cluster ICNB *Anabaena* classification scheme proposed by Rippka in Bergey's Manual of Systematic Bacteriology (2001) does not include our Group IIB. Group IIB consists of *Anabaena circinalis* strains that have a much larger cell size than is recognized by Bergey's (Rippka, Castenholz and Herdman, 2001) and these clearly clustered with strong bootstrap support in Group II in both the *cpc*BA-IGS (Figs. 17-19) and *rpo*C1 (Figs. 22-24) trees and are most closely related to *A. flos-aquae*. The *rpo*C1 analysis, while suffering from low bootstrap support at several nodes in the MP and ML trees where the genus *Anabaena* branches from the other form genera in the Nostocaceae, agrees for the most part with that of Fergusson (2000) with respect to overall topology. Both studies clearly distinguish a cluster of *A. circinalis* with a minimum of two types (I and II) as suggested by Fergusson although our tree places *A. circinalis* (CCAP 1403/18-N. Ireland) with *A. spiroides* and *A. solitaria* (both Australian strains) from Fergusson's study (Figs. 22-24). An RFLP study of 16S rRNA is also consistent with our inclusion of

A. flos-aquae and *A. circinalis* in Group II and a clearly separate Group I that includes *A. cylindrica* (Lyra et al., 2001).

The *rpo*C1 data alone would suggest that either: a) CCAP 1403/18 is misclassified and that it belongs in a Group IIB species closely related to *A. circinalis* designated either *A. spiroides* or *A. solitaria*, or b) that the Australian strains designated *A. spiroides* and *A. solitaria* are actually morphological outlier *A. circinalis* strains that were misclassified. Fergusson (2000) did not include any taxonomic notes regarding the morphology of the strains used in his study; however, CCAP 1403/18 does fit the morphological description of *A. circinalis* and is quite similar in morphology to the *A. circinalis* strains from the Pacific Northwest. Given the short distance (~3%) in *rpo*C1 sequence between Types I & II and the CCAP 1403/18 cluster and the high bootstrap support for this clade both in the MP and ML trees (Figs. 22 and 24), it would appear appropriate to designate the CCAP 1403/18 cluster as *A. circinalis* Type III. The 16S rRNA RFLP data also support an additional *A. circinalis* type as *A. circinalis* (NIES 41) (Lyra et al., 2001). In that study, the *A. spiroides* (NIES 79) strains also clustered together in a clade sister to *A. flos-aquae*. Additional genetic evidence and examination of more taxa should clarify the relationship between strains within Group IIB and settle any question whether Group IIB contains more than one species. It is also interesting to note that only members of *A. circinalis* Type I have been shown to produce saxitoxin.

Anabaena pertubarta also clustered in our *rpo*C1 tree in both the MP and ML trees within the *A. circinalis* clade with high bootstrap support (Figs. 22 and 24). *A.*

pertubarta is quite distant (~6%) in *rpo*C1 sequence from both the *A. circinalis* and *A. flos-aquae* strains sampled but is clearly a member of Group II under our analysis. The *rpo*C1 tree from Fergusson (2000) places *A. pertubarta* between two *Anabaena* clades, but these two clades appear to correspond to our Group I and Group IIB. *A. flos-aquae* and *A. aphanizomenoides* from the Fergusson study cluster with a Group I member (UTEX 377) in the *rpo*C1 tree (Fig. 22) which is surprising given that the three *A. flos-aquae* strains from the Pacific Northwest and Europe clearly cluster with Group II in both our *cpc*BA-IGS and *rpo*C1 trees (Figs. 17 and 22). Our *rpo*C1 tree topology, however, is based on very low bootstrap values for Group I in both the MP and ML analysis. It is therefore possible that the Australian *A. flos-aquae* and *A. aphanizomenoides* could shift to form what would be a larger Group II clade or an additional subset of Group II if additional taxa from Group II were added to the *rpo*C1 dataset. Given the high bootstrap support placing *A. pertubarta* in Group IIB, it is unlikely that additional taxa will change *A. pertubarta*'s position in our *rpo*C1 tree, but additional Group II taxa may shed light on whether *A. pertubarta* should retain its status as a separate species or be subsumed into *A. circinalis*.

The sole *A. spiroides* representative in the *cpc*BA-IGS tree (UTEX 1552) is found in Group I as opposed to the placement of a different *A. spiroides* strain in Group II of the *rpo*C1 tree. An examination of the *cpc*BA-IGS tree shows the *A. circin*alis Type II Australian strain ACC-R08 clearly separated from the *A. circinalis* Type III strain CCAP 1403/18. CCAP 1403/18 is nearly identical in *cpc*BA-IGS sequence with one of the

Pacific Northwest *A. circinalis* genotypes. The second *A. circinalis* Pacific Northwest genotype is sister to CCAP 1403/18 (Type III) but without any representatives from Type I, and the exact relationship between all three types is uncertain. Additional work must be undertaken to determine if both Pacific Northwest *A. circinalis* genotypes belong in Type III. The morphology of the *A. circinalis* Pacific Northwest strains fits that of *A. circinalis* rather than *A. spiroides*. Fig. 13 shows the typical spherical heterocyst 12 μm in diameter with a pair of cylindrical akinetes 30 μm long by 18 μm in width.

Rippka (2001) removed *Anabaena spiroides* from *Anabaena* because the trichome properties of *A. spiroides* are similar to *Cyanospira* but left *A. spiroides* unclassified because of the significant ecophysiological differences between *Cyanospira* and *A. spiroides* (most notably *Cyanospira*'s higher salt requirement). No phylogenetic data were available to compare *Cyanospira* with *Anabaena* in this study; however, both *cpc*BA-IGS and *rpo*C1 trees place *Anabaena spiroides* strains from Israel and Australia clearly within the genus *Anabaena*. *A. spiroides* (UTEX 1552) clustered with Group I *Anabaena* strains in the *cpc*BA-IGS tree and the Australian *Anabaena spiroides* clustered within Group IIB in the *rpo*C1 tree. Based on morphology (helical trichome, spherical cells intermediate in size to *A. flos-aquae* and *A. circinalis*) and habit (planktonic, gas vacuolated), it is more likely that the true *A. spiroides* is a Group II member and that any non-planktonic strains characterized as *A. spiroides* belong in Group I or may be misidentified *Nostoc* strains that either have not been shown to produce hormogonia or are mutants that have lost these characters in culture.

Current evidence also does not support the inclusion of *A. spiroides* in the genus *Cyanospira*. *Cyanospira* and *Anabaenopsis* cluster tightly in 16S rRNA trees and both *Anabaenopsis* and *Cyanospira* are found in the *Nostoc/Nodularia* branch of the 16S rRNA tree (Wilmotte and Herdman, 2001). This is consistent with our *rpo*C1 trees (Figs. 22, 23 and 24) placing *Anabaenopsis* in the *Nodularia/Nostoc* branch although with weak bootstrap support. Fatty acid analysis was used to determine the placement of a strain originally identified as *Anabaenopsis circularis* (ATCC 27895=PCC 6720), moved to *Nostoc* by Rippka (Rippka et al., 1979) and transferred by Castenholz into *Anabaena* (Castenholz, 1989) into the genus *Nostoc* (Caudales and Welles, 1992). *Anabaenopsis* should be reexamined given the 16S rRNA data linking this genus with *Cyanospira* and the increasing support that *Anabaenopsis* belongs in the *Nostoc/Nodularia* cluster.

Our *rpo*C1 tree and other studies clearly place *A. spiroides* strains in the *Anabaena* clade (Fergusson and Saint, 2000; Lyra et al., 2001). Based on ecology, morphology and the phylogenetic evidence currently available, it is likely that *Cyanospira* and *Anabaenopsis* are representatives of a distinct species and possibly merit a separate genus (Rippka, Castenholz and Herdman, 2001). It is possible that the morphology commonly associated with *A. spiroides* is represented by disparate genotypes created via evolutionary convergence and that strains previously designated *A. spiroides* will be subsumed within other *Anabaena* species. The most probable explanation of the taxonomic confusion in temperate regions is that the morphotype represented as *A. spiroides* is often confused with both *A. flos-aquae* and *A. circinalis* as

its cell dimensions are intermediate between the two, all three species are planktonic and the only significant difference noted under the ICBN is the rounded akinete ascribed to *A. spiroides* vs. cylindrical akinetes in *A. flos-aquae* and *A. circinalis*. This view is supported by work both in Australia (*rpo*C1)(Fergusson and Saint, 2000) and Europe (16S rRNA)(Lyra et al., 2001).

In addition to the confusion within the *Anabaena* clades of the *cpc*BA and *rpo*C1 trees, the placement of several *Anabaena* strains outside Groups I and II indicate that *Anabaena* is paraphyletic. The strain names are based on taxonomic assignment by culture collections and from the literature and may be subject to differences in opinion based on the use and weighting of morphological characters or other evidence. Several studies that have included *Anabaena* strains from different regions have also noted an *Anabaena* paraphyly (Lu et al., 1997; Lyra et al., 2001). The number of strains classified under the ICNB is significantly smaller than the number of taxa under study by those working with the ICBN as only axenic strains are typically used by the ICNB. This discrepancy in available taxa has also translated into a discrepancy in the number of strains that appear to be misclassified with a much higher number of questionable assignments coming from the ICBN.

Most of the suspect *Anabaena* strains in the *cpc*BA-IGS tree are found in the clade that contains *Nostoc* and *Nodularia*. None of the *Anabaena* strains from this study appeared within the *Nodularia* branch of the *cpc*BA tree suggesting that the morphological characters (primarily disc type cell shape) used to distinguish this genus

may be sound. The *Nostoc* reference strain used in our *cpc*BA study, *Nostoc* PCC 7120, was shown in DNA-DNA reassociation studies to be a member of *Nostoc* Cluster 3.1 after having been classified as an *Anabaena* strain for a number of years (Rippka, Castenholz and Herdman, 2001) and both our *cpc*BA and *rpo*C1 trees also place PCC 7120 in the *Nostoc* cluster which is most closely related to *Nodularia* which is in agreement with prior studies (Lyra et al., 2001; Wilmotte and Herdman, 2001). PCC 7120 does not produce hormogonia with gas vesicles like the other *Nostoc* strains in Cluster 3.2, but PCC 7120 does have the gas vesicle structural protein *gvp*A gene necessary to form gas vesicle walls in hormogonia and does not express them in culture (Damerval et al., 1989).

A second problematic *Anabaena* isolate clustering in *Nostoc*, UTEX 1444 =PCC 7937, is neither planktonic nor gas vacuolate, and it is therefore difficult to understand why UTEX characterized it as an *A. flos-aquae* strain when the planktonic character is central to this species' classification. The Pasteur collection designated this strain a *Nostoc* and given its similarity in *cpc*BA sequence to *Nostoc* PCC 7120, this study confirms UTEX 1444's assignment to *Nostoc*. Sequence similarity to PCC 7120 suggests that it belongs in *Nostoc* Cluster 3, an atypical cluster that does not exhibit the distinct *Nostoc* developmental cycle under standard growth conditions. A study differentiating *Anabaena* from *Nostoc* based on cellular fatty acid content shared several strains with our study and grouped UTEX 1444 with *Nostoc* based on fatty acid content; suggesting it be

named *Nostoc variabilis* (Caudales and Welles, 1992). Thus morphology, fatty acid content and *cpc*BA analyses all strongly support the placement of UTEX 1444 in *Nostoc*.

The third problematic isolate, *A. flos-aquae* (UTEX 2558) is so close in *cpc*BA-IGS sequence to UTEX 1444 that they are almost certainly strains of the same species (Fig. 19). UTEX 2558 is one of two Montana lake isolates (the second isolate, UTEX 2557, produces anatoxin) with gas vacuolate trichomes, cells 5-6 μm in length and 3 μm in width, and intercalary ellipsoidal heterocysts slightly larger than vegetative cells (Kangatharalingam et al., 1991). Given the planktonic nature and habit of this strain, it is surprising that it clusters with PCC 7120 and UTEX 1444 in the *Nostoc* clade of the *cpc*BA trees (Figs. 17-19). This indicates that the presence of gas vesicles in mature trichomes (as opposed to hormogonia) is not a valid character for distinguishing *Anabaena* from *Nostoc*. The genes for gas vesicle formation are clearly present in *Nostoc*, and the genetic switch that governs expression in hormogonia versus mature trichomes may be subject to constitutive expression and likely reversion as well. UTEX 2558 also exhibits heterocyst aggregation, a character found in other genera such as *Aphanizomenon*, *Gloeotrichia* and *Rivularia* (Prescott, 1982) but not known to occur in *Anabaena flos-aquae* or *Nostoc* strains. This character may need to be reexamined in the context of phylogenetic evidence as these genera are added to our taxonomic studies. Examination of UTEX 2558 for hormogonia should also be undertaken to establish whether this character is present in the strain as is currently required under the ICBN for placement in *Nostoc*.

The position of the two Montana planktonic isolates within the genus *Nostoc* is also supported by the *rpo*C1 tree. The toxin producing isolate, UTEX 2557, clusters with *Nostoc commune* (UTEX 584) in the *rpo*C1 tree along with *Anabaena bergii* as part of a larger *Nostoc* clade that includes *Nostoc* PCC 7120 (Figs. 22-24). The exact placement of UTEX 2557 in the *rpo*C1 tree within the *Nostoc/Nodularia* branch is not clear because of low bootstrap support in the internal branches, but the *Nostoc/Nodularia* clade including UTEX 2557 is clearly separated from the major *Anabaena* clades. The other *Anabaena* species in the *Nostoc* clade, *Anabaena bergii* (an Australian isolate), is nearly identical to an *Aphanizomenon ovalisporum* strain from Israel (Fergusson and Saint, 2000) by 16S rRNA and *rpo*C1 sequence, and both strains produce the toxin, cylindrospermopsin. Placement of UTEX 2557 and *Anabaena bergii* strains in the *Nostoc* clade adds to the toxic *Nostoc* strains reported in the literature, and the diversity of *Nostoc* toxins as cylindrospermopsin and anatoxin are new to this genus (previously reported toxic *Nostoc* strains are microcystin producers (Chorus and Bartram, 1999)). One other toxic (mirocystin) *Nostoc* strain has been reported, but no molecular marker data are yet available and this strain does not appear to be gas vacuolate in culture in any stage of development (Beattie et al., 1998; Beattie, personal communication). These results indicate that there is no utility in distinguishing *Nostoc* and *Anabaena* based on the presence of gas vesicles whether in mature trichomes or in hormogonia and that cultures that do not produce hormogonia may have lost this capability or simply may not express it in laboratory conditions.

The fifth problematic *Anabaena* isolate is *Anabaena randhawae* (UTEX 1823), a strain isolated from a rainwater pond in India. UTEX 1823 is separated with high bootstrap support from the two *Anabaena* clusters (Groups I and II) in the *cpc*BA trees (Figs. 17-19) and is sister and most closely related to the *Nostoc* and *Nodularia* strains included in the *cpc*BA study. It is, however, clearly distant and distinct from the *Nostoc* and *Nodularia* clusters and represents a "loner" sequence that cannot be placed at this time in *Nostoc*, *Nodularia* or *Anabaena*. Caudales (1992) fatty acid profile for UTEX 1823 also suggests that this strain is distinct from both *Anabaena* and *Nostoc* although the fatty acid data appear to place UTEX 1823 closer to *Anabaena*. UTEX 1823's Class A fatty acid content was higher and outside the standard deviation of all tested *Anabaena* (*Nostoc* Class A fatty acid content is lower than *Anabaena*), and its ratio of class C to class A fatty acids was below the standard deviation for the *Anabaena* strains examined (Caudales and Welles, 1992). UTEX 1823 may represent a new genus, but additional taxa from *Nostoc*, *Nodularia* and *Anabaena* should be examined with the *rpo*C1 marker, 16S rRNA and DNA-DNA reassociation to clarify UTEX 1823's phylogenetic placement.

The recorded species diversity of planktonic *Anabaena* from lowland Puget Sound lakes is surprisingly low given the plethora of planktonic *Anabaena* species noted from temperate regions (Geitler, 1932). The 1999 summer study sampled Puget Sound lakes from the Canadian border to the south end of Puget Sound and on both sides of Puget Sound. Sampling included shallow lakes (2 meter) that did not mix and deeper

lakes (27 meters) that mixed and were selected based on appropriate pH conditions (non-acidic) to support cyanobacteria bloom development. During most of the summer and early fall the surface temperature of the lakes ranged from 20-23°C and pH typically rose from a pH of 7 to between 8 and 9 over the season.

Several *Anabaena* species were identified in local ecological studies including that of Pieterse on four Seattle, WA area lakes in 1973 (Pieterse, 1974). The Pieterse study noted five species of *Anabaena* (*A.* sp., *A. flos-aquae, A. circinalis, A. spiroides*, and *A. verrucosa*) although Pieterse was unsure of the *A. verrucosa* identification because the morphotype did not correspond to the cell dimensions in the literature, and this morphotype was only found in Lake Washington. *A. verrucosa* is characterized by straight trichomes with cylindrical cells 3-4 μm wide and 4-8 μm long with cylindrical heterocysts 3-4 μm wide and 5-8 μm long and cylindrical akinetes (Whitton et al., 2000). The straight trichome morphotypes observed during our study of 8 Puget Sound lowland lakes did not match the *A. verrucosa* description, and it is therefore likely that Pieterse either misclassified the Lake Washington strain (it is likely the straight variant of *A. circinalis* we identified in our study), or the strain is no longer present.

The *A. spiroides* morphotype that Pieterse documented appears to be the small-celled *A. circinalis* morphotype identified in our *cpc*BA-IGS work. Our *cpc*BA-IGS data indicates that two *A. circinalis* strains accounted for all helical/coiled phenotypes present in lowland Puget Sound lakes. Pieterse provided no description or micrographs of any strains that can confirm our hypotheses, but only *A. flos-aquae* and *A. circinalis* were

recorded from Green Lake in the late 1980s (Barbiero, 1991) suggesting that the discrepancy is a question of the subjectivity in assigning species designations rather than a change in the species composition over time.

A limnology study examining cyanobacterial bloom dynamics found that *A. flos-aquae* blooms from 8-21°C and *A. spiroides* blooms from 18-22°C (Hutchinson, 1967). Pieterse (1974) found that the Green Lake morphotype he designated *A. spiroides* bloomed from 17.4-17.6°C, *A. circinalis* bloomed from 19-22°C, and *A. flos-aquae* bloomed from 10-13°C (incomplete data). Our study contradicts Pieterse's *A. flos-aquae* data. Pacific Northwest *A. flos-aquae* bloomed from 8-23°C with light blooms recorded in April of 1999 on Lake Goodwin and Lake Stevens at 8°C and 8.1 pH. *A. flos-aquae* was also the co-dominant or dominant cyanobacterium at the highest range of lake temperatures, confirming Hutchinson's wide range in blooming conditions for this species. *A. flos-aquae* was found in all of the lowland Puget Sound lakes we sampled.

Our study also discovered the presence of two distinct Pacific Northwest *A. flos-aquae* genotypes with different ecological requirements but identical phenotypes. The toxic strain (AL89) from American Lake appears to bloom only at low temperatures (8-10°C) in the winter months after a lake turnover event that results in high phosphorus availability in conjunction with favorable light (sunny) and wind (light) conditions. Monitoring American Lake in 1998 found the non-toxic *A. flos-aquae* strain present throughout the year but only blooming from spring through fall. This finding is consistent with the work of Collier (1996) and Jacoby (1994) in documenting the timing

of toxic blooms at American Lake and the phosphorus budget that drives this unique winter blooming strain. During winters with lower phosphorus availability, toxic events were not noted despite the presence of *A. flos-aquae* in the water column (Collier, 1996).

It appears that the toxic strain has a narrow niche that may not be present in most Puget Sound lakes as we only detected the toxic strain from American Lake and Black Lake, Idaho. Collier (1996) documented a toxic winter bloom at 8-12°C in 1996 but no toxic blooms in the winter of 1994-5 despite what are typically thought to be more favorable *Anabaena* conditions (lower transparency, higher pH, lower nitrogen concentrations, higher chl *a*, and less abundant zooplankton). The only identifiable difference was that SRP (phosphorus) was much higher in 1996 than in 1995. Water management authorities would be prudent to identify Pacific Northwest lakes that reach winter SRP levels approaching 30 μg/L and sample for the toxic *A. flos-aquae* strain after spikes in SRP.

Our field collections were focused on the summer/fall season when most cyanobacteria blooms occur. We did however obtain several isolates of the toxic *A. flos-aquae* from winter blooms and cultured the isolates to sequence their *cpc*BA-IGS region. Despite differences in pigmentation, growth rate and toxicity, all of the isolates were identical in *cpc*BA-IGS sequence. Some of the phenotypic variation therefore appears to be based on genetic differences, but the *cpc*BA-IGS region lacks the resolution to distinguish these differences. Chorus (1999) notes that even in single species blooms, a mixture of genetic subgroups can be present. Some may be toxic and others may be non-

toxic, and the number of strains within a species can be in the hundreds. Microsatellites or other fingerprinting methods could prove useful in examining diversity at this level (Rouhiainen et al., 1995), and probes specific for toxin encoding regions are also under development.

We noted pigment differences in some summer *A. flos-aquae* trichomes, but we were unable to get sequence from the lightly pigmented phenotype. The toxic strain did not grow well at 20°C in culture and was often pale in color. No formal growth studies were conducted to assess the optimum temperature of the toxic strain, but it was clear from the performance of the non-toxic strain in culture that it grew more rapidly than the toxic strain at 20°C.

Anabaena circinalis was also very common in lowland Puget Sound lakes although the helical morphotype was typically a co-dominant member of a bloom while the linear morphotype formed monospecific blooms in several instances. The first seasonal appearance of *A. circinalis* in PNW lakes in 1999 occurred at 20°C in early July. Both the helical and linear *A. circinalis* morphotype were abundant in Long Lake at 21°C at 8.5 pH. This temperature range is consistent with Hutchinson's data for *A. spiroides* and suggests that Hutchinson (1967) was actually monitoring *A. circinalis* rather than *A. spiroides*. Pieterse comments on a succession of *A. spiroides* over *A. circinalis* in 1973 when the pH in Green Lake dropped from 9.7 to 8.6 (Pieterse, 1974). We noted a succession in *A. circinalis* genotypes from mid-summer into early fall (September) on Green Lake with the pH at 9.3 in late August and 8.1 in early October. It is possible that

the two strains of *A. circinalis* favor different pH conditions and carbon availability. We did not note any environmental conditions that appeared to favor the helical morphotype over the linear morphotype.

Our *cpc*BA-IGS results (Figs. 20 & 21) indicate that there are three major *A. flos-aquae* genotypes present in lowland Puget Sound lakes. All of the lakes sampled from May to October contained a non-toxic genotype related to UTEX 2391 and CCAP 1403/13D. Even pooling of multiple trichomes from a Green Lake *A. flos-aquae* bloom and cloning of the resultant PCR product showed no significant variation in *cpc*BA-IGS sequence. One filament from a Black Lake August bloom did exhibit a unique *cpc*BA-IGS sequence that clustered in the *A. flos-aquae* clade, but unfortunately no record was made that can distinguish this strain from the common strain morphologically. The third genotype (AL89) was toxic, not found during the summer/fall season, and its exact systematic placement is unclear as the MP and ML trees disagree although morphologically it clearly fits the *A. flos-aquae* description.

Pieterse (1974) states that *Anabaena lemmermannii* was the dominant cyanobacterium in Lake Washington in 1933. This observation points out the confusion in differentiating between *A. flos-aquae* and *A. lemmermannii* both of which are reported to produce anatoxin (Chorus and Bartram, 1999). *Anabaena lemmermannii* Richter 1903 is described as having dense or loosely coiled trichomes, is free floating and solitary or bundled with mucilaginous envelope, gas vacuolate, with cylindrical cells 5.1-7.3 μm in width and 7.6-11.9 μm in length, heterocysts spherical or ellipsoidal and 6.2-8.7 μm in

width and 7.5-11 μm in length, and akinetes cylindrical with rounded ends 6.8-10.3 μm in width and 13.4-36.9 μm in length on both sides of heterocysts (Li, Watanabe and Watanabe, 2000b). Akinetes and heterocysts are situated in the center of trichome bundles when mature, and interestingly this is the same habit reported for UTEX 2558 which was identified as *A. flos-aquae* but is a *Nostoc* genotype based on *rpo*C1 and *cpc*BA trees.

Li (2000) claims that the most important *A. lemmermannii* character is that the akinetes are adjacent to the heterocysts. Unfortunately we were not able to produce akinetes in cultured UTEX 2558. Neither *A. flos-aquae* genotypes from the Pacific Northwest produced aggregate heterocysts, nor did they produce akinetes adjacent to heterocysts. Without careful documentation, the overlap between *A. lemmermannii* and *A. flos-aquae* can cause misidentification, and it is very likely that they are not closely related phylogenetically. Heterocyst aggregation is present in several genera and does not appear to be a "good" phylogenetic character. Li (2000) distinguished 20 planktonic species of *Anabaena* based on axenic culturing and inducement of akinetes, but while certain morphological characters may be constant, most of the morphological characters do not correspond to the phylogenetic relationships between strains.

The results of this study are a useful beginning in providing the preliminary data and sufficient descriptive results to expand our knowledge of *Anabaena* both globally and from local waterbodies. Tamas (2000) recommended that *Nostoc* and *Anabaena* be merged on the basis of the results of a *nif*H sequence phylogeny where *Anabaena* and

Nostoc shared multiple branches of the *nif*H tree. While Tamas is likely correct that traditional morphological criteria such as hormogonia formation cannot be correlated with the *Nostoc/Anabaena* distinction supported by current molecular based phylogenies, their study incorrectly concludes that a molecular based phylogeny with separate *Anabaena* and *Nostoc* clades will not emerge from a polyphasic taxonomy that includes the use of field collected material with akinetes and hormogonia that may not show in culture. It is important to note that under the ICBN the genus *Anabaena* is conserved and any genus in conflict with *Anabaena* would be removed.

There are numerous factors that may render morphological characters unusable for phylogenetic purposes. Morphology may be the product of multiple evolutionary origins (convergence), selective maintenance of traits, or simply a manifestation of the ease of genetic conversion (Wilmotte, 1994). Our study certainly places the ICBN use of trichome symmetry, akinete and heterocyst character and cell shape into serious question. It is also clear that the ICBN system based on *A. oscillariodes* and *A. flos-aquae* as subgenera is as incomplete as the ICNB's failure to include our Group IIB taxa in its three cluster system. A major effort in documenting morphology and adding new *Nostoc*, *Anabaena* and other "Nostocacean" taxa sequences to the Genbank database will be needed to remove faulty historical information from the system and provide accurate descriptions and molecular data to solve this phylogenetic puzzle.

At the same time, efforts to understand the distribution of species and strains are contributing powerful tools to begin to unravel the ecology of the scum ecostrategeists.

Hayes (1997) found two *Nodularia* genotypes across 9 Baltic Sea sampling stations with approximately an equal biomass but non-uniform distribution. Bolch (1999) examined nucleotide variation within *Nodularia* morphospecies groups and inferred a <1% *cpc*BA-IGS difference within these groups with individual bloom events appearing clonal. Two distinct ecotypes of *Prochlorococcus* have also been documented at one location (Moore, Rocap and Chisholm, 1998). Some of our *Anabaena* genotypes appear to be distributed widely while others remained distinct (toxic *A. flos-aquae* and second *A. circinalis* genotype). All *Anabaena* genotypes were distributed across the Puget Sound basin and could also be found as co-dominants in a bloom event. It appears that the cyanobacteria vary group by group in the breadth of their distribution and the population of strains that are supported locally. At least with respect to planktonic *Anabaena* in the Puget Sound region, a few major genotypes dominate local lakes and *cpc*BA-IGS shows that strain variation does exist but techniques with greater resolution will be required to fully document the existing diversity.

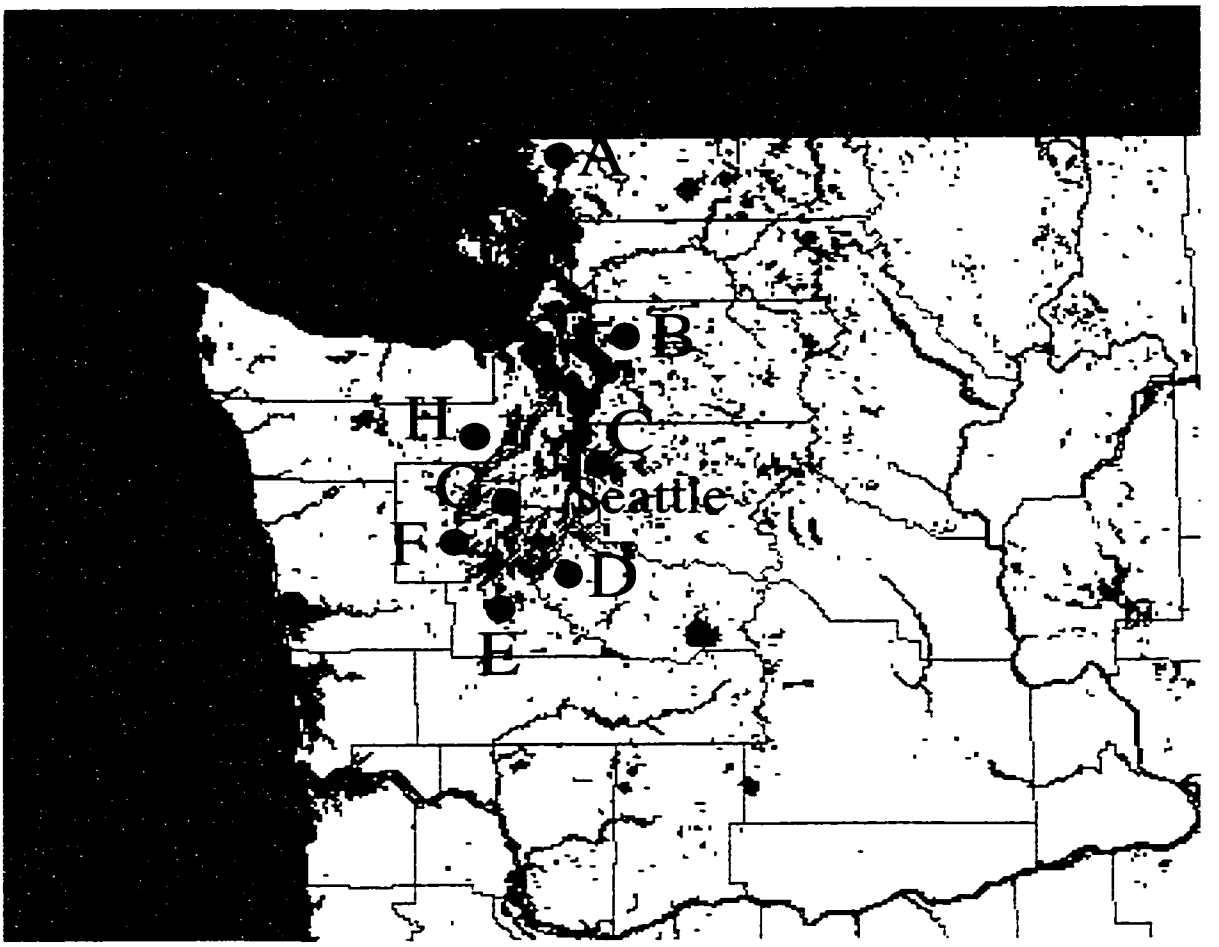

Figure 8. Map of lowland Puget Sound, Washington lakes sampled monthly in 1999 and 2000. All lakes had public access and supported planktonic cyanobacteria. A) Lake Terrell B) Lake Goodwin C) Green Lake D) American Lake E) Black Lake F) Spencer Lake G) Long Lake H) Lake Leland. Samples were also taken periodically from other local lakes including Lake Stevens, Big Lake, Lake Steilacoom, and Spanaway Lake.

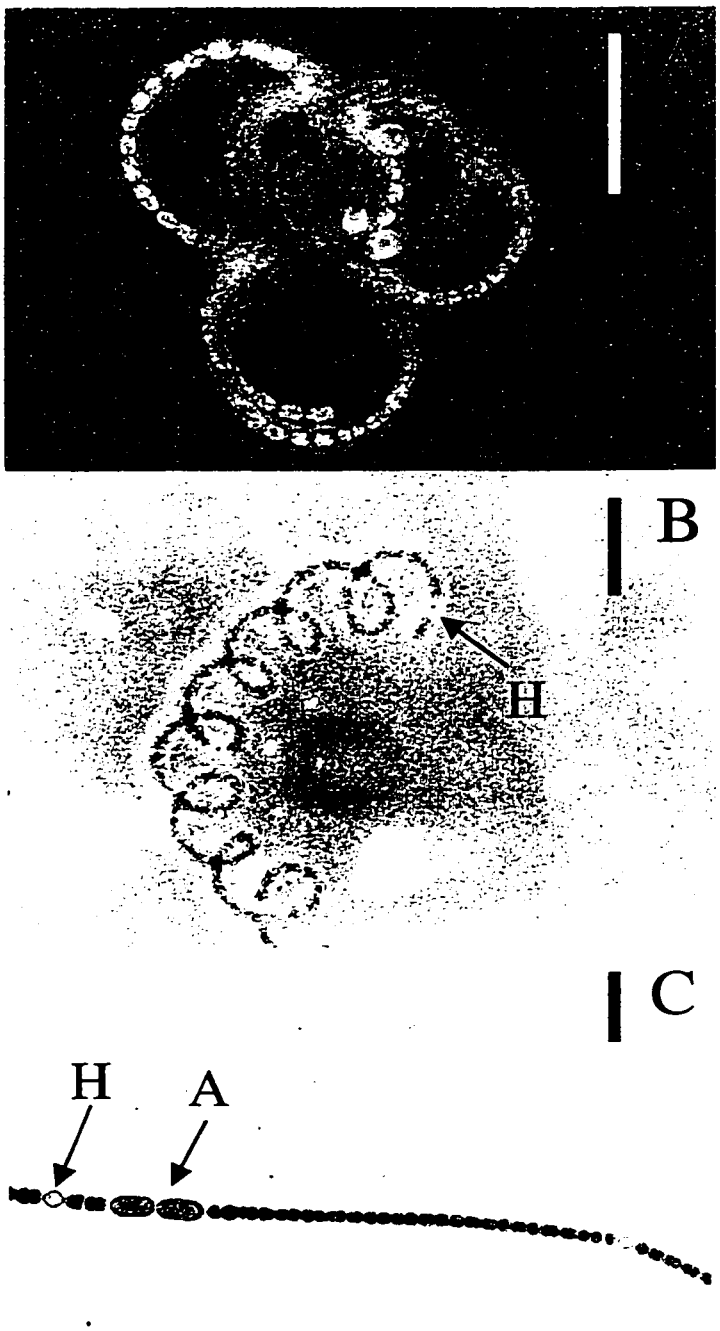

Figure 9. Planktonic *Anabaena* phenotypes in lowland Puget Sound lakes as seen by light microscopy. (a) *A. flos-aquae* from Green Lake, WA; (b) *A. circinalis* coiled/helical morphotype from Green Lake, WA; (c) *A. circinalis* straight form from Green Lake. Bar = 50 μm. H=Heterocyst. A=Akinete.

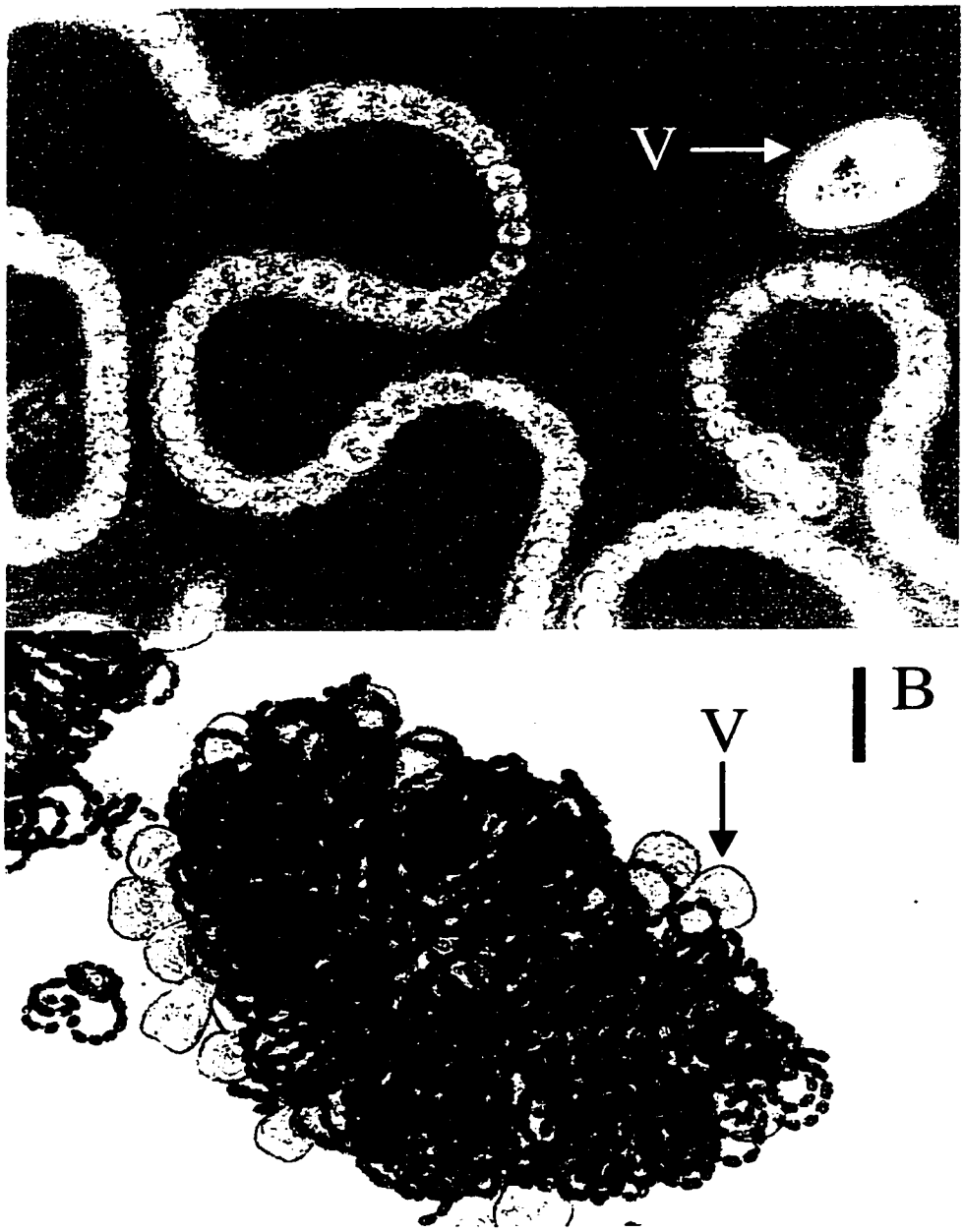

Figure 10. *Anabaena* with *Vorticella* attached as seen by light microscopy. This ciliate appeared to only attach to dense mature *Anabaena* filaments. (a) *A. circinalis*; (b) *A. flos-aquae*. Bar = 50 μm. V= *Vorticella*

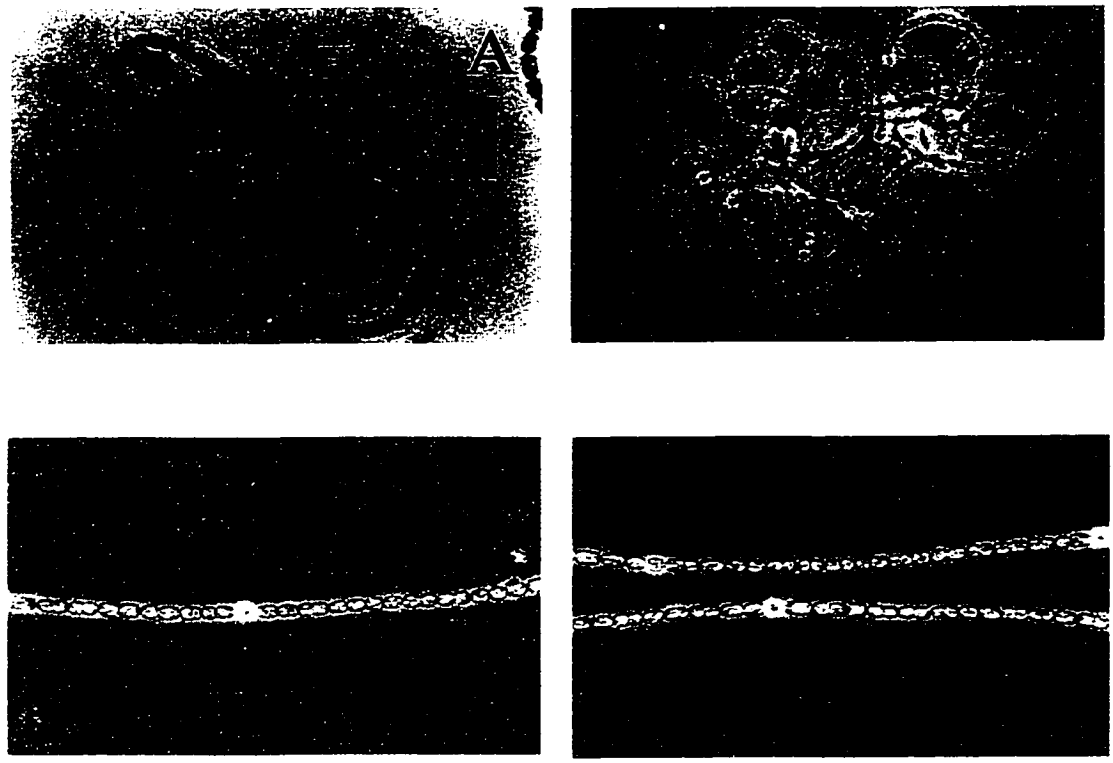

Figure 11. Two phenotypically identical strains of *Anabaena flos-aquae* from American Lake, WA as seen by light microscopy from field and cultured material. These two strains are quite distinct in *cpc*BA-IGS sequence. No akinetes were found in AL97 wild material to compare with the toxic AL89 strain. (a) *Anabaena flos-aquae* (AL97) non-toxic strain from American Lake, WA; (b) *Anabaena flos-aquae* (AL89) toxic strain from American Lake, WA; (c) *Anabaena flos-aquae* (AL97) in culture; (d) *Anabaena flos-aquae* (AL89) in culture. Bar = 50 μm. H = Heterocyst, A = Akinete.

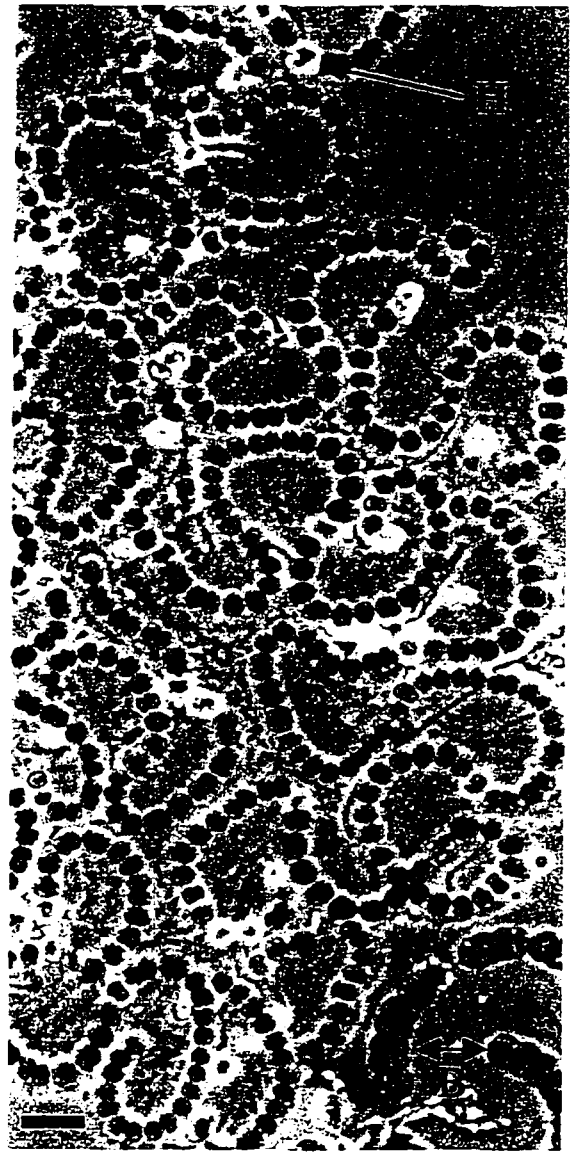

Figure 12. Small and large-celled phenotype of *Anabaena circinalis* present in lowland Puget Sound lakes as seen by light microscopy. Despite the large difference in size, the general cell shape and heterocysts are similar and *cpc*BA-IGS sequence confirmed that these two variants are *Anabaena circinalis*. The large celled phenotype is in right bottom corner with the label "B". H = Heterocyst. Bar = 30 μm.

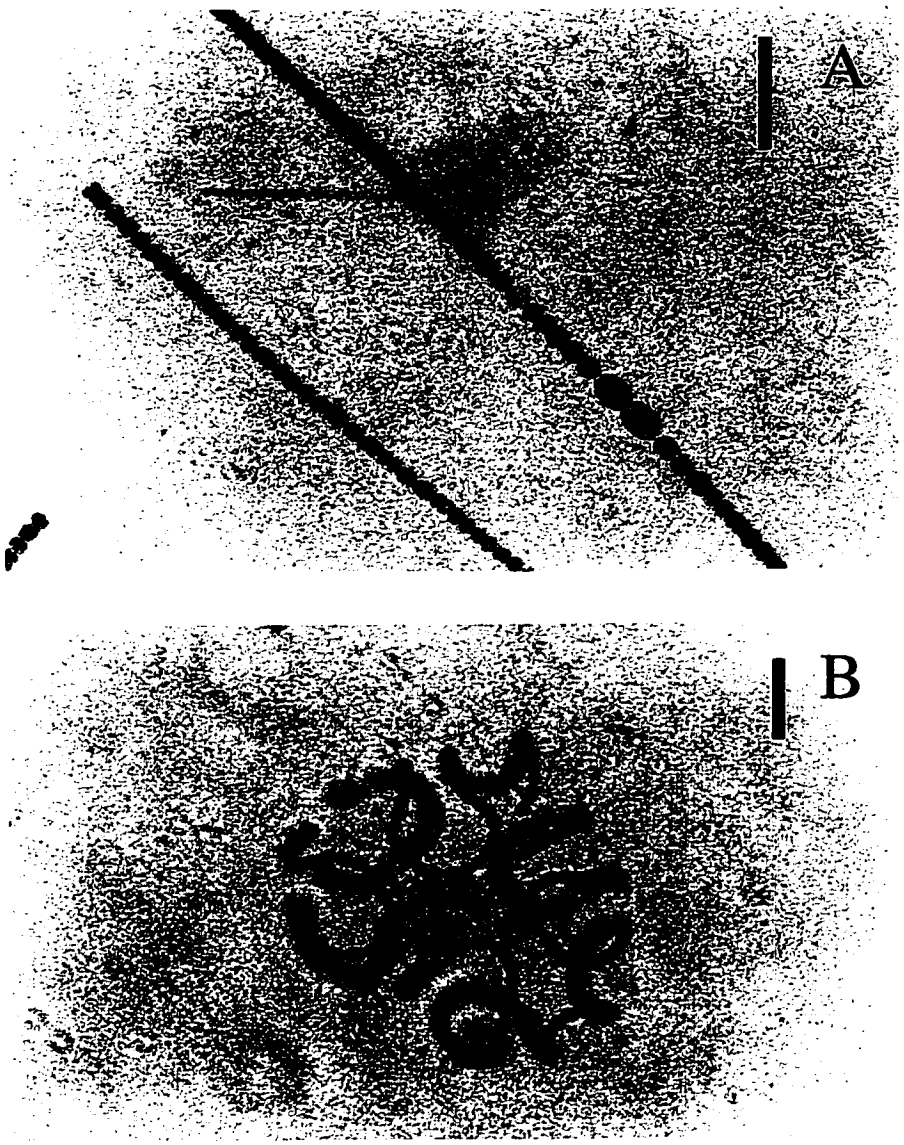

Figure 13. Two lowland Puget Sound lake *Anabaena circinalis* phenotypes with identical *cpc*BA-IGS sequence as seen by light microscopy. The first is a common linear form found in local lakes while the second is an equally common coiled habit with copious mucilage in a matrix that supports a significant bacterial flora. (a) *A. circinalis* "linear form" from Green Lake, WA; (b) *A. circinalis* "mucilaginous form" from Lake Union, WA. Bar = 50 μm.

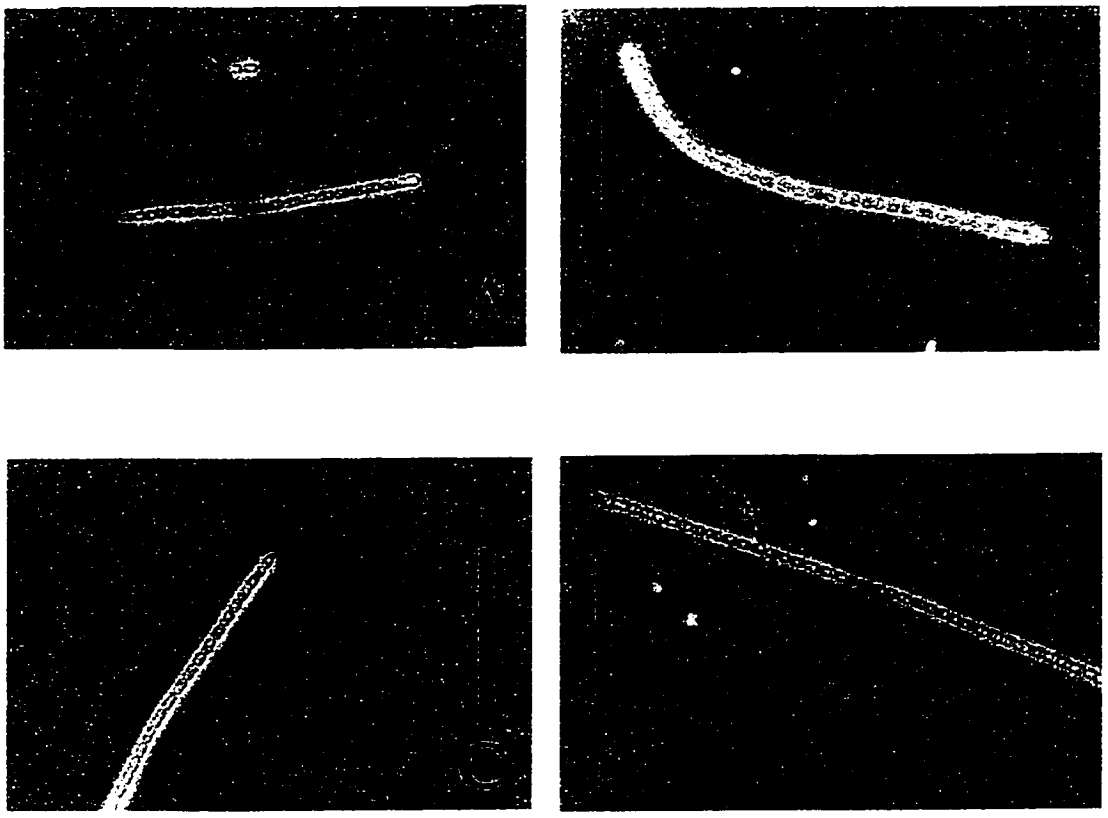

Figure 14. *Anabaena* culture collection strains from *Anabaena* Group I as seen by light microscopy. These strains shared very similar morphology (small cells with tapered terminal cells) in culture but produced no heterocysts during this study. These strains are not generally associated with large planktonic cyanobacteria blooms. (a) *Anabaena inaequalis* (UTEX 380); (b) *Anabaena sphaerica* (UTEX 1619); (c) *Anabaena cylindrica* (UTEX 1403/30); and (d) *Anabaena inaequalis* (UTEX 381). Bar = 50 μm.

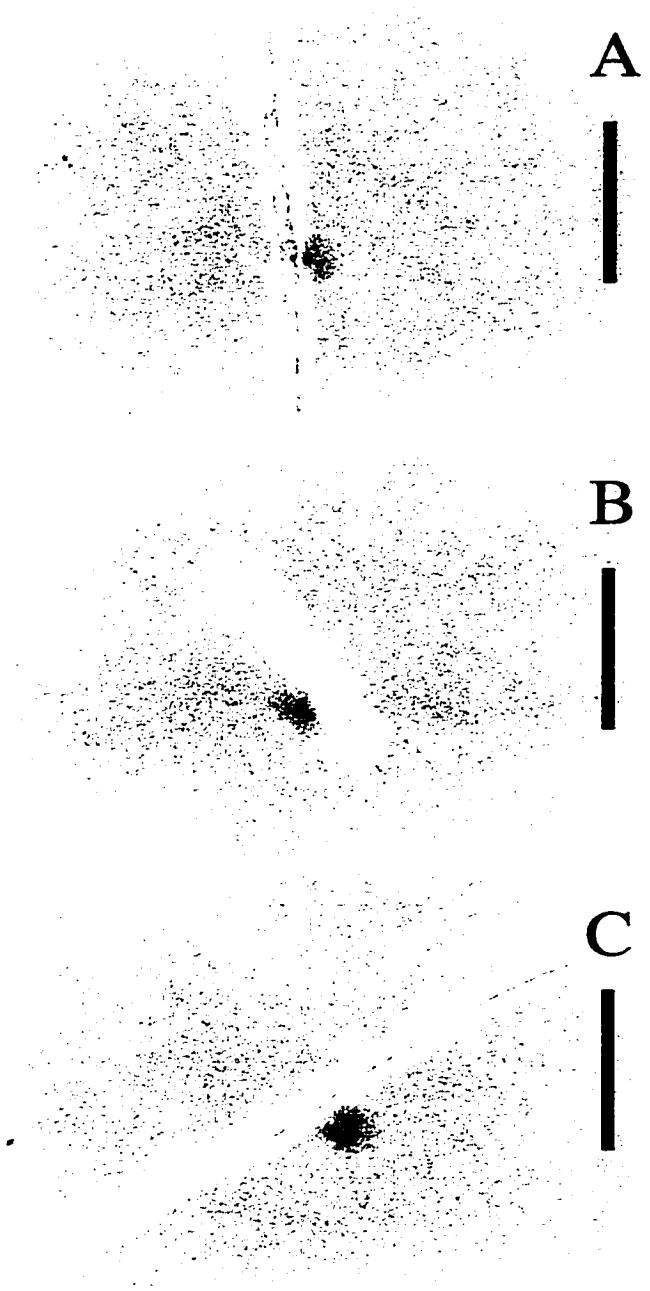

Figure 15. Micrographs of *Anabaena* strains from *Anabaena* Group II. The *Anabaena circinalis* strain (B) has typical spherical cells about 10 μm in diameter while the *Anabaena flos-aquae* strains are rectilinear and only 4-5 μm in width. Differences in cell size and shape are useful field characters as well. (a) *A. flos-aquae* (CCAP 1403/13B); (b) *A. circinalis* (CCAP 1403/18); and (c) *A. flos-aquae* (CCAP 1403/13D). Bar = 50 μm.

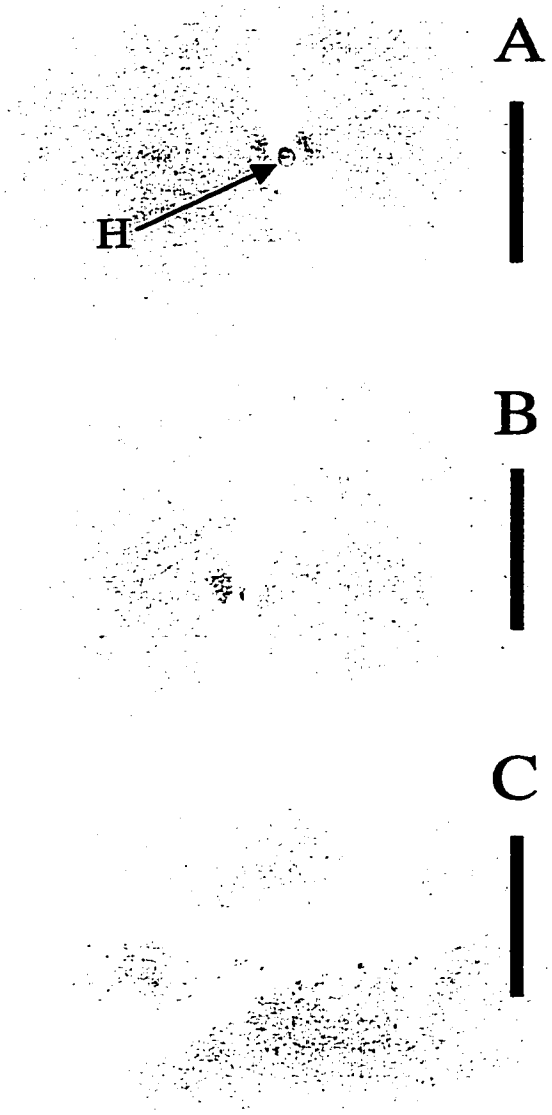

Figure 16. *Anabaena* culture collection strains clustering in the *Nostoc/Nodularia* Clade as seen by light microscopy. These strains should be reassigned to *Nostoc* (UTEX 2557 and UTEX 2558) and *Nodularia* (UTEX 1823) based on *cpc*BA-IGS sequence and life history information. (a) UTEX 2558; (b) UTEX 2557; and (c) UTEX 1823. Bar = 50 μm. H = Heterocyst.

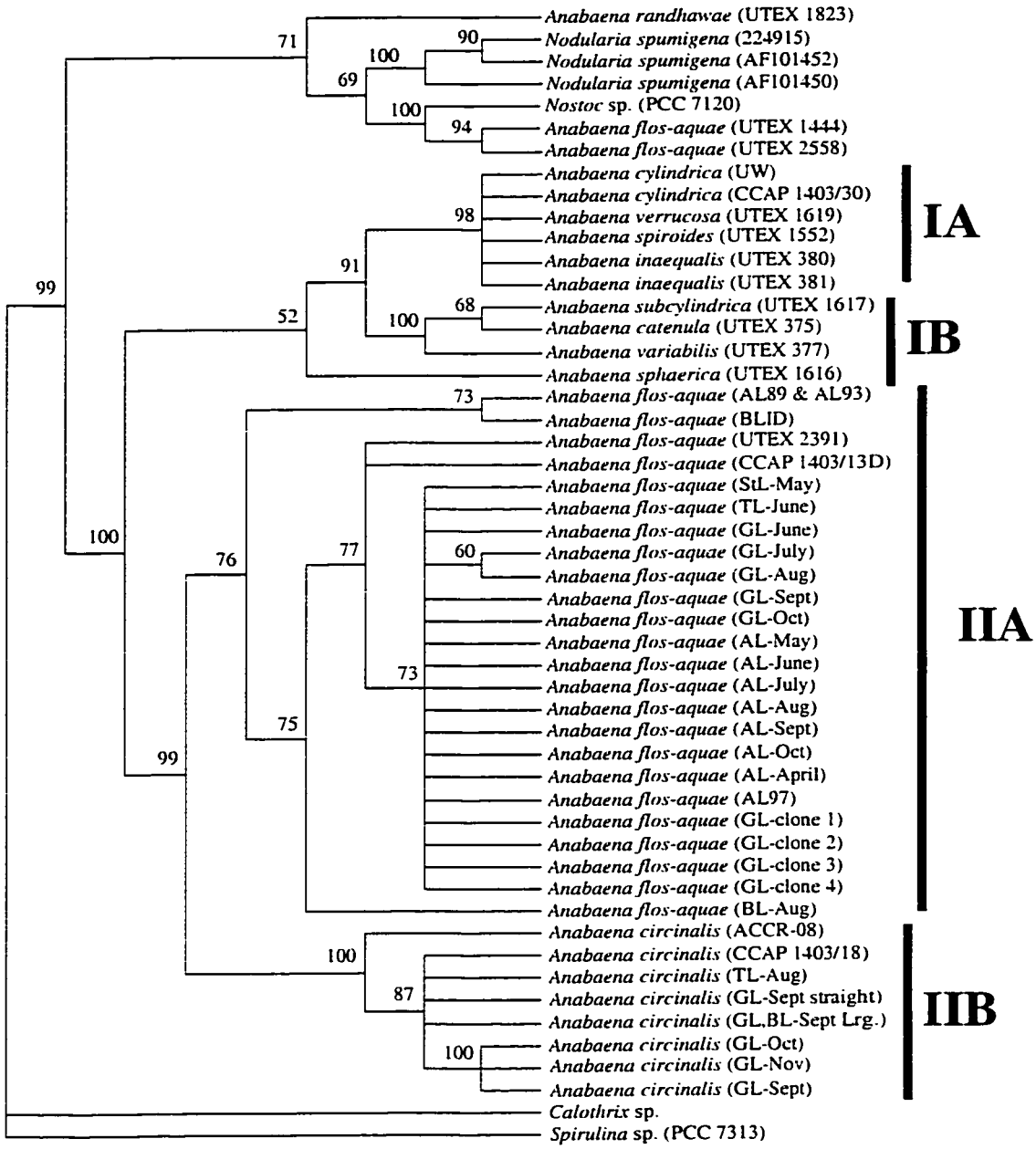

Figure 17. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *Anabaena* and sister group *cpc*BA-IGS sequences excluding 67 base pairs in the IGS region (320-386). Note the two major clusters of *Anabaena* (I and II) with *Nostoc* and *Nodularia* in a sister clade. Group IIB is composed of 3 clusters of *A. circinalis* strains that include a distinct local autumn strain. Group IIA includes distinct clusters of toxic and non-toxic *Anabaena flos-aquae* strains. The summer *A. flos-aquae* flora shows little divergence across the lowland Puget Sound. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). <50% not labeled.

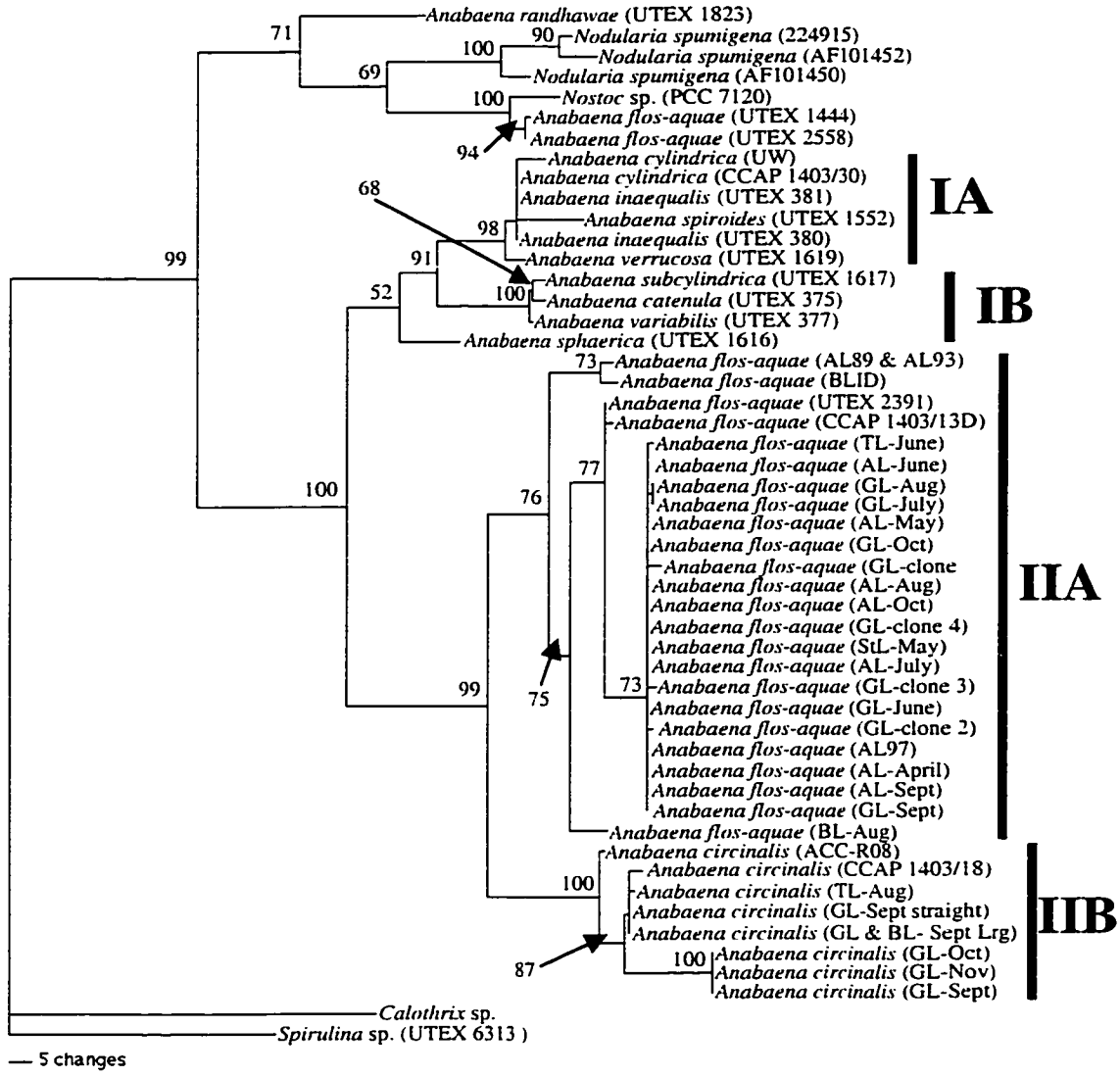

Figure 18. Phylogenetic tree inferred from a heuristic search using maximum likelihood from *Anabaena* and sister group *cpc*BA-IGS sequence data (-ln =4316.05322) excluding 67 base pairs in the IGS region (320-386). Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. The two major groups (I & II) of *Anabaena* are confirmed with the local planktonic flora composed of only a few *A. flos-aquae* (IIA) and *A. circinalis* (IIB) strains. The toxic Pacific Northwest *A. flos-aquae* is distinct from the dominant spring-fall *A. flos-aquae* strain. At least 3 *A. circinalis* strains are represented in the tree with two strains from Puget Sound lowland lakes.

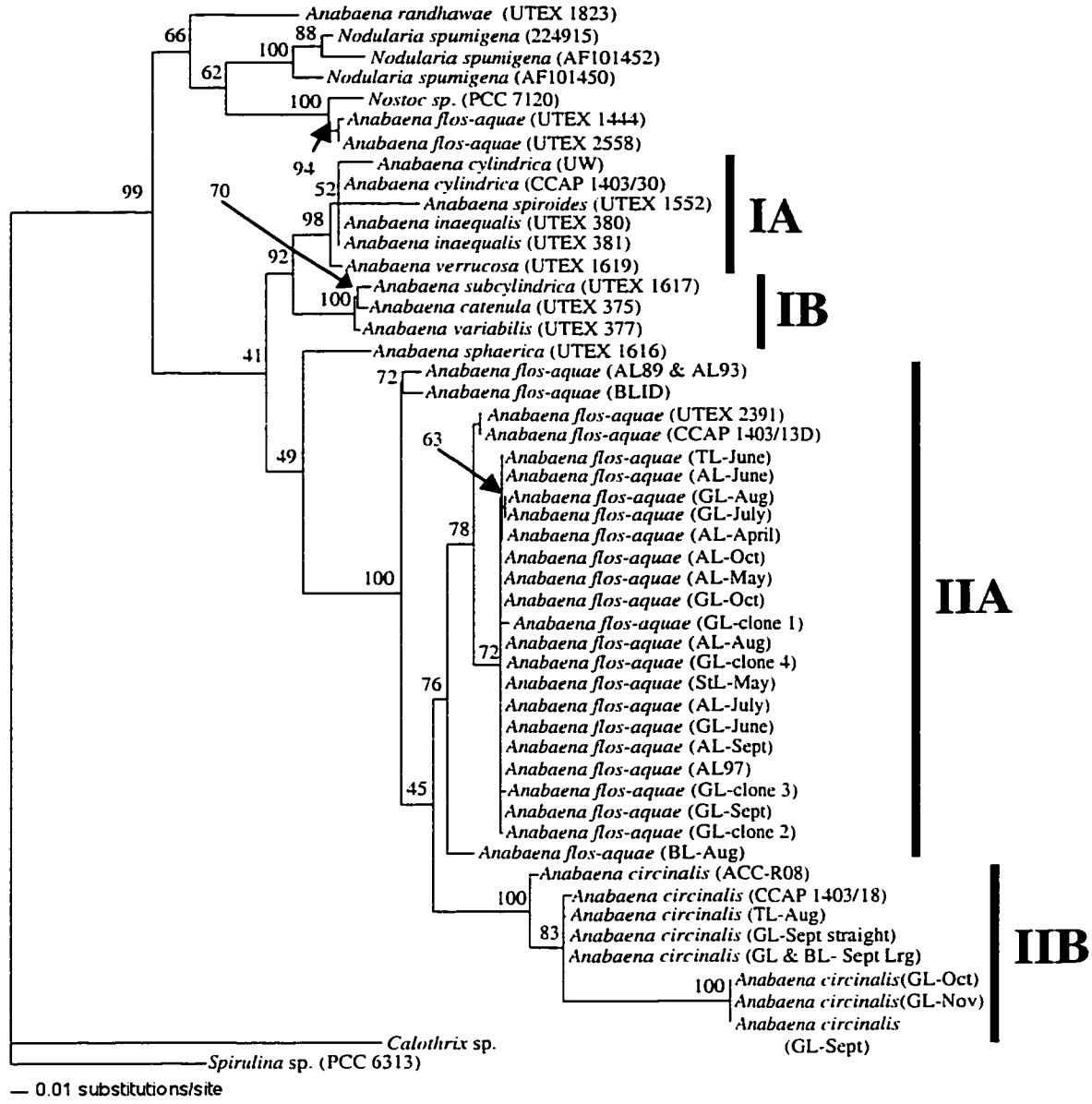

Figure 19. Phylogram based on maximum parsimony analysis of Anabaena and sister group *cpc*BA-IGS sequences excluding excluding 67 base pairs in the IGS region (320-386). Tree shown is one of 865 most parsimonious trees recovered in a heuristic search. Numbers above the nodes are labeled with bootstrap values for parsimony (1000 replicate samples) and nodes with less than 50% bootstrap values are not labeled. Tree length = 683 steps; CI = 0.647; RI = 0.849; RC = 0.549; HI = 0.353. Scale bar for 5 nucleotide changes is shown at bottom.

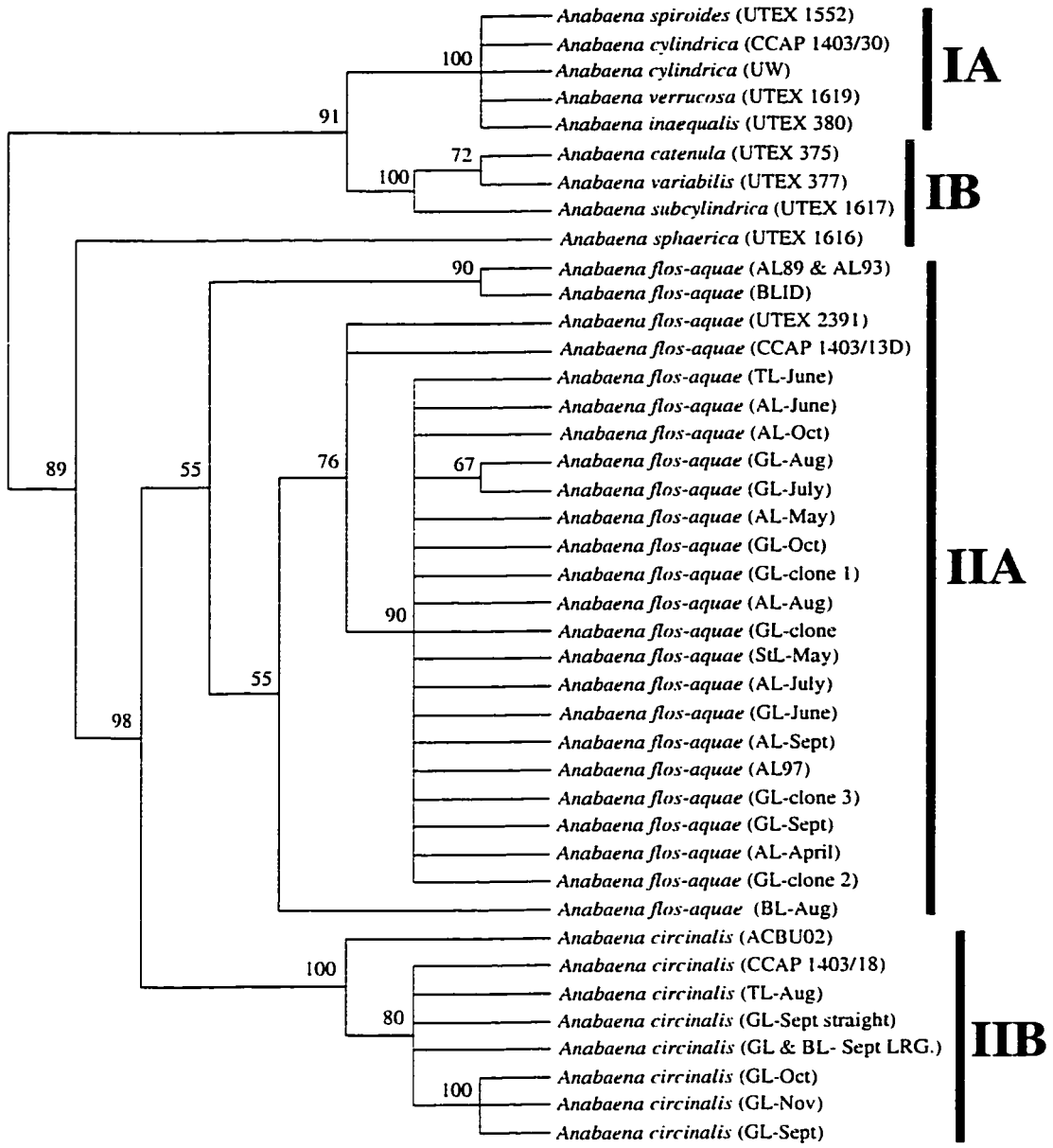

Figure 20. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *Anabaena cpc*BA-IGS sequences (663 bp). Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Tree length = 316, CI = 0.780, RI= 0.918, HI = 0.220, (998 equally parsimonious trees). *A. circinalis* (IIB) and *A. flos-aquae* (IB) are distinct clades. All *A. flos-aquae* filaments collected from Pacific Northwest lakes in the spring-fall except one (BL-Aug.) had nearly identical *cpc*BA-IGS sequence. The two fall-winter toxic (AL89 & BLID) strains were genetically distinct from the other Pacific Northwest *A. flos-aquae* strains.

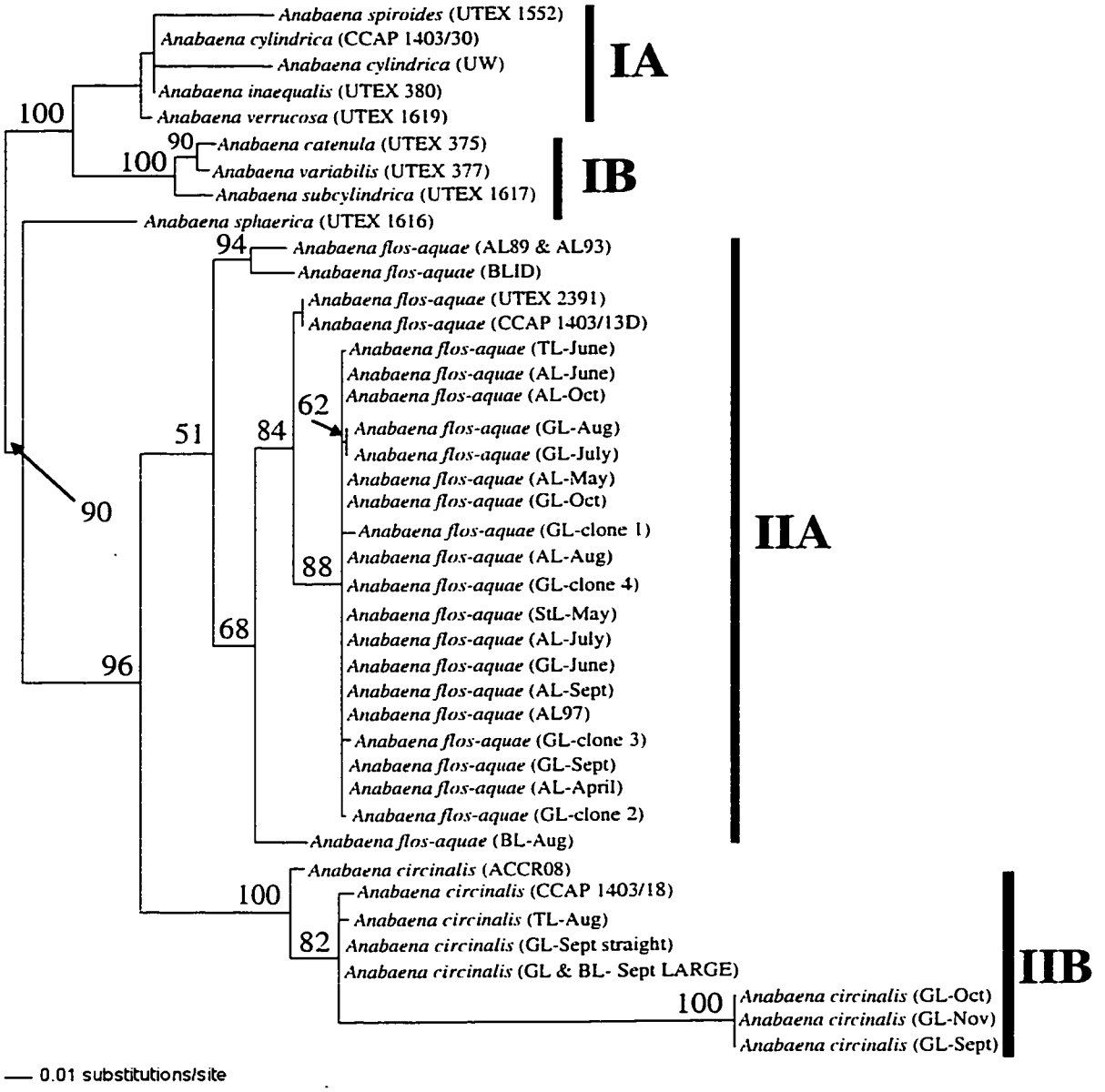

Figure 21. Phylogenetic tree inferred from a heuristic search using maximum likelihood from *Anabaena cpc*BA-IGS sequence data (-ln = 2637.14860). Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). Two distinct strain clusters of *A. circinalis* from Pacific Northwest lakes exist. The first strain cluster includes a U.K. strain as well as 3 Pacific Northwest phenotypes (linear, colied and large) that are identical in *cpc*BA-IGS sequence. The second cluster (GL Sept-Nov.) is quite distinct in sequence but not morphologically.

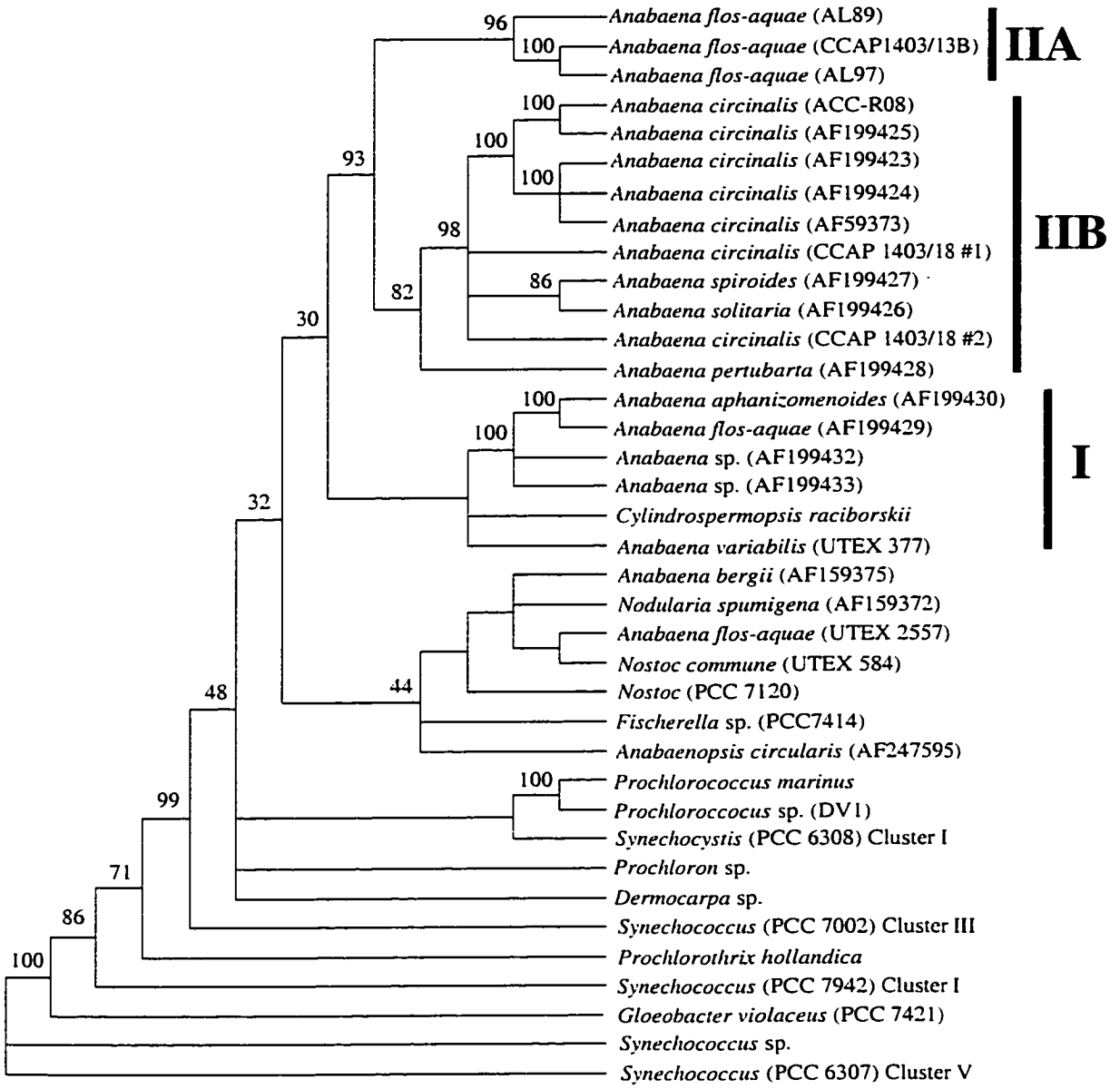

Figure 22. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of cyanobacteria *rpo*C1 sequences (585 base pairs). 299 parsimony informative positions were found. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). This marker confirms a monophyletic *Anabaena* Group II containing *A. flos-aquae* and *A. circinalis*, but indicates that *Anabaena* Group I may include several genera and that the placement of the *Nostoc/Nodularia* clade is unclear. Heterocystous cyanobacteria are also confirmed as a monophyletic clade.

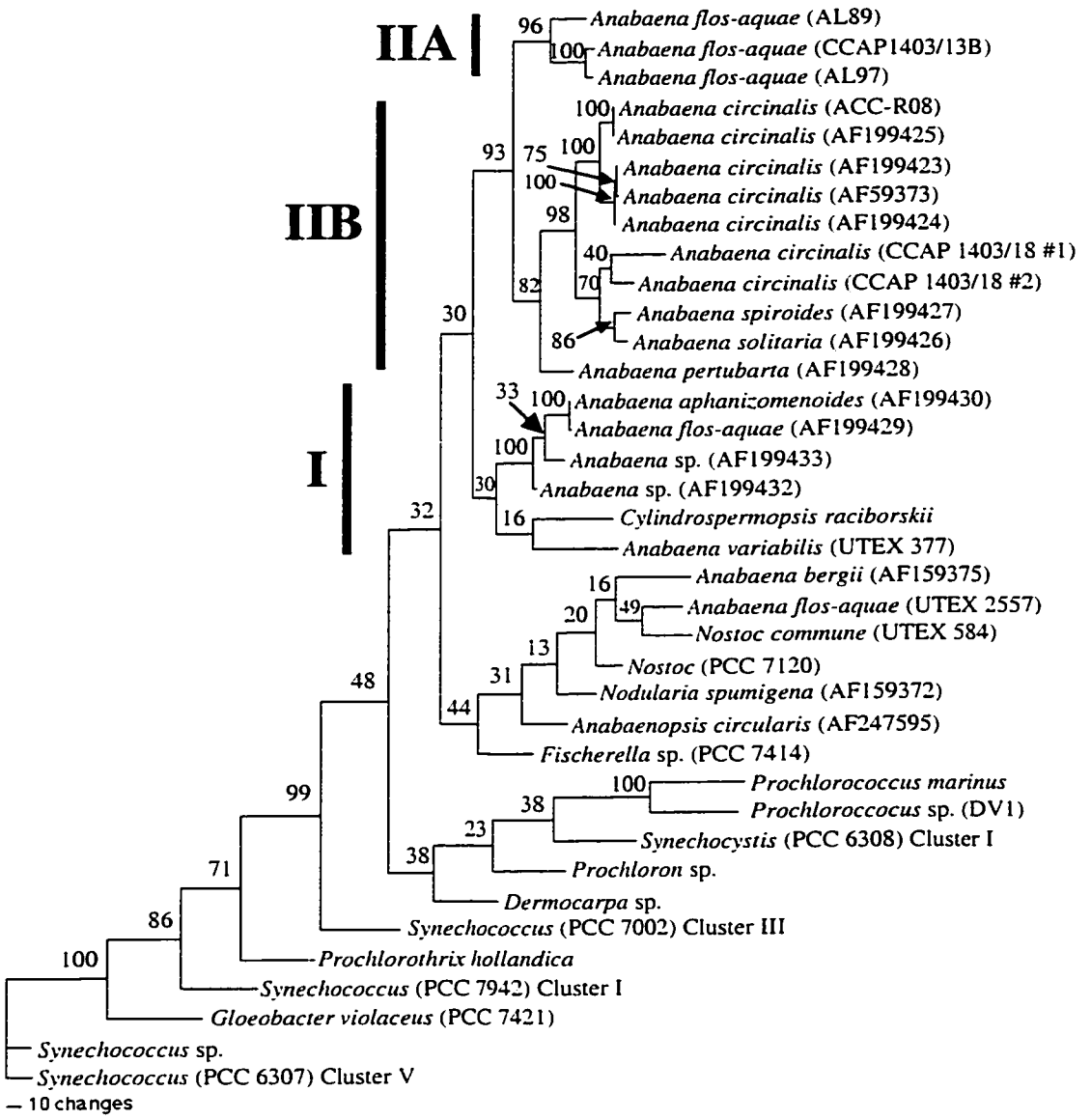

Figure 23. Phylogram based on maximum parsimony analysis of cyanobacteria *rpo*C1 sequences. Tree shown is one of 17 most parsimonious trees recovered in a heuristic search. Numbers above the nodes are labeled with bootstrap values for parsimony (1000 replicate samples). Tree length = 2031 steps; CI = 0.319; RI = 0.554; RC = 0.177; HI = 0.681. Scale bar for 10 nucleotide changes is shown at bottom. All trees supported a distinct *Anabaena* Group II.

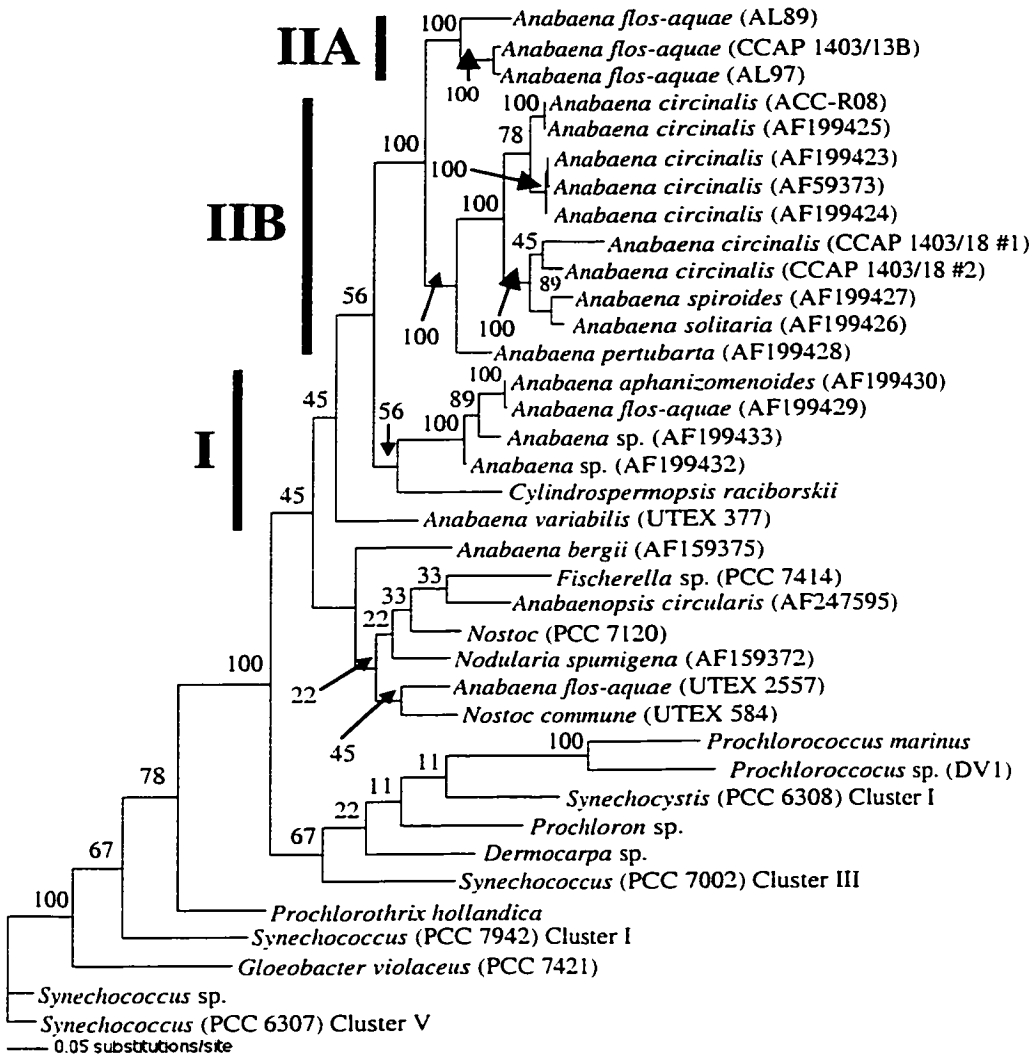

Figure 24. Phylogenetic tree inferred from a heuristic search using maximum likelihood from cyanobacteria *rpo*C1 sequence data (-ln = 9718.70416). Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). Scale bar for 0.05 substitutions per site is shown at bottom. Analysis also supports a distinct *Anabaena* Group IIA & IIB with a sister *Anabaena* Group I that may include *Cylindrospermopsis*. Other heterocystous genera form a sister clade to *Anabaena*.

Table 2. Cyanobacteria strains included in the *Anabaena* study.

Taxon	Strain	Toxicity	Source	Accession Number
Anabaena				
aphanizomenoides Forti	ANA280A	No	Australia	AF199430
bergii Komárek	ANA283A	No	Australia	AF159375
catenula (Kützing) Bornet et Flahault	UTEX 375	No	Netherlands	
circinalis Rabenhorst	ANA175A		Australia	AF199423
circinalis Rabenhorst	ANA019A		Australia	AF199424
circinalis Rabenhorst	ANA350C		Australia	AF199425
circinalis Rabenhorst	ACC-R08	No	Australia	
circinalis Rabenhorst	CCAP 1403/18 #1	No	N. Ireland	
circinalis Rabenhorst	CCAP 1403/18 #2	No	N. Ireland	
circinalis Rabenhorst	GL-Sept	?	Green Lake, WA	
circinalis Rabenhorst	GL-Sept Linear	?	Green Lake, WA	
circinalis Rabenhorst	GL-Sept Large	?	Green Lake, WA	
circinalis Rabenhorst	GL-Oct	?	Green Lake, WA	
circinalis Rabenhorst	GL-Nov	?	Green Lake, WA	
circinalis Rabenhorst	TL-Aug	?	Terrell Lake, WA	
circinalis Rabenhorst	BL-Sept	?	Black Lake, WA	
cylindrica Lemmermann	UW	No	Unknown	
cylindrica Lemmermann	CCAP 1403/30	No	Saudi Arabia	
flos-aquae Brébisson ex Bornet et Flahault	ANA354A	No	Australia	AF199429
flos-aquae (Lyngbe) Brébisson	UTEX 2557	Yes	Hebgen Lake, MT	
flos-aquae (Lyngbe) Brébisson	UTEX2558	No	Hebgen Lake, MT	
flos-aquae Brébisson ex Bornet et Flahault	AL89	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL89 N39	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL89 61L	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL89 55D	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	BLID	Yes	Black Lake, ID	
flos-aquae Brébisson ex Bornet et Flahault	AL93	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL97	No	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-May	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-June	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-July	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-Aug	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-Sept	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	AL-Oct	?	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL-June	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL-July	?	Green Lake, WA	

Table 2. Continued

Taxon	Strain	Toxicity	Source	Accession Number
Anabaena				
flos-aquae Brébisson ex Bornet et Flahault	GL-Aug	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL-Sept	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL-Oct	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL- clone 1	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL- clone 2	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL- clone 3	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GL- clone 4	?	Green Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	StL-May	?	Steilacoom Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	TL-June	?	Terrell Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	BL	?	Black Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	GwL	?	Lake Goodwin, WA	
flos-aquae Brébisson ex Bornet et Flahault	BiL	?	Big Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	SpL	?	Spencer Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	LoL	?	Long Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	LeL	?	Leland Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	SeL	?	Lake Stevens, WA	
flos-aquae Brébisson ex Bornet et Flahault	CCAP 1403/13B	No	UK	
flos-aquae Brébisson ex Bornet et Flahault	CCAP 1403/13D	No	Wales, UK	
flos-aquae (Lyngbe) Brébisson	UTEX 1444	No	Mississippi	
flos-aquae Brébisson ex Bornet et Flahault	UTEX 2391	Yes	Alberta, Canada	
inaequalis (Kützing) Bornet et Flahault	UTEX 380	No	Netherlands	
inaequalis (Kützing) Bornet et Flahault	UTEX 381	No	Netherlands	
pertubarta Cronberg et Kómarek	ANA221E	No	Australia	AF199428
randhawae Venkatamaran	UTEX 1823	No	India	
soliataria Klebahn	ANA207A	No	Australia	AF199426
sphaerica Bornet et Flahault	UTEX 1616	No	India	
spiroides Klebahn	UTEX 1552	No	Fish pond, Israel	
spiroides Klebahn	ANA292C	No	Australia	AF199427
subcylindrica Borge	UTEX 1617	No	Nashville, TN	
variabilis Kützing	UTEX 377	No	Netherlands	
verrucosa Boye-Petersen	UTEX 1619	No	Austin, TX	
sp.	ANA238C	No	Australia	AF199432
sp.	ANA255C	No	Australia	AF199433
Anabaenopsis				
circularis (G. S. West) Woloszynska et Miller		No	Japan	AF159374

Table 2. Continued

Taxon	Strain	Toxicity	Source	Accession Number
Cylindrospermospsis				
raciborskii Woloszynska		Yes	Australia and Brazil	AF159371
Nodularia				
spumigena Mertens ex Bornet et Flahault	PCC 73104	?	Canada	AF159372
spumigena Mertens ex Bornet et Flahault	BC9427	?	Baltic Sea	AJ224915
spumigena Mertens ex Bornet et Flahault		?	Australia	AF101452
spumigena Mertens ex Bornet et Flahault		?	Australia	AF101450
Nostoc				
commune Vaucher	UTEX 584	No	Scotland	M29747
sp.	PCC 7120	No	North America	
Calothrix				
sp.		?	--	
Fischerella				
ambigua (Näg.) Gomont	UTEX 1903	?	--	
sp.	PCC 7414	?	--	Z11153
Outgroup Taxa				
Dermocarpa sp.		?	--	U522341
Gloeobacter violaceus Rippka, Waterbury et Cohen-Bazire		?	--	U52340
Prochlorococcus marinus Chisholm		?	--	Z11160
Prochlorococcus sp.	DV1	?	--	Z11159
Prochloron sp.		?	--	Z11161
Prochlorothrix hollandica Burger-Wiersma		?	--	Z11154
Spirulina sp.	PCC 7313	?	--	AJ401188
Synechococcus sp.	PCC 7002	?	--	
Synechococcus sp.	PCC 7942	?	--	
Synechococcus sp.	PCC 6307	?	--	
Synechococcus sp.		?	--	
Synechocystis sp.	PCC 6803	?	--	

CHAPTER 3: THE PHYLOGENETIC PLACEMENT OF *APHANIZOMENON* AND *ANABAENA* WITHIN THE FAMILY NOSTOCACEAE, ORDER NOSTOCALES

ABSTRACT

A phylogenetic analysis of *Anabaena* and *Aphanizomenon* was constructed from Pacific Northwest, U.S.A. direct PCR lake samples (single filaments) and isolates from the UTEX, SAG and CCAP collections using DNA sequence from the *cpc*BA-IGS and *rpo*C1 regions. Sequences from over 17 species, including several toxin producing strains, representing a variety of morphotypes were examined using 381 bp of DNA from the *cpc*BA-IGS region and 584 bp from the *rpo*C1 region. *Aphanizomenon flos-aquae* blooms in lowland Puget Sound lakes appeared limited to one genotype and phenotype during the summer and fall, compared with several common *Anabaena* phenotypes composed of four genotypes.

The *cpc*BA-IGS and *rpo*C1 trees placed all of the *Aphanizomenon* strains in the *Anabaena* clade with high bootstrap support. A distinct clade of planktonic *Anabaena flos-aquae* Brébisson ex Bornet et Flahault 1886, *Aphanizomenon gracile* (Lemm.) Lemmermann and *Aphanizomenon flos-aquae* Ralfs ex Bornet et Flahault 1886 strains from the Northern hemisphere was also highly supported in the *cpc*BA-IGS and *rpo*C1 trees and designated *Anabaena* Group IIA. A revised *Anabaena flos-aquae* is proposed based on *Anabaena*'s priority under the International Code of Botanical Nomenclature. The traditional morphological characters used to distinguish *Anabaena* and *Aphanizomenon* are invalid, and the description of *Anabaena flos-aquae* is expanded to

include the variation found within *Anabaena* Group IIA. The *Anabaena flos-aquae* clade consists of several genotype clusters that upon further study may merit subspecies status. Only one of the clusters contained toxic strains.

INTRODUCTION

The genera *Anabaena* Bory ex Bornet et Flahault 1886 and *Aphanizomenon* Morren ex Bornet et Flahault 1886 are members of the family Nostocaceae Bornet et Flahault 1886 in the order Nostocales (Komárek and Anagnostidis, 1989). Taxonomists have acknowledged that *Aphanizomenon* has two serious problems: 1) the generic delimitation from *Anabaena*; and 2) the reliability of criteria used to define *Aphanizomenon* species (Komárek and Kovacik, 1989). The relationship between these genera is poorly understood in part due to the overlap in morphological characters among species assigned to these genera (Komárek and Kovacik, 1989). Recent phylogenetic evidence has also cast doubt on the current classification of *Anabaena* and *Aphanizomenon* as well as the other members of the Nostocales (Wilmotte and Herdman, 2001). The phylogenetic relationship between *Anabaena*, *Nostoc* and *Nodularia* is discussed in greater detail in Chapter 2 and illustrates the failure of the current morphology-based taxonomic system to accurately distinguish these genera. This failure also suggests that the division of *Anabaena* and *Aphanizomenon* may be suspect.

Anabaena and *Aphanizomenon* are isopolar, non-branching trichomes with nitrogen-fixing heterocysts, and spores called akinetes (Komárek and Anagnostidis, 1989). The public typically associates them with blooms that can create thick scums on the water surface under appropriate conditions in eutrophic lakes, ponds and reservoirs (Paerl and Tucker, 1995). *Aphanizomenon* and planktonic strains of *Anabaena* are scum-forming ecostrategists that use gas vesicles in combination with carbohydrate ballast to control access to light and nutrients by moving up and down in the water column and can often be found together or successively in a water body (Chorus and Bartram, 1999).

Anabaena and *Aphanizomenon* are widely distributed throughout the world although *Aphanizomenon* is not common in tropical regions (Hindak, 2000). *Anabaena* can be found not only in lakes and estuarine conditions but also in soils and as a symbiont in several plants while *Aphanizomenon* appears to be restricted to the planktonic habit (Lee, 1999). Both genera thrive in limited ecological conditions where they tend to dominate seasonally to the exclusion of other organisms when environmental conditions are stable and may be found in mixed cyanobacteria assemblages that often also include *Microcystis* (Paerl, 1988).

Anabaena and *Aphanizomenon* have been implicated in toxic blooms in lakes and reservoirs. Some *Anabaena* strains produce: a) microcystin, a serine/threonine protein phosphatase 1 and 2A inhibitor (Honkanen et al., 1990); b) saxitoxin, an alkaloid nerve cell sodium channel blocker; c) anatoxin a, an acetyl choline mimic; or d) anatoxin a (S), an anticholinesterase (Chorus and Bartram, 1999). Several *Aphanizomenon* strains have

also been shown to produce the neurotoxins, anatoxin and saxitoxin (Chorus and Bartram, 1999). No correlation between phylogenetic relationships and toxin production has been identified in any of the cyanobacteria (Tillett, Parker and Neilan, 2001), but toxins can be traced to particular strains, and this information is critical in understanding the ecological conditions and distribution of strains that pose risks to the public's health.

An understanding of the identity and succession of planktonic cyanobacteria is critical in expanding our knowledge of cyanobacteria ecology (Lyra et al., 2001). Toxicity risk assessment, water quality management and restoration efforts will remain limited in their success unless we can identify, track and predict the response of the scum ecostrategists (Chorus and Bartram, 1999). The first task in understanding cyanobacteria diversity is to distinguish phenotypic plasticity from true genotypic differences. Unfortunately, the morphological and genetic information available for planktonic cyanobacteria is currently limited (Li and Watanabe, 2001). This represents a serious public health problem when accurate identification of *Anabaena* and *Aphanizomenon* species is necessary to determine the presence of toxin producing strains.

Construction of a classification system that provides an accurate description of the phylogenetic relationship between organisms and a field identification key is difficult, as the current concept of a "species" in the cyanobacteria has no distinct boundaries. As prokaryotes, cyanobacteria are clonal yet may exchange genetic information across genera through horizontal transfer (Rudi, Skulberg and Jakobsen, 1998) or other mechanisms. Without a phylogenetic framework for these organisms, we lack the ability

to understand key ecological relationships such as the degree to which any exchange of genetic information occurs between genera or the ecophysiological parameters that govern succession of cyanobacteria strains in waterbodies. This study addresses the questions related to the first order inquiry: What is the phylogenetic relationship between strains of cyanobacteria traditionally assigned by morphology to the genera *Aphanizomenon* and *Anabaena*?

The heterocystous clade of cyanobacteria is is well supported in several phylogenetic studies (Giavannoni et al., 1988; Wilmotte and Herdman, 2001), but the phylogenetic picture within the heterocystous cyanobacteria is unclear. Wilmotte (2001) identifies the need for more detailed studies on the genotypic relationships within the heterocystous cyanobacteria based on the disagreement of recent phylogenetic data with current generic and species assignments and suggests that numerous genera including *Nostoc*, *Nodularia*, and *Anabaena* may need to be reclassified (Lu et al., 1997; Lyra et al., 2001; Wilmotte and Herdman, 2001). This taxonomic issue is also discussed by Rippka in Bergey's Manual of Systematic Bacteriology (Rippka, Castenholz and Herdman, 2001).

Cyanobacteria taxonomy stands at a cross-road where botanists under the International Code of Botanical Nomenclature (ICBN) and bacteriologists under the International Code of Nomenclature of Bacteria (ICNB) are converging on a polyphasic taxonomy for the cyanobacteria that will resolve the significant taxomomic issues that remain in this group of bacteria (Wilmotte and Herdman, 2001). The historical

descriptions of nearly all of the known cyanobacteria morphotypes were conducted under the ICBN and are accompanied by generic and species epithets primarily based on morphology. The ICBN system constitutes a key that allows the practical application of morphological characters to the field identification of cyanobacteria that in many instances may not mirror the true phylogeny of the organisms. Despite its flaws, it remains the only current means to identify and communicate information about species until a more accurate system can be constructed. As long as the morphological characters used to identify strains are not plastic, this system remains useful perhaps, not phylogenetically, but from a field identification and risk assessment perspective.

Botanists have embraced the utilization of phylogenetic evidence to determine the validity of the current taxonomy and develop new objective characters (morphological, chemical, molecular, etc.) in a polyphasic taxonomy. The difficulty in isolating and maintaining axenic cultures of cyanobacteria has slowed the application of molecular techniques to many taxa including the members of the Nostocales (Castenholz, 1992). Bacteriologists recommend the application of methods such as DNA-DNA hybridization and 16S rRNA sequencing for use in systematic revision (Brenner et al., 2001), but these techniques are also dependent on culturing limitations. Efforts to provide 16S rRNA data without having to culture strains has made recent progress (Neilan et al., 1997a), but DNA-DNA hybridization still requires a large quantity of axenic cells. It is simply not possible at this time to obtain a sample from the field, isolate all the strains that may be present, and culture all of them successfully for DNA extraction.

The use of molecular markers with greater phylogenetic resolution than 16S rRNA also appears necessary in order to bring a broader range of phylogenetic tools into use to determine population level questions such as the relatedness of strains within a population (Palenik, 1994). The 16S rRNA region has proven a poor marker for distinguishing some cyanobacterial lineages at the species level and offers limited to no utility in distinguishing clusters of strains (Neilan, Jacobs and Goodman, 1995). Molecular markers other than 16S rRNA are also needed to examine higher order phylogenetic questions in the cyanobacteria such as generic or familial relationships. DNA-DNA hybridization is unable to resolve relationships at these levels (Brenner, Staley and Krieg, 2001), and 16S rRNA data have been unable to resolve higher order relationships such as the apparent rapid radiation of the cyanobacteria lineage billions of years ago (Wilmotte and Herdman, 2001).

The increasing volume of molecular data from heterocystous strains supports a significant revision in the nomenclature of heterocystous genera and species although the evidence at this time is incomplete and not actionable because of the limited number of strains available (Rippka, Castenholz and Herdman, 2001). Molecular markers such as *cpc*BA-IGS (Bolch, Blackburn, Neilan and Grewe, 1996), 16-23S-ITS rRNA (Lu et al., 1997), *nif*H1 (Zimmerman and Culley, 1991), *rpo*C1 (Palenik and Swift, 1996) and *rbc*L (Rudi, Skulberg and Jakobsen, 1998) have been developed to examine the phylogenetic relationships within the Nostocales as well as other cyanobacterial lineages. Rudi (1998) discusses the risks in choosing a gene that may be paralogous, a duplication/pseudogene

or possibly transferred laterally among taxa. 16S rRNA is known to exist in multiple copies in taxa examined to date while *rpo*C1 and *cpc*BA are thought to be single copy genes (Bolch et al., 1999; Palenik, 1994). In addition, both genes are unique among the prokaryota thus allowing the amplication of DNA by PCR from unicyanobacterial samples without the need to render samples axenic or develop specific PCR primers for the cyanobacteria (Neilan et al., 1994; Palenik, 1994).

In light of recent 16S rRNA evidence, Bergey's Manual of Systematic Bacteriology eliminated all higher order systematic organization within the heterocystous cluster and only uses temporary form genera to describe the heterocystous cyanobacteria (Castenholz, 2001). Many of the form genera are also suspect phylogenetically and the future status of *Anabaena* and *Aphanizomenon* is left open by Bergey's Manual of Systematic Bacteriology (Rippka, Castenholz and Herdman, 2001). Bacteriologists have long understood that phylogenetics offers the most accurate classification system (Brenner, Staley and Krieg, 2001), but there is some debate regarding the necessity of DNA-DNA hybridization where polyphasic taxonomic evidence strongly supports the establishment of a species. The standard definition of a genomospecies includes all strains with 70% or greater DNA-DNA relatedness (Wayne, Brenner and Colwell, 1987). Brenner (2001) recommends that a new species not be named if it cannot be differentiated from other genomospecies by some phenotypic property but Wayne (1997) provides for the designation of subspecies status where a name already exists for one of

the genomospecies in question. Polyphasic taxonomy as applied to nomenclature is therefore a combination of DNA-DNA relatedness and phenotypic separation.

Bory de St. Vincent first described the genus *Anabaena* Bory de St. Vincent (1822) in his Dictionnaire Classique d'Histoire Naturelle (Bory de St Vincent, 1822). Charles Morren described the genus *Aphanizomenon* Morren (1838) sixteen years later (Morren, 1838). The ICBN recognizes Bornet and Flahault's 1886 monograph as the first valid publication and description with priority for nomenclatural purposes of Nostacalean genera including *Anabaena*, *Aphanizomenon*, *Nostoc* and *Nodularia* (Greuter et al., 2000). It is also important to note that *Anabaena* is a conserved genus under the ICBN and therefore assumes priority over any other Nostocalean genus if a phylogenetic conflict occurs (Greuter et al., 2000). The first valid publication date recognized by the ICNB (Sneath, 1992) is the Approved Lists of Bacterial Names in 1980 (Skerman et al., 1980) and no cyanobacteria genera from the Nostocales are currently validly published under the ICNB.

Aphanizomenon flos-aquae was described by Linneaeus in 1753 as *Byssus flos-aquae*, and Bory's 1822 description of several *Anabaena* species also includes a paragraph that describes *Byssus flos-aquae* from dried herbarium samples that correspond to *Aphanizomenon flos-aquae* (Bory de St. Vincent, 1822). Bory's paragraph however, does not appear to be a formal description, and he explicitly disclaims making a formal description although he states that he believes *Byssus flos-aquae* is an *Anabaena*. Bornet and Flahault (1886) record Bory's work as having assigned *Byssus flos-aquae* to

Anabaena flos-aquae, but this appears to be the sole instance where *Aphanizomenon flos-aquae* is recorded as *Anabaena flos-aquae*. Of the *Anabaena* species formally described by Bory, only *Anabaena oscillarioides* remains in the genus today and represents the type species for the genus (Komárek and Anagnostidis, 1989). *Anabaena flos-aquae* was first described by Brébisson in 1835 (Brebisson and Godey, 1835), and its phylogenetically close sister taxon, *Anabaena circinalis* was described by Rabenhorst in 1852. Both of these species are entitled to Bornet and Flahault's 1886 publication date as they are validly described in that monograph as well.

Morren's description of *Aphanizomenon* clearly illustrates and describes the type species *Aphanizomenon flos-aquae* although he named the species, *Aphanizomenon incurvum* (Morren, 1838). A combination of Morren's generic epithet (*Aphanizomenon*) and the specific epithet *flos-aquae* was proposed by Ralfs in 1850 and revised by Bornet and Flahault in 1886 by a division of the genus into two species (*Aphanizomenon flos-aquae* and *Aphanizomenon incurvum*) distinguished by straight vs. curved trichomes (Bornet and Flahault, 1886). The first valid description of *Aphanizomenon flos-aquae* is therefore 1886. Drouet's proposed division of *Aphanizomenon* into *Calothrix* and *Rhaphidiopsis* based on the end cell shape (ovoid and conical vs. cylindrical and hemispherical) of trichomes met with great skepticism (Hindak, 2000), and most botanists now recognize a number of *Aphanizomenon* species based on morphological distinctions developed by Komárek (Komárek and Anagnostidis, 1989).

The history of *Aphanizomenon gracile*'s nomenclature is also quite interesting. The basonym for *Aphanizomenon gracile* is *Anabaena flos-aquae* var. *gracile* Lemmermann 1898, and the synonym is *Aphanizomenon flos-aquae* f. *gracile* (Lemmermann) Elenkin, 1938. Lemmermann moved *Aphanizomenon gracile* from *Anabaena* to *Aphanizomenon* in 1907 and retained the varietal epithet *gracile* as the species epithet. Subsequent work by Elenkin shifted *Aphanizomenon gracile* into *Aphanizomenon flos-aquae* and Komárek later reinstated *Aphanizomenon gracile* by placing it into *Aphanizomenon* Group III with a number of other strains with a solitary trichome habit. It is interesting to note that both *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* have been described historically as *Anabaena flos-aquae*.

Anabaena and *Aphanizomenon* were originally placed in the same family, Nostocaceae, but were moved to different families in the 1950s and 60s (Hindak, 2000). The most recent designation recognized by Komárek returned the genera to the family Nostocaceae, in the subfamily Anabaenoideae (Bornet et Flahaut) Kirchner, 1900 (Komárek and Kovacik, 1989; Geitler, 1930-1932; Huber-Pestalozzi 1938; Komárek & Anagnostidis, 1989; Komárek and Anagnostidis, 1989). The Nostocaceae is characterized by uniseriate, isopolar filaments with heterocysts and akinetes. The various Nostocaceae genera are separated based on trichome symmetry as produced by heterocysts and akinetes, cell shape of vegetative cells, heterocysts and akinetes, and features such as habit (planktonic, motility, etc.). A more detailed discussion of Nostocaceae taxonomy is found in Chapter 2.

Aphanizomenon is distinguished from *Anabaena* by the presence of several morphological features: 1) elongated and narrow terminal cells that are often hyaline (it is recognized that narrowing also often occurs in some *Anabaena* spp.); 2) fascicle-like colonies (this feature is present in the type species, *Aphanizomenon flos-aquae*, but absent in all other species and is known in *Anabaena affinis* as well); 3) trichome structure (subsymmetric structure vs. metameric structure in *Anabaena*); and 4) heterocyst development (based on only one study using one species from each genus that indicated *Aphanizomenon* heterocysts disappear completely at a threshold level of nitrogen in the environment). *Aphanizomenon* is also generally described as having 1-3 heterocysts per trichome that are not symmetric, while *Anabaena* has many heterocysts per trichome that are usually symmetric. *Aphanizomenon* terminal cells are often generally elongated or attenuated although certain *Anabaena* species also have this trait. Komárek also created a genus, *Trichormus*, to separate out all of the apoheterocystic strains previously in *Anabaena*. Unfortunately, for every character listed above, at least one exception exists that forces Komárek to admit that no sharp boundary exists between the two genera. Komárek does claim however that individual species can be distinguished despite the overlap (Komárek and Kovacik, 1989).

Komárek distinguishes four (4) groups of *Aphanizomenon* species: 1) *flos-aquae, flexuosum*-motile fascicle-like aggregates with parallel trichomes; 2) *elenkinii, issatschenkoi, ovalisporum*-solitary, continually narrowing trichome with narrow, elongated and hyaline terminal cells with blunt or sharp ends; 3) *gracile, volzii, minderi-*

solitary free floating filaments rarely joined in small fascicles with facultative gas vacuoles; and 4) *bergii, aphanizomenoides*- solitary trichomes with gas vacuoles and metameric trichomes containing many heterocysts/trichome. In contrast, Komárek recognizes only two groups of *Anabaena* (*Anabaena oscillarioides* group and the *Anabaena flos-aquae* (planktonic) group) while Rippka (2001) recognizes at least three groups. Chapter 2 demonstrates that both Rippka and Komárek have underestimated the diversity within *Anabaena*.

New techniques in molecular analysis now offer the means to ascertain genetic relatedness from single filaments of cyanobacteria and promise to accelerate the pace of identification and resolution of taxonomic issues within the heterocystous cyanobacteria. This is the first study to employ a single filament, single PCR reaction to examine strains of *Anabaena* and *Aphanizomenon* from the field. *Anabaena* and *Aphanizomenon* strains were compared using *cpc*BA-IGS and *rpo*C1 as molecular markers. Both markers have been used to examine cyanobacteria diversity (Bolch, Blackburn, Neilan and Grewe, 1996; Fergusson and Saint, 2000; Neilan, Jacobs and Goodman, 1995; Palenik and Swift, 1996) and represent the largest dataset for comparison of Pacific Northwest, U.S.A. strains to other regional strains. The *cpc*BA-IGS and *rpo*C1 gene markers allow the use of direct PCR from field samples containing bacterial contaminants as no other prokaryotes contain either gene (Bolch et al., 1996; Palenik, 1992). Phylogenetic trees were constructed using parsimony and maximum likelihood analysis to examine the

phylogenetic relationship between *Anabaena* and *Aphanizomenon* and the diversity of *Aphanizomenon* in the Pacific Northwest.

MATERIALS AND METHODS

SAMPLING

Sampling was designed to include a broad representation of *Aphanizomenon* strains from a range of geographic locations available from culture collections and planktonic representatives from the Pacific Northwest. Pacific Northwest *Anabaena* and culture representatives were collected as detailed in Chapter 2. *Aphanizomenon* culture isolates were obtained from the University of Texas Culture Collection (UTEX) (Starr and Zeikus, 1993), the Culture Collection of Algae and Protozoa (CCAP), and Sammlung von Algenkulturen (SAG). All cyanobacteria strains collected and sequenced are listed in Tables 2 & 3 and include Accession numbers for sequences obtained from Genbank as well as any known information on origin and toxicity and any sister and outgroup taxa used in this study.

Culture isolates were grown in either BG-11 or BG-11_0 Medium (Rippka et al., 1979). Field samples were frozen at –20°C for later DNA isolation or placed in growth chambers until used for direct filament PCR. All strains were maintained at 100 micromoles photons·m^{-2}·sec^{-1} on a 16L:8D hour light/dark cycle at 20°C.

MICROSCOPY

Black and white photomicrographs were obtained using a Zeiss compound microscope with a Nikon M-35 1S photomicrographic camera using TMAX 400 film. Color microphotographs were obtained using a Zeiss Photomicroscope II microscope and Kodachrome 64 slide film. Color prints were made using a Nikon slide scanner to digitize slides, and the images were printed with an HP Deskjet 970 printer.

DNA ISOLATION AND SEQUENCING

Two methods were employed in isolating cyanobacteria DNA for PCR: 1) DNA from culture isolates was obtained by centrifuging approximately 0.5 ml log phase culture and processing the pelleted cells. Pellets were either immediately extracted or frozen at -20°C and extracted at a later date. DNA was isolated from fresh or frozen material either according to a modified Instagene method intermediate to that of Bolch et al. (1996) and Neilan et al. (1995) (reagents and washes per Bolch but temperature, time and vortexing per Neilan) or using a xanthogenate-SDS extraction protocol detailed by (Tillett and Neilan, 2000) but incubating all samples for two hours at 60°C. 2) Approximately 20 μl of field-collected material were pipetted onto a depression slide and examined at 40x under a dissecting microscope. The longest intact individual filaments representing one phenotype were pipetted (Eppendorf ultramicropipetter) into a second depression slide containing 100 μl of BG-11 medium and this washing procedure was repeated 5 times. Single filaments were pipetted in a 1 μl liquid volume into 0.6 ml eppendorf tubes and frozen for later PCR or placed on ice until addition of PCR reaction

mix. DNA from culture isolates was examined by gel analysis and quantitated with approximately 5 ng used for each 25 μl PCR reaction.

PCR reaction conditions were optimized for each molecular marker primer set and for each type of DNA amplified (whole cell direct filament PCR vs. extracted DNA from culture isolates). Double stranded PCR reaction mix for *cpc*BA-IGS and *rpo*C1 of extracted DNA consisted of 10 mM Tris-HCl, pH. 9.2, 25 mM KCl, 3.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 μg BSA, 0.0125 units of Taq polymerase, 0.1 mM of each primer, and 1 μl total DNA template either undiluted or diluted up to 1:100 in a 25 μl reaction volume. PCR reaction mix for direct filament PCR consisted of 10 mM Tris-HCl, pH 8.8, 25mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 9.4 μg BSA, 0.0125 units Taq polymerase, 0.4 mM each primer. 3.75 μl glycerol was substituted in some reactions for BSA in direct filament PCR, and concentration of primers was doubled in some reactions to remedy DNA derivitization problems. PCR reaction mix was added to the 1 μl filament mix and immediately frozen or amplified. A PTC-100 Programmable Thermal Controller (MJ Research, Inc.) was used for PCR amplification.

Primers used for *cpc*BA-IGS amplification and sequencing are described in (Bolch et al., 1996) (forward *cpc*βF: 5'-GGCTGCTTGTTTACGCGACA-3' and reverse *cpc*αR: 5'-CCAGTACCACCAGCAACTAA-3'). This primer set amplified a ~610 bp fragment that included the 3' end of the β subunit of phycocyanin, an approximate 100 bp ITS region, and the 5' end of α subunit of phycocyanin. Primers used for *rpo*C1 PCR amplification are described in (Palenik, 1994) (forward C: 5'-

GAGCTCYAWNACCATCCAYTCNGG -3' and reverse N: 5'-GGTACCNAAYGGNSARRTNGTTGG –3') and yielded a ~623 bp fragment.

The reaction profile for *cpc*BA-IGS amplification of filaments was: 1) an initial denaturation at 96°C for 4 minutes followed by; 2) 94°C for one minute; 48°C for one minute; 72°C for two minutes for 38 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for *cpc*BA-IGS amplification of extracted DNA was: 1) an initial denaturation at 96°C for 4 minutes followed by; 2) 94°C for one minute; 52°C for one minute; 72°C for two minutes for 35 cycles followed by; 3) final extension at 72°C for ten minutes. The reaction profile for the *rpo*C1 region amplification was the following: 1) an initial denaturation at 96°C for three minutes followed by; 2) 94°C for one minute; 50°C for one minute; 72°C for two minutes for 35 cycles followed by; 3) final extension at 72°C for 5 minutes.

Double stranded PCR products were cleaned using Qiaquick PCR Purification Kit (Qiagen Inc., Chatworth, CA) or electrophoresed in a 1.5% agarose gel (SeaKem, FMC), stained with ethidium bromide, and the appropriate sized bands were cut and cleaned using Quiquick Gel Purification or Quiex II (Quigen Inc., Chatworth. CA) and quantified by spectrophotometry. All *rpo*C1 PCR products and PCR products from samples that provided poor data from direct sequencing or samples suspected to contain more than one strain were purified and ligated into the cloning vector pGEM-T Easy (Promega). Transformation was performed using the *E. coli* JM109 strain. The recombinant plasmids were verified by restriction digestion followed by agarose electrophoresis. A minimum

of 4 clones from each sample was sequenced using the T7 and SP6 primers provided by Promega. Direct PCR sequencing was accomplished with the same primers as used in the PCR reaction. DNA was sequenced using the cycle sequencing method for both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Foster City, CA) and analyzed on an ABI 377 automated sequencer. DNA sequences were assembled using Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI).

DATA ANALYSIS

Sequence alignment for *cpc*BA-IGS and *rpo*C1 was performed using ClustalX (Thompson et al., 1997) and subsequently adjusted manually to correct errors and to align difficult portions of the intergenic spacer region in *cpc*BA-IGS. Due to the number of insertions and deletions in the intergenic region of *cpc*BA and the difficulties in aligning large data matrices, *cpc*BA-IGS sequence alignment was performed in several steps. First, taxa within *Anabaena/Aphanizomenon* were aligned, and this alignment was adjusted manually. Regions of the intergenic spacer in *cpc*BA-IGS contained numerous insertions and deletions that made confident alignment with the outgroup taxa impossible and the unalignable IGS region of the outgroup taxa, *Calothrix* and *Spirulina,* was therefore removed and substituted with a "missing" designation and aligned. A new Clustal X alignment was created by combining the *Anabaena/Aphanizomenon* Clustal X alignment with the outgroup taxa alignment, and this combined alignment was then adjusted manually to represent the best estimate of the positional homology. A second

alignment of only *Anabaena* Group II taxa (See Chapter 2) was constructed to examine the relationships of Group IIA taxa, and this alignment used the entire *cpc*BA-IGS region.

A *cpc*BA amino acid alignment was also created by identifying the open reading frame of the Beta and Alpha subunits of *cpc* and translating the partial sequence of these two subunits into their respective amino acids. The amino acid sequences of *cpc*B and *cpc*A were then combined for each strain, and the strains were aligned using Clustal X and adjusted manually.

tRASA was performed on the aligned sequences to determine phylogenetic signal (Lyons-Weiler, 2001). Outgroups were identified and tested for suitability in the *cpc*BA-IGS and *rpo*C1 analyses. Recent work by Lyons-Weiler has demonstrated that inappropriate outgroup usage can degrade phylogenetic signal and result in inaccurate tree topology (Lyons-Weiler and Hoelzer, 1997). A negative tRASA is one of the symptoms of long branch attraction (Lyons-Weiler, Hoelzer and Tausch, 1996), and declining power curves and high taxon variance ratios are also evidence of problematic taxa (Lyons-Weiler and Hoelzer, 1997). Taxa violating these criteria were removed from the alignments.

The *cpc*BA-IGS and *rpo*C1 data were analyzed using PAUP* (version 4.0b8)(Swofford, 1999). Both parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981) analyses were conducted. Regions of ambiguous alignment were

excluded from analyses. Gaps were introduced in the alignment in order to optimize positional homology (submitted to EMBL).

One thousand heuristic searches with random sequence addition were conducted with all characters weighted equally with TBR swapping and MULPARS in effect for parsimony analyses. Five hundred bootstrap replications were conducted to assess relative group support (Felsenstein, 1985), and each bootstrap replicate used ten heuristic searches with random sequence addition.

For maximum likelihood analyses, a substitution model where all base transformations are equally likely and a discrete gamma distribution with three rate classes to account for rate heterogeneity were used. Ten heuristic searches with random sequence addition were conducted with MULPARS turned off (DeBry and Olmstead, 2000). The tree with the maximum likelihood was retained to represent the best estimate of phylogenetic patterns. One hundred bootstrap replications were conducted under maximum likelihood criteria to assess relative group support.

RESULTS

Only one Pacific Northwest *Aphanizomenon* morphotype/genotype was found in contrast to the several morphotypes and genotypes of planktonic *Anabaena* found in the Pacific Northwest. Lake Terrell, Horseshoe Lake and Green Lake all contained the typical fascicle *Aphanizomenon flos-aquae* morphotype that could often be seen by the naked eye (Fig. 25A), and all three samples were identical in *cpc*BA-IGS genotype. *Aphanizomenon flos-aquae* was abundant from spring through autumn in lowland Puget

Sound lakes often as monotypic blooms but also as a common representative in mixed blooms composed of other common cyanobacteria genera such as *Anabaena, Microcystis, Coelosphaerium,* and *Gloeotrichia.*

Vegetative cells of the Pacific Northwest *Aphanizomenon flos-aquae* morphotype/genotype were gas vacuolate, cylindrical shaped, approximately 5-6.6 μm in width by 6-11.5 μm in length with cylindrical shaped heterocysts slightly larger than vegetative cells and long cylindrical akinetes approximately 7-10 μm wide and 50-75 μm long. Straight trichomes were found in fascicles, a condition which is representative of the common species *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault (1886) (Figs. 26a and b).

The morphology of some of the *Aphanizomenon* strains from culture collections appeared inconsistent with their description in the literature. Komárek (1999) warns that up to 50% of culture collection isolates have been contaminated or misidentified. One example is *Aphanizomenon flos-aquae* NH5 which clearly has a morphology associated with *Aphanizomenon gracile* (Fig. 25b). Most of the cultures did not contain heterocysts or akinetes, which are necessary to apply the current *Aphanizomenon* morphological taxonomy. It is also common for cyanobacteria morphology to vary considerably from their field habit under culture conditions. Figure 27 shows the morphology of *Anabaena flos-aquae* (AL89) in the field and in culture. Reference data are often difficult to obtain because culture collections typically do not describe the original characteristics of deposited strains other than by assigning a species name that is meant to confer certain

morphological attributes. The lack of deposit descriptions makes comparisons with field material difficult.

Aphanizomenon flos-aquae NH5 is strictly solitary in habit rather than found in fascicles of trichomes as described for the type strain (Fig. 25b). NH5 cells also showed greater constriction and smaller cell size than the type *A. flos-aquae* consistent with the description of *Aphanizomenon gracile* rather than that described for *A. flos-aquae*. *A. flos-aquae* (CCAP 1401/1) was originally deposited as *A. gracile* at CCAP and changed to *A. flos-aquae*. The CCAP 1401/1 strain, however, is also solitary and fits the description of *A. gracile*. The Pacific Northwest *A. flos-aquae* strain and *A. flos-aquae* (UTEX 2384) were nearly identical in habit in culture and formed fascicles of trichomes. The *A. flos-aquae cpc*BA-IGS sequences obtained from Genbank are described as coming from fascicle colonies (Barker et al., 2000) whereas the *rpo*C1 *Aphanizomenon* sequences obtained from Genbank are not described except for that of *Aphanizomenon ovalisporum*, whose sequence is identical to *Anabaena bergii* but clusters in the *Nostoc/Nodularia* clade (Fergusson and Saint, 2000).

RASA was performed on both the *cpc*BA-IGS and *rpo*C1 alignments to confirm adequate phylogenetic signal (Lyons-Weiler, Hoelzer and Tausch, 1996). The tRASA for the unrooted *cpc*BA-IGS data was 14.241532 with 347 degrees of freedom. The tRASA for *cpc*BA-IGS rooting with the outgroup taxa, *Calothrix* and *Spirulina*, was 15.558668 with 402 degrees of freedom. The tRASA for the unrooted *rpo*C1 data was 11.570422 with 662 degrees of freedom. The tRASA for *rpo*C1 rooting with the outgroup taxon,

Gloeobacter violaceus, was 23.053431 with 626 degrees of freedom. The tRASA values for the *rpo*C1 and *cpc*BA datasets contained sufficient phylogenetic signal for the analysis. Rooting improved phylogenetic signal, but all of the alignments contained sufficient phylogenetic signal under the tRASA test.

Sequences from 26 taxa in the *cpc*BA-IGS study, 52 taxa in the *cpc*BA amino acid study and 41 taxa in the *rpo*C1 study (Table 3) provided a total aligned length of 381 bp for *cpc*BA-IGS, 215 amino acids for *cpc*BA and 584 bp for *rpo*C1 (Submitted to EMBL). Nine gaps ranging from 1 to 22 bases long were introduced into the *cpc*BA-IGS *Anabaena* alignment to account for unique sequences found in the IGS region. One parsimony informative gap was included in the *cpc*BA-IGS analysis as a two-state character. Three gaps (two single gaps and one two amino acid gap) were introduced into the *cpc*BA amino acid alignment and treated as missing. Five gaps, ranging from 1 to 4 bases long, were introduced into the *rpo*C1 alignment and treated as missing.

In the *cpc*BA-IGS dataset, 149 sites were variable, of which 89 sites were parsimony informative. Parsimony analyses resulted in 36 most parsimonious trees of 245 steps (CI=0.792, RI=0.851, HI=0.208 for informative characters). The Maximum Parsimony (MP) Strict Consensus of the 36 trees is shown in Fig. 28. Maximum likelihood analyses yielded four trees of Maximum Likelihood (ML) (-lnL= 1666.3899) (Fig. 29). The ML Strict Consensus tree is shown in Fig. 30. The overall topology of the ML Strict Consensus tree (Fig. 30) is similar to that of the MP Strict Consensus tree (Fig. 28). MP Tree #1 of 36 trees is included for reference to the distance between strains (Fig.

31). Differences between the ML tree and the MP strict consensus tree are restricted to resolution of taxa at the tips of the trees' topologies and in areas with weak bootstrap support and short branch lengths.

Both the MP and ML Strict Consensus trees support a clade of mixed *Anabaena flos-aquae* and *Aphanizomenon* strains designated Group IIA and a clade of *Anabaena circinalis* designated Group IIB separated from the *Calothrix/Spirulina* outgroup with high bootstrap support. Moderate bootstrap support confirms a division of Group IIA into at least two clades. The first clade contains two Pacific Northwest toxic strains of *Anabaena flos-aquae* (AL89 and Black Lake), an *Aphanizomenon gracile* strain (SAG 31.79), and two *Aphanizomenon* strains (CCAP 1401/1 and NH5). The second clade contains several non-toxic *Anabaena flos-aquae* (AL97, AL93, Green Lake, Black Lake) strains and strains of *Aphanizomenon flos-aquae* from the Iowa (Norman, Crooked and Little Crooked Lakes), Canada (UTEX 2384), the Baltic Sea and the Pacific Northwest (Green Lake). These two Group IIA clades are consistent with *Anabaena* Group IIA in Chapter 2.

The ML strict consensus tree appears to have slightly higher resolution than the MP tree and separates *Anabaena flos-aquae* AL89 (anatoxin producer) and *Aphanizomenon* NH5 (saxitoxin producer) from *Aphanizomenon flos-aquae* strains SAG and CCAP 1401/1 as well as the toxic *Anabaena flos-aquae* from Black Lake, Idaho, with low to moderate bootstrap support. The MP strict consensus tree has a polytomy at this node. Both the MP and ML strict consensus trees also show a polytomy within the

second clade within Group IIA. The MP and ML analyses cluster one of the non-toxic Pacific Northwest *Anabaena flos-aquae* genotypes (AL97) with a Baltic Sea *Aphanizomenon flos-aquae* and an *Aphanizomenon flos-aquae* strain (Norman Lake) and also support a second cluster of North American *Aphanizomenon flos-aquae* strains although the ML tree places an *Anabaena flos-aquae* (Black Lake) strain in the second cluster with very low bootstrap support. Two culture collection *Anabaena flos-aquae* strains (UTEX 2391 and CCAP 1403/13D) are unresolved within Group IIA. Group IIB is sister to Group IIA in both MP and ML trees and contains numerous *Anabaena circinalis* strains from Australia, Europe and the Pacific Northwest. This is consistent with the findings in Chapter 2.

In the *cpc*BA amino acid data set, 215 amino acids were aligned, of which, 49 sites were parsimony informative. Parsimony analyses resulted in 15 most parsimonious trees of 438 steps (CI=0.723, RI=0.883, HI=0.277 for informative characters). The MP Strict Consensus of the 41 trees is shown in Fig. 32. The MP Strict Consensus tree's *Anabaena* clade made up of the three Groups identified in Chapter 2 (Group I, Group IIA, and Group IIB) is highly supported. All of the *Aphanizomenon* strains cluster within Group IIA with *Anabaena flos-aquae*. The *Anabaena circinalis* cluster, Group IIB, remains sister to Group IIA and the Group I *Anabaena* strains are represented by two branches of strains that are unresolved at the node that separates *Anabaena* from other genera. *Nodularia*, *Nostoc*, a *Fischerella* strain (sequenced in this study and also

available in Genbank) and several misclassified *Anabaena* strains are found in a second major Nostoacalean clade consistent with the results in Chapter 2.

After confirming the relationship between Group IIA and Group IIB taxa in the *cpc*BA-IGS dataset, a *cpc*BA-IGS tree for Group IIA was tested using two *Anabaena circinalis* strains from Group IIB as an outgroup. In this data set, 253 base pairs were aligned, of which, 39 sites were parsimony informative. Parsimony analysis resulted in 2 most parsimonious trees of 63 steps (CI=0.841, RI=0.905, HI=0.159 for informative characters). The MP Strict Consensus of the 2 trees is shown in in Fig. 33. Maximum likelihood analyses yielded one tree of Maximum Likelihood (-lnL= 705.65368) (Fig. 34). The overall topology of the ML tree is similar to that of the MP Strict Consensus tree and MP tree number 1 (Fig. 35). Differences between the ML tree and the MP trees are restricted to resolution of taxa at the tips of the trees' topologies and in areas with weak bootstrap support and short branch lengths.

All of the *cpc*BA-IGS trees strongly support 4 genotypic clusters within Group IIA with one cluster represented by a single strain (*Anabaena flos-aquae*-Black Lake August). The first clade contains *Aphanizomenon flos-aquae* strains from the Baltic Sea and Indiana with three strains of *Anabaena flos-aquae* (AL97, UTEX 2391 and CCAP 1403/D). Sister to this cluster is a clade containing the two toxic Pacific Northwest *Anabaena flos-aquae* strains and three solitary habit *Aphanizomenon* strains. The lone taxon (*Anabaena flos-aquae* Black Lake August) sits between the two clusters described

above and the final cluster that contains 4 *Aphanizomenon flos-aquae* strains from North America.

In the *rpo*C1 data set, 351 sites were variable, of which, 299 sites were parsimony informative. Parsimony analyses resulted in 1 most parsimonious tree of 2060 steps (CI=0.315, RI=0.582, HI=0.685 for informative characters). The MP tree is shown in Fig. 36. Maximum likelihood analyses yielded one tree of maximum likelihood (-lnL= 7476.10474). The overall topology and bootstrap support of the ML tree (Fig. 37) is similar to that of the MP tree. Differences between the ML tree and the MP tree are restricted to areas with weak bootstrap support and short branch lengths.

Both the ML and MP *rpo*C1 trees highly support an *Anabaena* Group IIA that includes Pacific Northwest *Anabaena flos-aquae* strains, the European *Anabaena flos-aquae* strain and all the *Aphanizomenon* strains sequenced in this study. Moderate bootstrap support places *Aphanizomenon gracile* (SAG 31.79) closest to *Anabaena flos-aquae* (AL89) and *Aphanizomenon flos-aquae* (NH5) closest to *Aphanizomenon flos-aquae* (UTEX 2384). Two other *Anabaena flos-aquae* strains are sister to the mixed *Aphanizomenon/Anabaena* pairings and Group IIB is sister in the MP and ML trees to Group IIA with high bootstrap support. Group IIB contains all of the *Anabaena circinalis* strains as well as three Australian strains designated *A. spiroides, A. solitaria* and *A. pertubarta* and this relationship is discussed in detail in Chapter 2.

Anabaena Group I from the *cpc*BA-IGS analysis was represented by only one strain, *A. variabilis* (UTEX 377), in the *rpo*C1 trees and clusters with weak support in a

mixed clade containing *Cylindrospermopsis raciborskii*, *Aphanizomenon gracile* and other Australian *Anabaena flos-aquae* strains. All of the strains in this clade except for UTEX 377 were sequenced in another study that did not describe strain morphology (Fergusson and Saint, 2000). The strains may or may not represent the species designated and are from the Southern Hemisphere. The low bootstrap support for this clade is primarily due to the movement of UTEX 377 and *Cylindrospermopsis* in and out of the clade into a separate cluster or into a clade containing other heterocystous genera. Removal of UTEX 377 and *Cylindrospermopsis* from the analysis places the remaining members of this clade sister to Group II with much higher support (80%) but did not place them within Group II (data not shown).

The ML and MP trees place the remaining sequences from heterocystous strains just outside the Group I *Anabaena* lineage with low bootstrap support that is also indicative of the uncertainty associated with the UTEX 377 and *Cylindrospermopsis* strains. This clade includes members of *Nostoc, Nodularia, Anabaenops*is and *Fischerella* with low internal bootstrap support placing *Anabaena bergii* with *Nodularia*, UTEX 2557 with *Nostoc* and *Anabaenopsis* with *Fischerella.* The heterocystous cyanobacteria are separated from the non-heterocystous cyanobacteria with moderate support with a clade containing *Dermocarpa*, *Prochlorococcus* and a Cluster I *Synechococcus* as the closest relatives from the strains examined and *Gloeobacter violaceous* as the most distant taxon.

DISCUSSION

The phylogenetic evidence from this study indicates that the morphotypes commonly recognized as *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* are phylogenetically within the genus *Anabaena*. A well-defined and highly supported group of planktonic *Anabaena flos-aquae* and *Aphanizomenon* strains was found in both the *cpc*BA-IGS and *rpo*C1 analyses (Figs. 28-37). The *cpc*BA amino acid tree (Fig. 32) also clearly separates a large *Anabaena* clade from a *Nostoc/Nodularia* clade with high bootstrap support. The *Anabaena flos-aquae/Aphanizomenon* cluster (Group IIA) is found within the *Anabaena* clade sister to a cluster of *Anabaena circinalis* strains (Group IIB) that together compose an exclusively planktonic clade we call *Anabaena* Group II. Group II is sister to *Anabaena* Group I in the *cpc*BA trees and the entire *Anabaena* clade containing Groups I & II is clearly distinct from the other Nostocalean genera. The relationship between *Anabaena* Groups I & II is discussed in greater detail in Chapter 2 and the inclusion of the *Aphanizomenon* strains appears to have had little or no effect on the topology of the *cpc*BA-IGS or *rpo*C1 trees from Chapter 2.

Our placement of *Aphanizomenon* within the genus *Anabaena* is consistent with the findings of previous phylogenetic studies that questioned whether the current division of *Anabaena* and *Aphanizomenon* is merited (Wilmotte and Herdman, 2001). Unfortunately, these studies were unable to answer this question because they either examined too few taxa from the Nostocales or the available taxa were misidentified and thus produced no conclusive findings regarding the *Aphanizomenon/Anabaena* question.

A recent 16S rRNA-ITS RFLP study placed *Aphanizomenon gracile* sister to 3 *Anabaena* strains in a clade that created a paraphyly between two *Nostoc* clades (Lu et al., 1997). Chapter 2 discusses the current confusion in assigning strains to the genera *Nostoc* and *Anabaena*, and it is unclear whether Lu correctly identified the strains in that study as the strains were not described. Lu's *Aphanizomenon/Anabaena* clade only contains planktonic strains, which is consistent with our planktonic *Anabaena* Group II designation.

A 3 hydroxy fatty acid composition chemotaxonomy study by Li (1998) showed *Aphanizomenon flos-aquae* f. *gracile* clustering tightly with *Anabaena flos-aquae* CCAP 1403/B and these two strains were members of a clade that included *Anabaena affinis, Anabaena solitaria, Anabaena ucrainica, Anabaena crassa* and *Anabaena smithii*. Our *cpc*BA-IGS data also indicate that *Aphanizomenon* is closely related to *Anabaena flos-aquae* CCAP 1403/D (a close relative of CCAP 1403/13B). The *rpo*C1 phylogeny is consistent with the *cpc*BA-IGS tree in that it places our *Aphanizomenon* strains and *Anabaena flos-aquae* CCAP 1403/13B in *Anabaena* Group IIA. Li's study did not contain any of the other Nostocalean genera such as *Nostoc* and *Nodularia* and therefore was unable to assess the phylogenetic placement of *Aphanizomenon* with respect to any Nostocalean genera.

A recent 16S rRNA RFLP phylogeny of planktonic cyanobacteria placed a *Nodularia/Nostoc* clade sister to an *Anabaena* clade that contained several strains of *Aphanizomenon* (with nearly identical RFLP patterns for *Aphanizomenon gracile* and

Aphanizomenon flos-aquae) (Lyra et al., 2001). As in our *rpo*C1 and *cpc*BA-IGS trees, their strains of *Anabaena circinalis* formed a sister clade to a clade containing *Anabaena flos-aquae* strains, but unlike our study this clade also contained a variety of other Group I *Anabaena* strains.

Lyra's 16S rRNA tree offers a lower resolution phylogenetic view showing an *Anabaena* clade containing *Aphanizomenon,* but *Anabaena cylindrica* resides on a branch of the 16 S rRNA tree outside both the *Anabaena* and *Nostoc/Nodularia* clades. This strain may simply be misidentified as an *Anabaena* strain as we also experienced a similar tree placement outside of the *Anabaena* and *Nostoc/Nodularia* clades with *A. cylindrica* (UTEX 1611) but removed the strain from our analysis because it failed the variance test under RASA. Lyra's REP/ERIC PCR tree also supports our *cpc*BA-IGS and *rpo*C1 analyses as 13 of 16 *Anabaena* strains were members of one large *Anbaena* clade that included 6 strains of *Aphanizomenon.* An internal clade comparable with our *Anabaena* Group I was identified, and all of the *Aphanizomenon* strains except one were located deep within several clusters of *Anabaena.* Three of the *Anabaen*a strains were found in a *Nostoc* clade, and it is likely that the errant *Anabaena* strains in the Lyra study were misidentified or are missing the morphological characteristics necessary for proper generic assignment (motility, gas vesicles) due to mutation or morphological anomalies from culturing as discussed in Chapter 2.

Fergusson's (2000) *rpo*C1 study included one Australian strain of *Aphanizomenon gracile* that clustered with two Australian *Anabaena flos-aquae* strains

within the larger *Anabaena* clade. The expanded *rpo*C1 analysis of this study places the Fergusson *Anabaena flos-aquae* and *Aphanizomenon* strains in a clade sister to our *Anabaena* Group II (*Anabaena flos-aquae, Aphanizomenon* and *Anabaena circinalis*) that clearly creates a larger *Anabaena* clade as opposed to an expanded *Nostoc/Nodularia* clade. Additional *Anabaena flos-aquae* from the Southern Hemisphere and descriptions of Fergusson's Australian strains may resolve the *Anabaena* Group II differences between our *Anabaena flos-aquae* strains and Fergusson's *Anabaena flos-aquae* strains. Misidentification of *Anabaena flos-aquae* strains is more common than previously thought as evidenced by the results of Chapter 2 where *Anabaena flos-aquae* UTEX 2557 and *Anabaena flos-aquae* UTEX 2558 were clearly found to be members of the genus *Nostoc*, and it would not be surprising to find that either the Fergusson strains were misidentified or that they represent a unique Australian or Southern Hemisphere lineage of *Anabaena* whose morphology has converged on that of the type *Anabena flos-aquae* from the Northern Hemisphere.

The low bootstrap support in the *rpo*C1 tree associated with *Anabaena variabilis* (UTEX 377) and *Cylindrospermopsis raciborskii* makes interpretation of the phylogenetic relationships where the *Anabaena* and *Nostoc/Nodularia* clades diverge difficult. This uncertainty was also found in a 16S rRNA analysis (Lyra et al., 2001). It is important to note that in all *rpo*C1 analyses (Maximum Parsimony, Maximum Likelihood, Neighbor Joining (not shown), and UPGMA (not shown)) the *Aphanizomenon* and *Anabaena* strains except for *Anabaena bergii/Aphanizomenon*

ovalisporum and *Anbaena flos aquae* (UTEX 2557) form a monophyletic group with the exception of the inclusion of *Cylindrospermopsis*. *Anabaena bergii* is actually synonymous with *Aphanizomenon ovalisporum* in 16S rRNA and *rpo*C1 sequence and will likely be reclassified (Fergusson and Saint, 2000). UTEX 2557's placement in *Nostoc* is discussed in Chapter 2. *Anabaena variabilis* (UTEX 377) as our only representative of *Anabaena* Group I from the *cpc*BA tree may resolve with greater support in the *rpo*C1 tree with the addition of other Group I strains. A16S rRNA tree with a limited set of taxa indicates that *Cylindrospermopsis raciborskii* is external to the *Anabaena* and *Nostoc/Nodularia* clades (Li et al., 2000a) while Fergusson's and our *rpo*C1 trees suggest that it is more likely within the *Anabaena* and *Nostoc/Nodularia* clades.

The lower bootstrap values of the *rpo*C1 phylogenetic studies at the *Anabaena/Nostoc* interface (Figs. 36-37) may be the result of a data set with an insufficient number of taxa in key positions (e.g., *Anabaena* Group I strains, *Cylindrospermopsis*, *Anabaenopsis*, *Fischerella, Nodularia*) to resolve the relationship between these two clusters of heterocystous cyanobacteria. Other possible explanations include horizontal transfer of genetic information that may confuse or incorrectly identify the relationship between taxa (Rudi, Skulberg and Jakobsen, 1998), multiple copies of *rpo*C1, or this node may represent a point of very rapid radiation between the genera with very little concomitant divergence in *rpo*C1 sequence. The overall consistency in

topology between the *cpc*BA and *rpo*C1 trees, however, argues against horizontal gene transfer as an explanation for the lack of resolution at this node.

Additional taxa, molecular markers including 16S rRNA sequence, and DNA-DNA hybridization as recommended under the International Code of Nomenclature of Bacteria (ICNB) will need to be examined in order to conclusively resolve the relationship between *Anabaena* and the *Nostoc/Nodularia* clade and the many other genera that appear to cluster at this interface. While *Anabaena* Group II is clearly separated from other genera in all the phylogenetic studies and the exact relationship of *Anabaena* Group I to the *Nostoc/Nodularia* clade is clear in the *cpc*BA tree, the interface between these genera is not clear in the *rpo*C1 tree perhaps because it only contains one *Anabaena* Group I strain. While well-supported clades exist in both the *rpo*C1 and *cpc*BA-IGS trees, the traditional morphological criteria used to delimit these taxa at the generic level is clearly invalid and new characters mapping phylogeny are not apparent at this time.

The relationship between strains within Group IIA differs slightly between the *rpo*C1 tree and the *cpc*BA-IGS trees, but it should be noted that the number of Group IIA taxa is much smaller in the *rpo*C1 tree than the *cpc*BA-IGS tree, and this may account for any discrepancy. The *cpc*BA-IGS trees (Figs. 33-35) contain 4 branches of genotypic clusters: Group IIA(4) includes only North American *Aphanizomenon flos-aquae* with the fascicle habit while Group IIA(2) is restricted to *Aphanizomenon* strains with morphology and a solitary habit consistent with the species *Aphanizomenon gracile*, and

also includes the toxic Pacific Northwest strains of *Anabaena flos-aquae*. This branch contains the only known toxin producers in Group IIA. Group IIA(1) contains 3 *Anabaena flos-aquae* strains and *Aphanizomenon flos-aquae* strains from the BalticSea and Indiana. This confirms Barker's finding that at least 2 *Aphanizomenon flos-aquae* genotypes can be found in North America (Barker et al., 2000), but interestingly the closest relative to the Baltic and Norman Lake, Indiana *Aphanizomenon flos-aquae* strains is a Pacific Northwest strain of *Anabaena flos-aquae* (AL97) followed by two other *Anabaena flos-aquae* strains (UTEX 2391 and CCAP 1403/13D). The mixing of *Anabaena* and *Aphanizomenon* morphotypes across all of the clusters except for Group IIA-4 confirms the lack of utility of the current morphology based distinctions between these genera.

Recent phylogenetic work on morphotypes of strains designated *Aphanizomenon* has shed some light on the genetic relationships between *Aphanizomenon* strains. Li (2000) used 16S rRNA to show that the saxitoxin producing *Aphanizomenon flos-aquae* NH5 was not genotypically within the *Aphanizomenon flos-aquae* cluster of strains. An *Aphanizomenon flos-aquae* from Klamath Lake, Oregon clustered with other *Aphanizomenon* strains with the fascicle habit from India, China, and Japan. The Klamath strain is also likely closely related if not identical to our Pacific Northwest *Aphanizomenon flos-aquae* strain. We were unable to PCR DNA from the Klamath Lake strain (likely due to DOC inhibition) to compare its *cpc*BA-IGS sequence with our

Pacific Northwest strains. It should be noted that the morphology and habit of the Klamath strain is identical to our Green Lake *Aphanizomenon flos-aquae* strain.

Li (2000) found that two *Aphanizomenon* strains (an *Aphanizomenon gracile* and *Aphanizomenon* NH5) formed a monophyletic clade sister to the *Aphanizomenon flos-aquae* cluster and that an *Aphanizomenon issatchenkoi* strain was the most distant genotype of the *Aphanizomenon* strains sampled. A 94.3% similarity in 16S rRNA sequence was found across the *Aphanizomenon* strains indicating that *Aphanizomenon issatchenkoi* is likely a different species from *Aphanizomenon flos-aquae*. Similarity below 97.5% in 16S rRNA sequence clearly indicates that a species delimitation is merited (Stackebrandt and Goebel, 1994); however, DNA-DNA hybridization is usually performed to confirm that the 16S rRNA data is consistent with the species designation (Lachance, 1981). The two strains, *Aphanizomenon gracile* and *Aphanizomenon* NH5, were 97.7% similar in 16S rRNA sequence and thus may belong in the same species, and the difference between the *Aphanizomenon flos-aquae* strains and *Aphanizomenon gracile* stains is borderline (96.4-97.6%) and bacteriologists would accept more than one species provided that both the DNA-DNA hybridization data and phenotypes supported this delineation.

In comparison, *cpc*BA-IGS sequence should have greater phylogenetic resolution than 16S rRNA because the *cpc* noncoding IGS region is not subject to the evolutionary constraints of a gene coding region. This study's *Aphanizomenon* strains shared 92-93% similarity in *cpcBA*-IGS sequence between the *Aphanizomenon gracile* containing Group

IIA(2) and the North American *Aphanizomenon flos-aquae* Group IIA(4). *Aphanizomenon flos-aquae* strains from Groups IIA(1) and IIA(4), shared 93.4-95.6% similarity and Groups IIA(1) and IIA(2) shared 93.1-94.4% similarity. The lone strain in Group IIA(3) (*Anabaena flos-aquae*) shared 96.5% similarity with Groups IIA(4) and IIA(1), and 94% similarity with Group IIA(3). The similarity of Group IIA to *Anabaena circinalis* Group IIB ranged from 88.4-92.5% in *cpc*BA-IGS sequence and 91-92% in *rpo*C1 sequence.

If we recognize *A. circinalis* as a separate species from *A. flos-aquae* based on our *rpo*C1 and *cpc*BA-IGS data as well as morphology, a reasonable estimate for the dividing line between species in *cpc*BA-IGS and *rpo*C1 sequence is approximately 92% as compared with 16S rRNA at 97.5%. *Anabaena* Group IIA(4) is equivalent to Li's *Aphanizomenon flos-aquae* cluster, and both this study and Li's study sequenced *Aphanizomenon* NH5. The similarity of *rpo*C1 sequences for the entire *Anabaena* Group IIA is 96.5% with the sequence difference between *Aphanizomenon* NH5 and *Aphanizomenon flos-aquae* less than 1%. A 96.4-97.6% 16S rRNA, 92-93% *cpc*BA-IGS, and 99% *rpo*C1 sequence similarity in sequences does not clearly distinguish the Group IIA(2) and IIA(4) strain clusters as separate species, and the higher similarity between Groups IIA(1) and IIA(2) also indicates that there is no clear division within *Anabena* Group IIA to justify breaking any of the strain clusters out from a single species despite the morphological diversity present in the clade. Any attempt to do so would result in polyphyletic clade.

Komárek (1989) recognized 4 groups of *Aphanizomenon* based on filament shape, trichome structure, and terminal cells. Genera in the Nostocaceae were primarily distinguished based on trichome symmetry (Fig. 38). *Aphanizomenon* Group 1 includes strains with fascicle aggregations such as *Aphanizomenon flos-aquae* while Group 2 has narrow and elongated end cells. Group 3 *Aphanizomenon* strains have narrow end cells and subsymmetric trichomes with 1-3 heterocysts and Group 4 strains have narrow terminal cells but metameric trichomes similar to those of *Anabaena*. Li (2000) found that the 16S rRNA data supported this four-group system although in our view there only appear to be three clusters of genotypes. *Aphanizomenon* NH5 was placed in Group 4 based on its metameric trichome, akinetes next to heterocysts, and solitary habit. A subsymmetric strain of *Aphanizomenon gracile* was placed in Group 3 although it clustered tightly with NH5 in 16S rRNA sequence, and therefore we would lump Groups 3 and 4 phylogenetically. The most distant strain, *Aphanizomenon issatchenkoi* has solitary sharp ends and was placed by Li in Group 2 and designated a separate species from *Aphanizomenon flos-aquae* because its 16S rRNA similarity of 96.5% to the other *Aphanizomenon* strains was less than the 97.5% threshold typically applied by bacteriologists in separating species (Stackebrandt and Goebel, 1994).

We were unable to determine *Aphanizomenon issatchenkoi*'s phylogenetic position, as we had no samples from that strain. It may represent an expansion of Group IIA or another separate *Anabaena* clade. Unfortunately, Li's study contained no planktonic *Anabaena* strains representative of *Anabaena* Groups I or II as he used

Anabaena PCC 7120 which has been shown to be a *Nostoc* (See Chapter 2) and an *A. cylindrica* strain that clustered with *Nodularia* suggesting that the problematic *Anabaena cylindrica* PCC 7122 strain was used in the Li study. The Li study therefore contains no "good" *Anabaena* strains, and he was not able to place the *Aphanizomeon* strains in a greater phylogenetic context.

The 99.9% similarity in 16S rRNA sequence shown by Lyra (2001) between *Aphanizomenon* and several planktonic *Anabaena* strains strongly supports our *cpc*BA-IGS and *rpo*C1 findings that *Aphanizomenon* be subsumed into *Anabaena* in Group IIA as *Anabaena flos-aquae*. Lyra was unable to draw any conclusions based solely on one molecular marker and recommended DNA-DNA hybridization studies. While DNA-DNA hybridization studies are certainly merited to confirm these relationships, it is now apparent that several independent molecular markers and the 16S rRNA data unequivocally place *Aphanizomenon* in the genus *Anabaena*.

Ecological observations also hint at the close relationship between *Aphanizomenon* and *Anabaena flos-aquae* strains. Baker (1981) observed a continuum in morphological variation as measured by cell size and shape in five *Aphanizomenon* populations observed in Minnesota across the growing season and in diverse habitats. He concluded that only one species existed. Some filaments had multiple heterocysts and akinetes consistent with *Anabaena* morphology and some filaments contained both the typical cylindrical *Aphanizomenon* cells as well as *Anabaena*-like spherical cells. Baker (1981) also found that heterocyst and akinete size ranges were variable within a clonal

culture but that cell shape remained consistent from the field into culture. The percent of end cells also varied seasonally between and within populations. These observations are consistent with the morphological variability found in *Anabaena* Group IIA strains and suggest that populations (clusters of strains) are distinct yet overlap each other. Baker did not find a bimodal curve for cell size for *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* as indicated by Komárek and this question should be investigated further as it might support a subspecies designation for strains in Group II.

Aphanizomenon is found in the temperate regions of both hemispheres and occasionally in the tropics (Hindak, 2000). Temperature tolerance varies between populations as Baker (1981) found the highest growth rates at 10-18°C, but a tolerance range of 4-28°C for *Aphanizomenon* in one region and 6-11°C in another region in his study. Baker's results indicate the presence of multiple strains, but this study did not sample *Aphanizomenon* from water temperatures below 14°C, and it is therefore possible that additional *Aphanizomenon* strain diversity may be present in the Pacific Northwest.

At this time it is proposed that all of the *Anabaena* Group IIA strains be designated *Anabaena flos-aquae* based on *cpc*BA-IGS and *rpoC1* data from this study as well as the 16S rRNA data of Lyra (2001). A monotypic Group IIA is the only monophyletic solution given the diversity in morphology and the mixing of *Anabaena* and *Aphanizomenon* strains within the *cpc*BA-IGS Group IIA clade. The lone *Anabaena flos-aquae* strain that composes the 4th clade in *cpc*BA-IGS Group IIA may simply be an undersampled genotypic cluster, but the other 3 strain clusters are clearly supported in the

*cpc*BA-IGS tree. Group IIA is composed entirely of strains from northern temperate regions. Group IIA-4 consists solely of North American strains but this may be the result of inadequate sampling of other regional lakes as Li (2000) found a cluster of *Aphanizomenon flos-aquae* strains from Asia and North America. Groups IIA(1) and (2) include European and North American strains.

Under the ICBN, *Anabaena* is a conserved genus (Greuter et al., 2000) and therefore has priority over *Aphanizomenon*. The specific epithet *flos-aquae* is shared by both *Aphanizomenon* and *Anabaena* and was first validly published for both genera in Bornet and Flahault's 1886 monograph on the Nostocales (Bornet and Flahault, 1886). The specific epithet, *gracile*, was not validly published until 1907, and therefore *flos-aquae* has priority under the ICBN. The International Code of Nomenclature of Bacteria does not currently recognize any validly published cyanobacteria and this study recommends confirmation by DNA-DNA hybridization before validly publishing the revised *Anabaena flos-aquae* for ICNB purposes.

The emended description of *Anabaena flos-aquae* is as follows:

> Planktonic with gas vacuoules, trichomes solitary, coiled and forming a twisted entangled mass, or found as fascicle aggregates like flakes, colorless mucilage if present, trichomes subsymmetric to metameric, vegetative cells cylindrical, bent, ellipsoidal, or spherical from 2-8 μm wide to 5-15 μm long, heterocysts 2.5-9 μm wide and 5.5-20 μm long, akinetes adjacent or distant

from heterocysts, cylindrical or ellipsoidal and 4.5-13 μm wide and 7-80 μm long with smooth colorless walls.

The description above places *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* into the species *Anabaena flos-aquae*. A separate cluster of Southern Hemisphere (Australian) strains of *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* appear to belong in a separate *rpo*C1 clade outside *Anabaena* Groups IIA and IIB but still within the genus *Anabaena*. As the first valid publication and description of *Anabaena flos-aquae* is a European strain in Bornet and Flahault's 1886 monograph (Bornet and Flahault, 1886), Group IIA has priority over any Australian strains designated *Anabaena flos-aquae*, *Aphanizomenon flos-aquae* or *Aphanizomenon gracile* under the ICBN. The morphological similarities of the Australian strains are likely another incidence of convergence in form as appears to be common within the Nostocales.

The use of molecular markers such as *cpc*BA-IGS and *rpo*C1 is one step among many in efforts to better understand cyanobacterial diversity. These techniques allow us to narrow the search for appropriate strains to test the current taxonomy, to construct meaningful phylogenies in some cases, and are important components of a robust polyphasic taxonomy system that includes molecular markers, DNA-DNA hybridization and other phylogenetic tools that are used in an appropriate manner to answer specific phylogenetic questions. Without effective screens to examine strains from the field, it will be impossible to determine the true cyanobacterial diversity present. As whole

genomes are sequenced, the tools for examining phylogenetic questions will expand and the utility of specific techniques will become clearer.

Here, we have examined the diversity of *Aphanizomenon* and *Anabaena* from the Pacific Northwest region and incorporated these data with other primarily Northern hemisphere strains to examine the diversity of this lineage. The results indicate that significant reform is necessary in Nostocalean taxonomy and serious reconsideration of suitable morphological markers will be needed to identify members of *Anabaena* in the field. Additional taxa and putative species from other regions need to be examined to complete the picture and DNA-DNA hybridization for selected taxa at critical points in the phylogeny should be performed. The second order task will be to examine the rate of genetic transfer between cyanobacteria taxa as a possible evolutionary response to their environment. The rate of horizontal transfer among cyanobacteria is unknown and may be substantial in some taxa and not in others. The use of techniques such as single filament PCR promise to help answer these questions in future studies.

Figure 25. *Aphanizomenon* strains as seen by light microscopy. (a) *Aphanizomenon flos-aquae* in fascicle colonies with coiled and entangled *Anabaena flos-aquae* (AL97) from Green Lake, WA. These strains only differ by 4% in *cpc*BA-IGS sequence; (b)*Aphanizomenon flos-aquae* NH5 in typical solitary habit with heterocysts. Note the constriction of cells walls and heterocysts characteristic of *Aphanizomenon gracile*. NH5 differs in *cpc*BA-IGS sequence from AL97 above by only 5.6%. Bar = 50 μm. H = Heterocyst

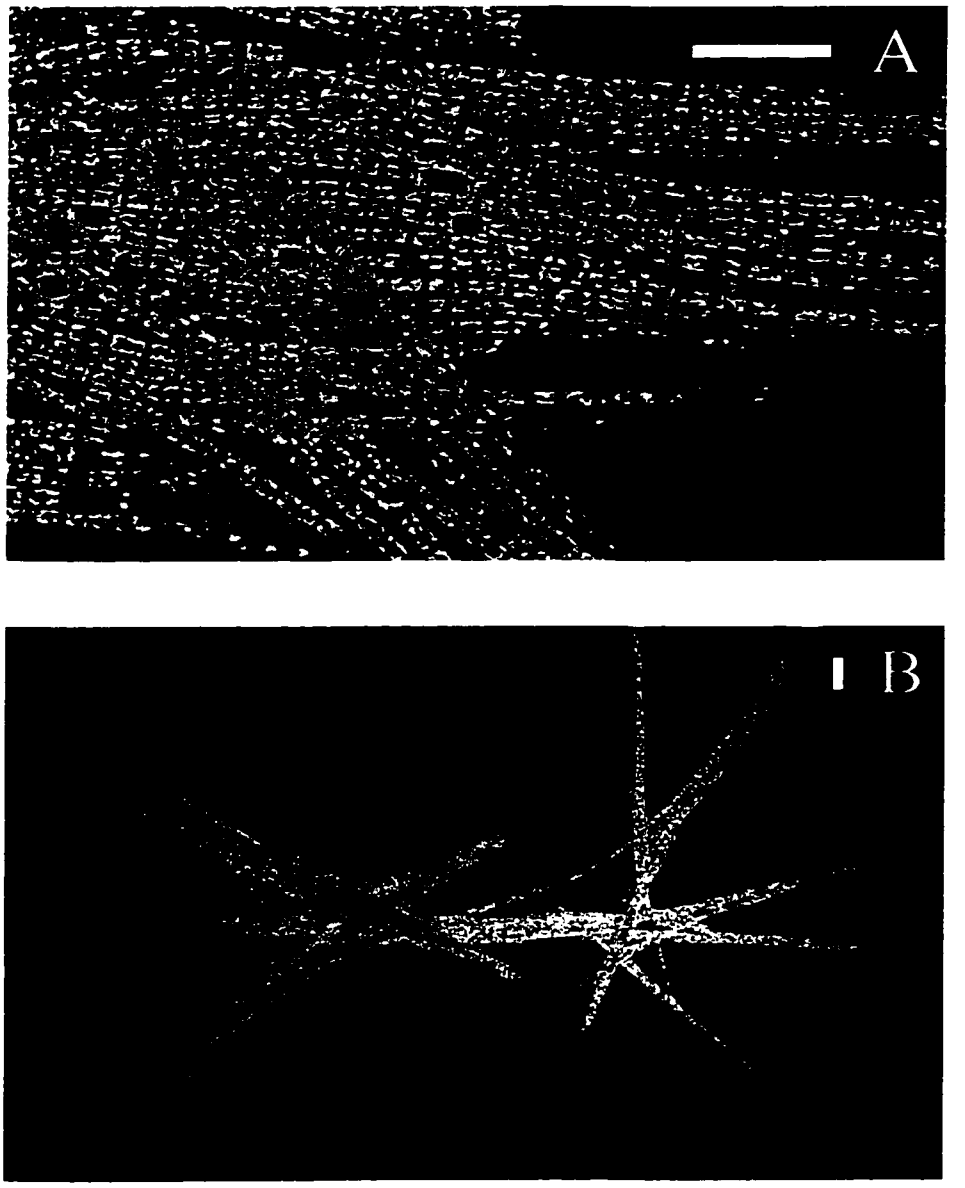

Figure 26. *Aphanizomenon flos-aquae* (UTEX 2384) under light microscopy. (a) UTEX 2384; (b) UTEX 2384. This strain is characteristic of all the strains in Group IIA(4) and to date are strictly North American. Bar = 25 μm.

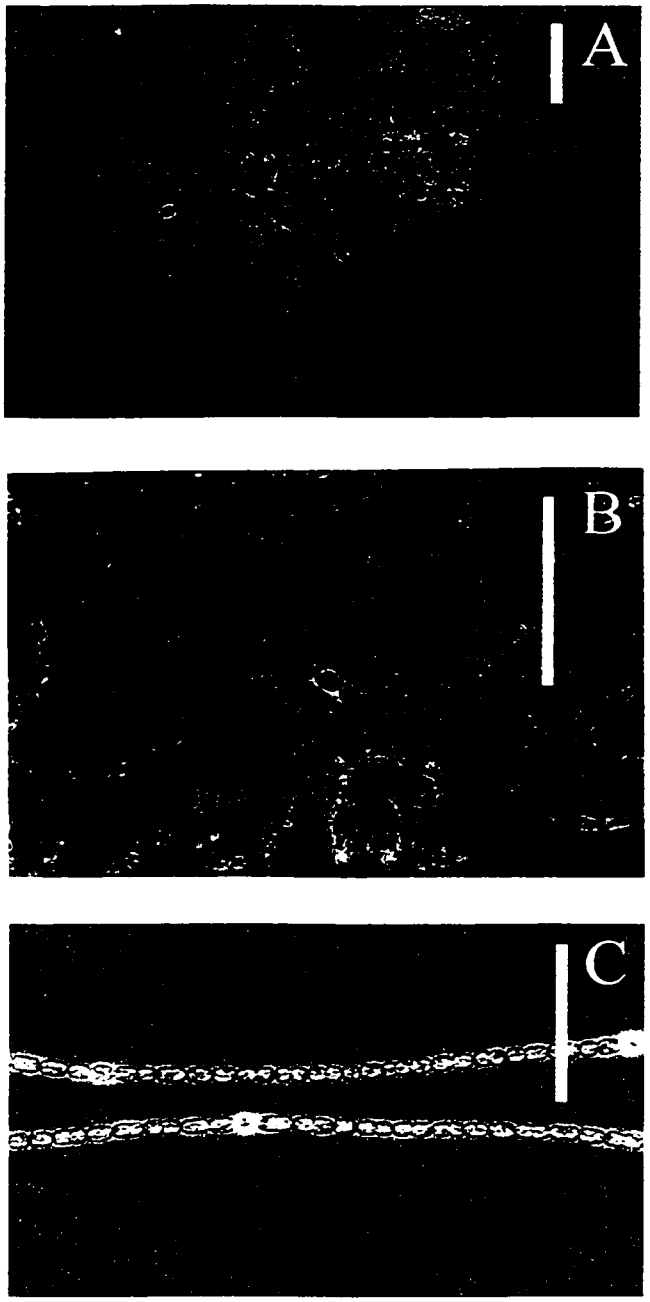

Figure 27. *Anabaena flos-aquae* (AL89) as seen by light microscopy. (a) *A. flos-aquae* from American Lake, WA; (b) *A. flos-aquae* from American Lake, WA. Note the cylindrical akinetes and clear heterocysts; (c) *A. flos-aquae* in culture. Note the linear form in culture. This toxic strain differs from *Aphanizomenon* NH5 in Fig. 25b by only 2.4% in *cpc*BA-IGS sequence. Bar = 50 μm.

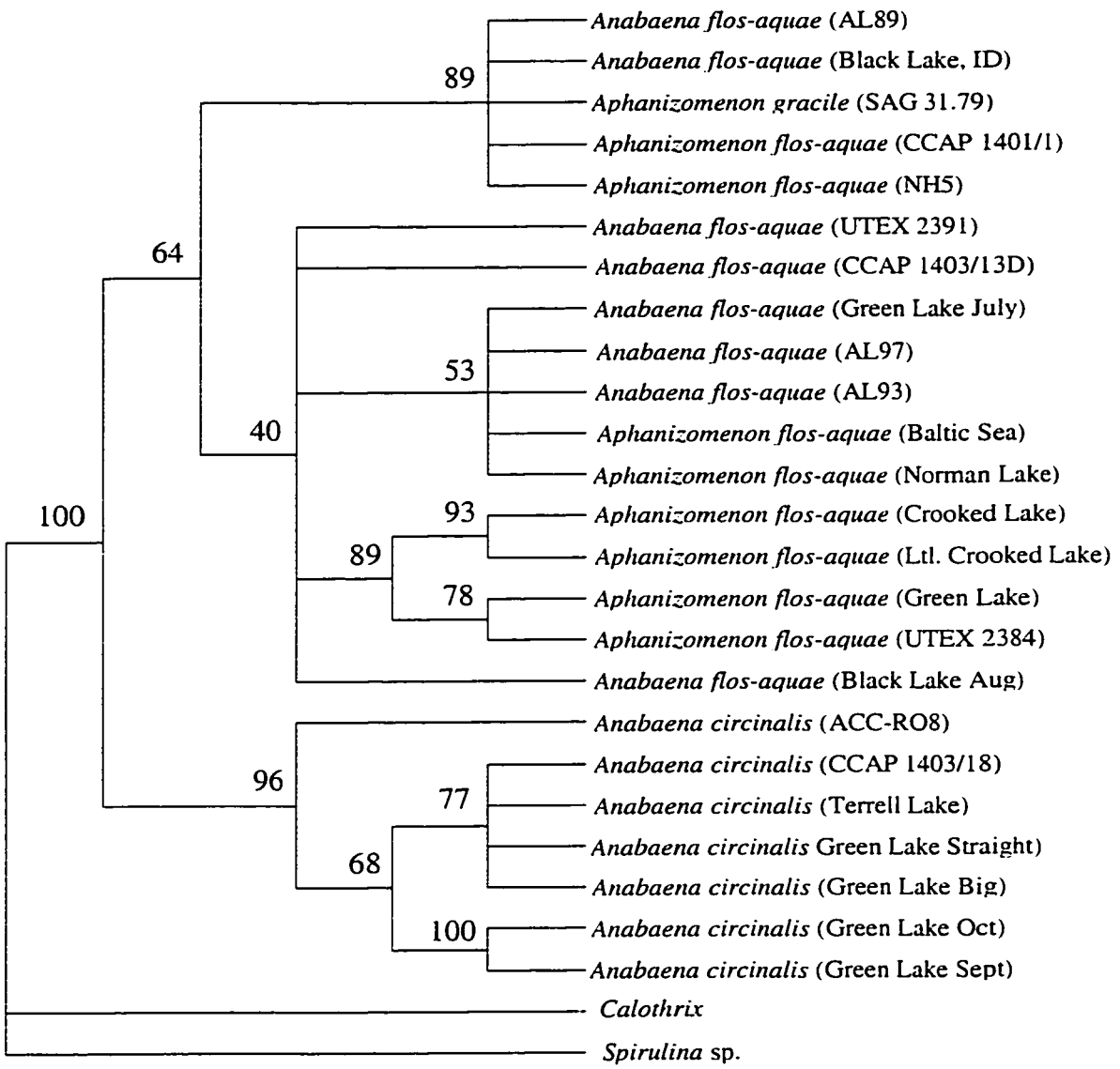

Figure 28. Maximum Parsimony Strict Consensus tree inferred from a heuristic search using maximum parsimony analysis (89 parsimony informative positions) of *Anabaena* and *Aphanizomenon cpc*BA-IGS sequences excluding unalignable portions of the IGS from the outgroup. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Nodes with bootstrap values of less than 50% are not labeled. A mixed clade of *Aphanizomenon* and *Anabaena flos-aquae* is clearly distinguished from a clade of *Anabaena circinalis* using a heterocystous genus (*Calothrix*) and a non-heterocystous strain as the outgroup.

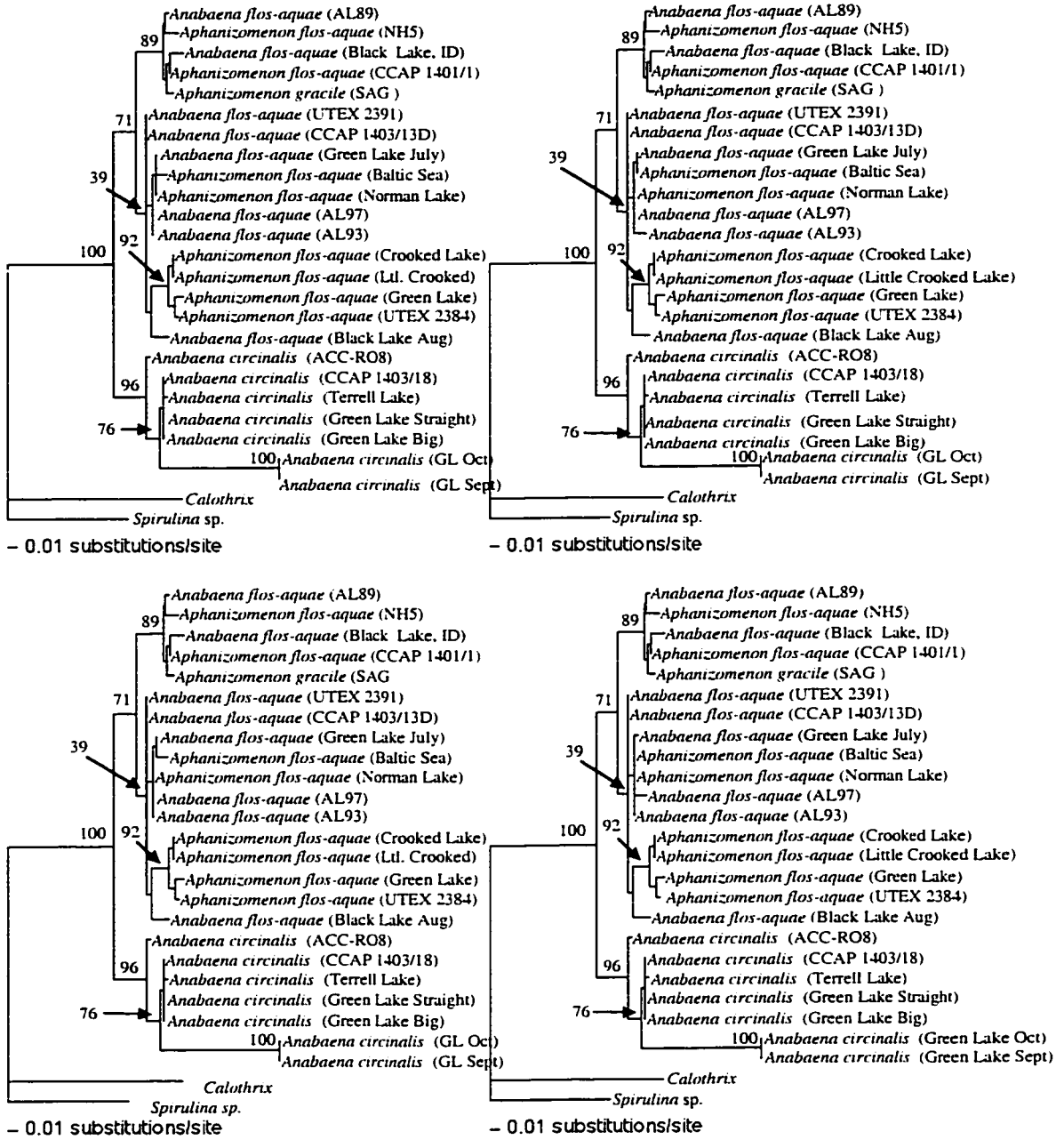

Figure 29. Four phylogenetic trees inferred from a heuristic search using maximum likelihood from *Anabaena* and *Aphanizomenon cpc*BA-IGS sequence data (-ln =1666.3899) excluding unalignable portions of the IGS region from the outgroup. Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). Nodes with bootstrap values below 20% are not labeled. All four trees support separate *Anabaena flos-aquae* and *Anabaena circinalis* clades with *Aphanizomenon* only in *A. flos-aquae*.

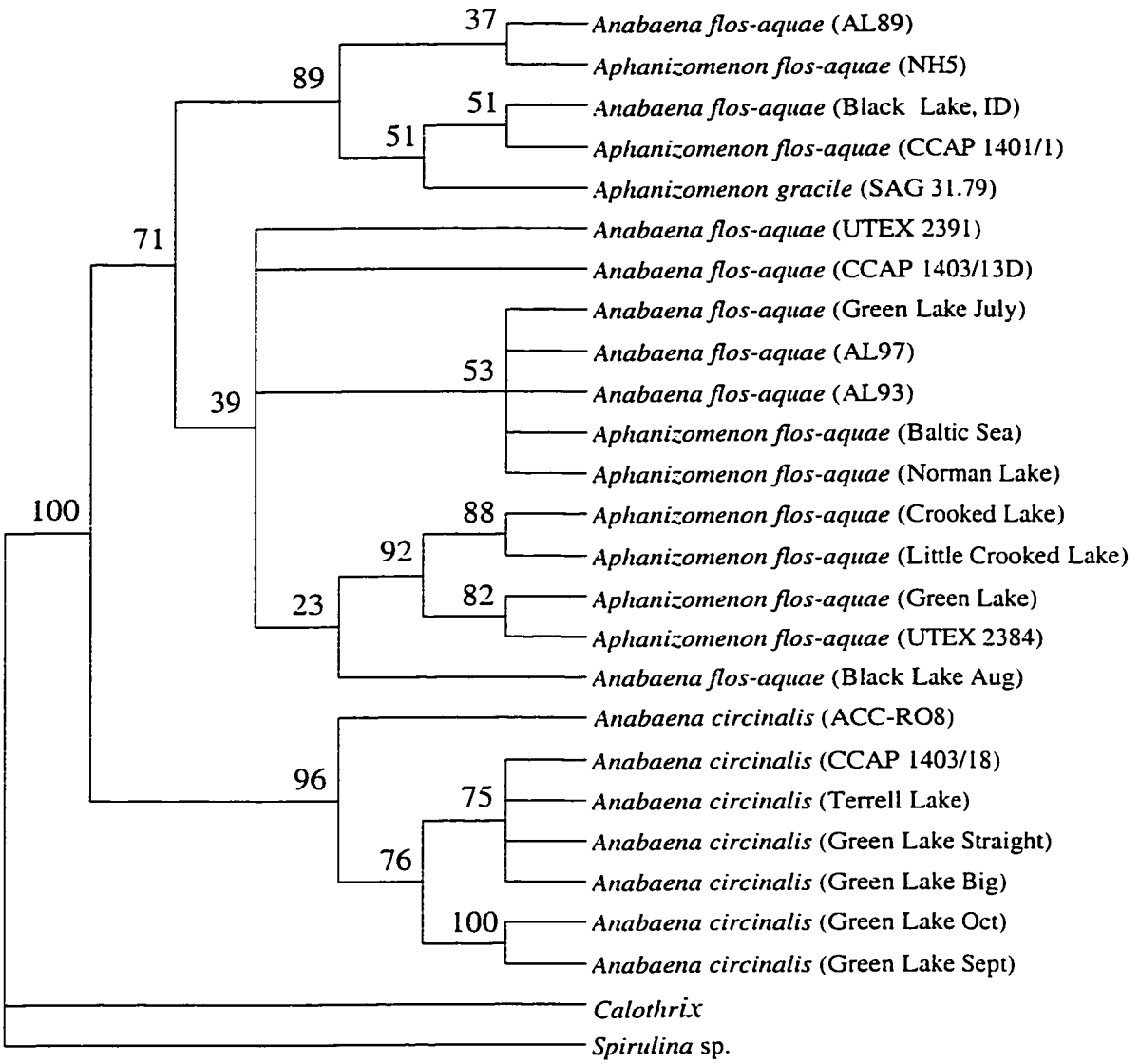

Figure 30. Maximum Likelihood Strict Consensus tree from a heuristic search using maximum likelihood from *Anabaena* and *Aphanizomenon cpc*BA-IGS sequence data (-ln =1666.3899). Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). *Aphanizomenon* strains all cluster in the *Anabaena flos-aquae* clade with high bootstrap support. Two major *A. flos-aquae* clades are supported in this tree with *Aphanizomenon* dispersed throughout the clade.

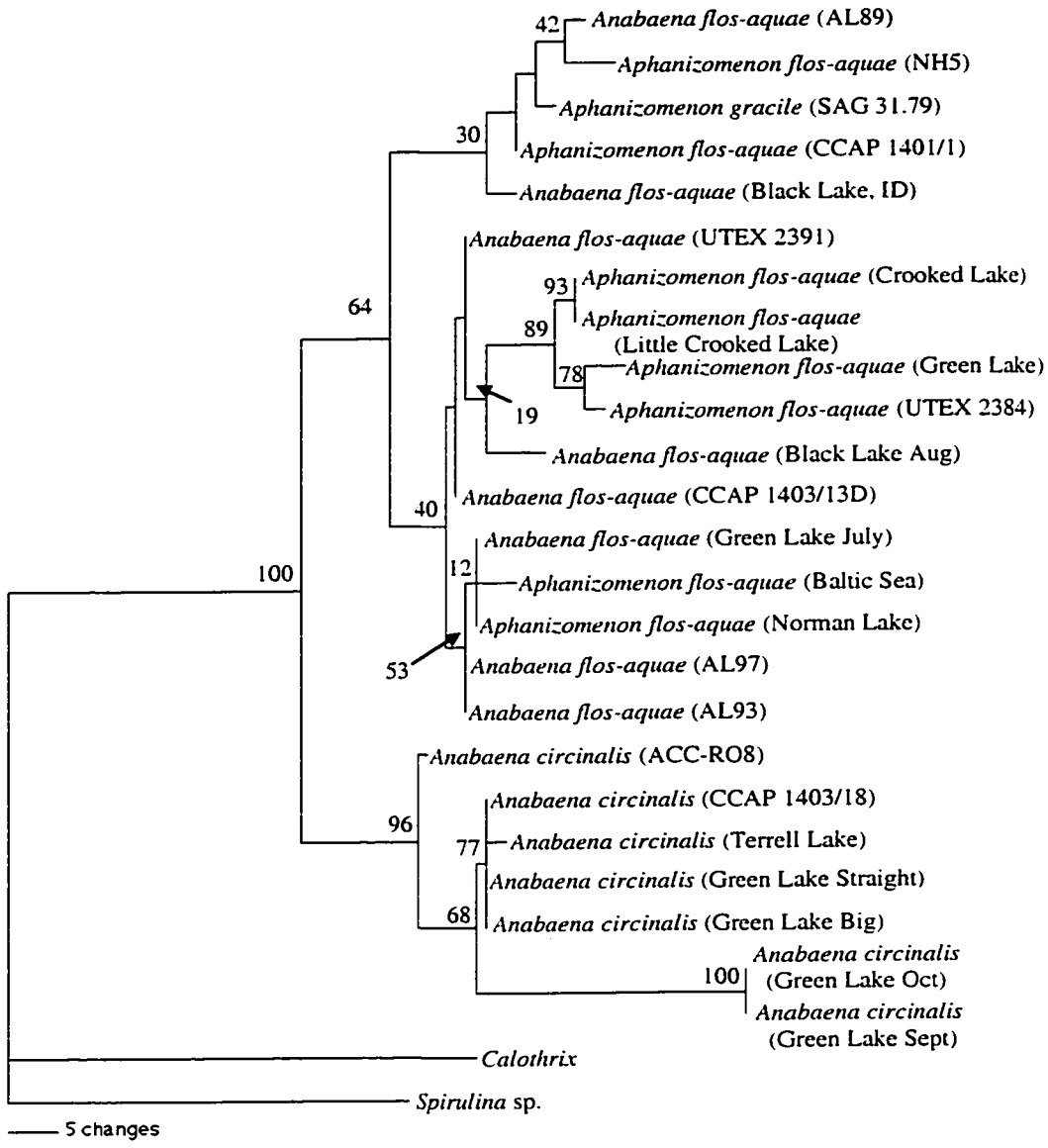

Figure 31. Phylogram based on maximum parsimony analysis of *Anabaena* and *Aphanizomenon cpc*BA-IGS sequences. Tree shown is one of 16 most parsimonious trees recovered in a heuristic search. Numbers above the nodes are labeled with bootstrap values for parsimony (1000 replicate samples). Bootstrap values <10% are not shown. Tree length = 245 steps; CI = 0.792; RI = 0.851; RC = 0.674; HI = 0.208. Scale bar for 5 nucleotide changes is shown at bottom. All trees supported inclusion of *Aphanizomenon* in the *Anabaena flos-aquae* clade. Moderate bootstrap support separates two *A. flos-aquae* clades.

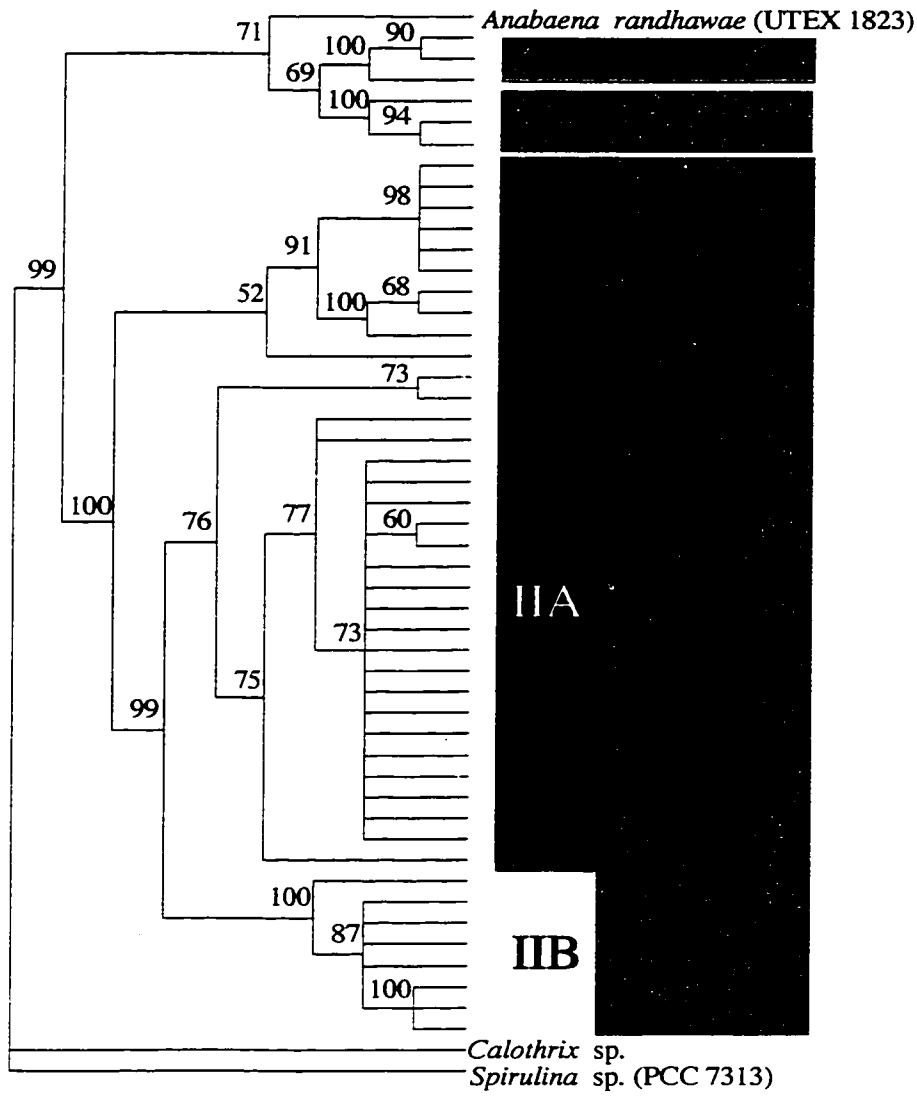

Figure 32. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *cpc*BA amino acid sequences. Two hundred fifteen amino acids produced 49 parsimony informative positions. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Nodes with bootstrap values of less than 50% are not labeled. An *Anabaena* clade composed of Groups I, IIA and IIB is highly supported with a sister clade that contains *Nostoc, Nodularia, Fischerella* and an assortment of misidentified *Anabaena* strains. Group II is entirely composed of planktonic strains. Group IIA includes *Anabaena flos-aquae* and *Aphanizomenon.* Group IIB includes *Anabaena circinalis.* Group I includes all other *Anabaena* spp.

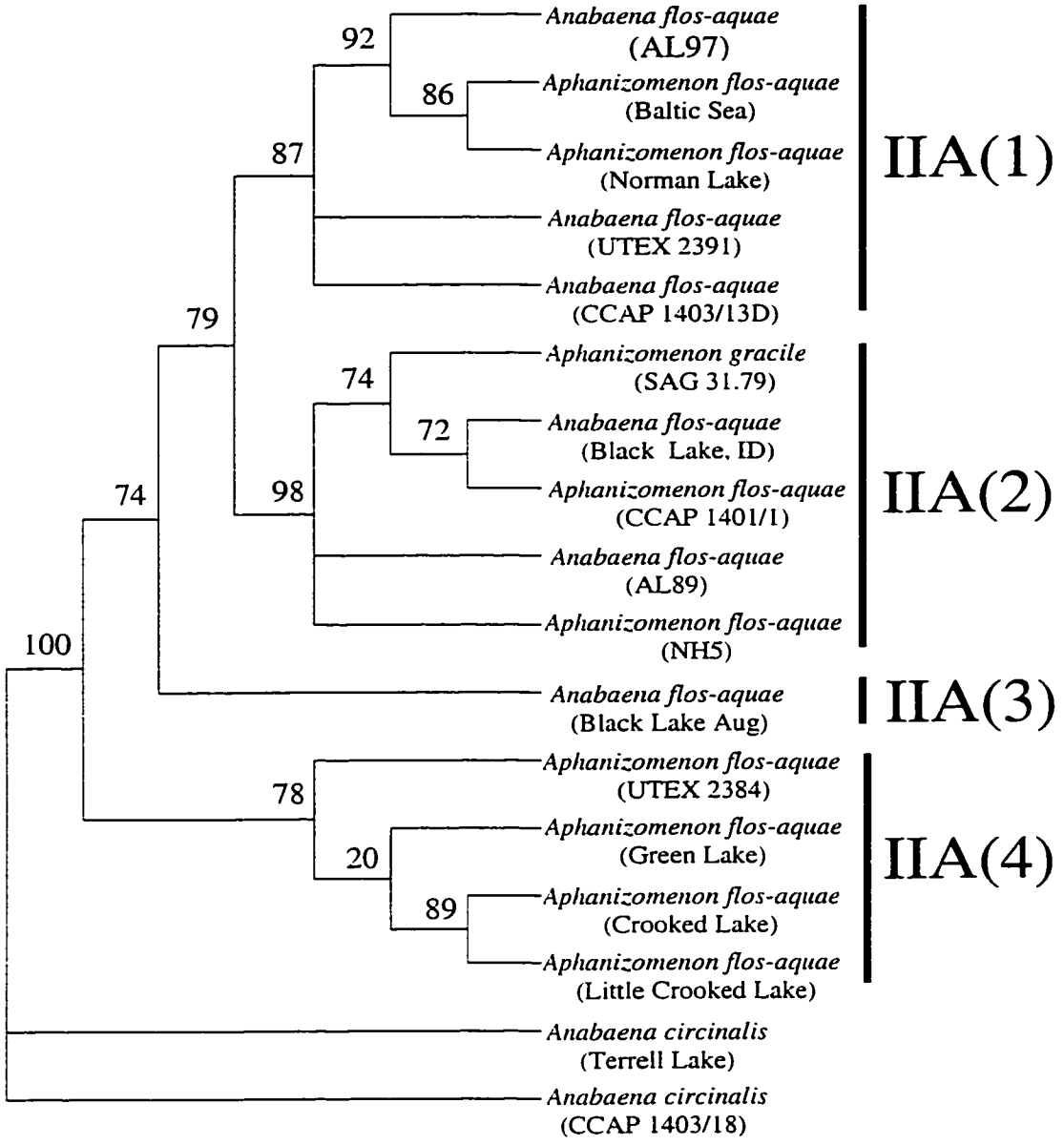

Figure 33. Strict consensus tree inferred from a heuristic search using maximum parsimony analysis of *Anabaena* and *Aphanizomenon cpc*BA-IGS sequences with *Anabaena circinalis* as the outgroup. Of the 253 characters, 39 were parsimony informative. Nodes are labeled with bootstrap values for maximum parsimony (1000 replicate samples). Nodes with bootstrap values of less than 50% are not labeled. Four genotype clusters are found within *Anabaena* Group IIA. Toxic strains have not been found in Group IIA(4) strains (entirely North American).

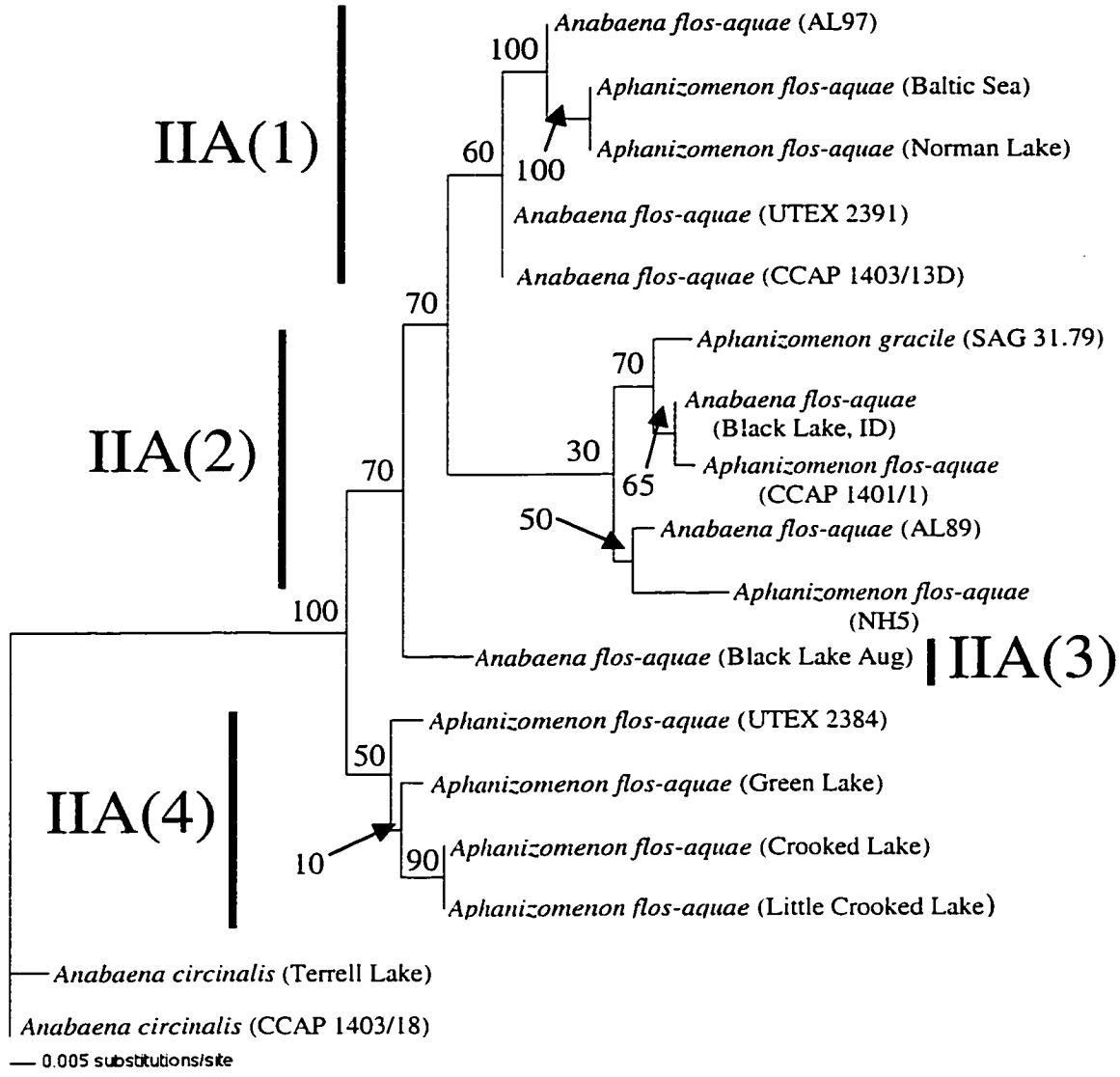

Figure 34. Phylogenetic tree inferred from a heuristic search using maximum likelihood from *Anabaena* and *Aphanizomenon cpc*BA-IGS sequence data with *Anabaena circinalis* as the outgroup (-ln = 705.656368). Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). Group IIA is consistent with an *Anabaena flos-aquae* designation and includes all *Aphanizomenon* strains.

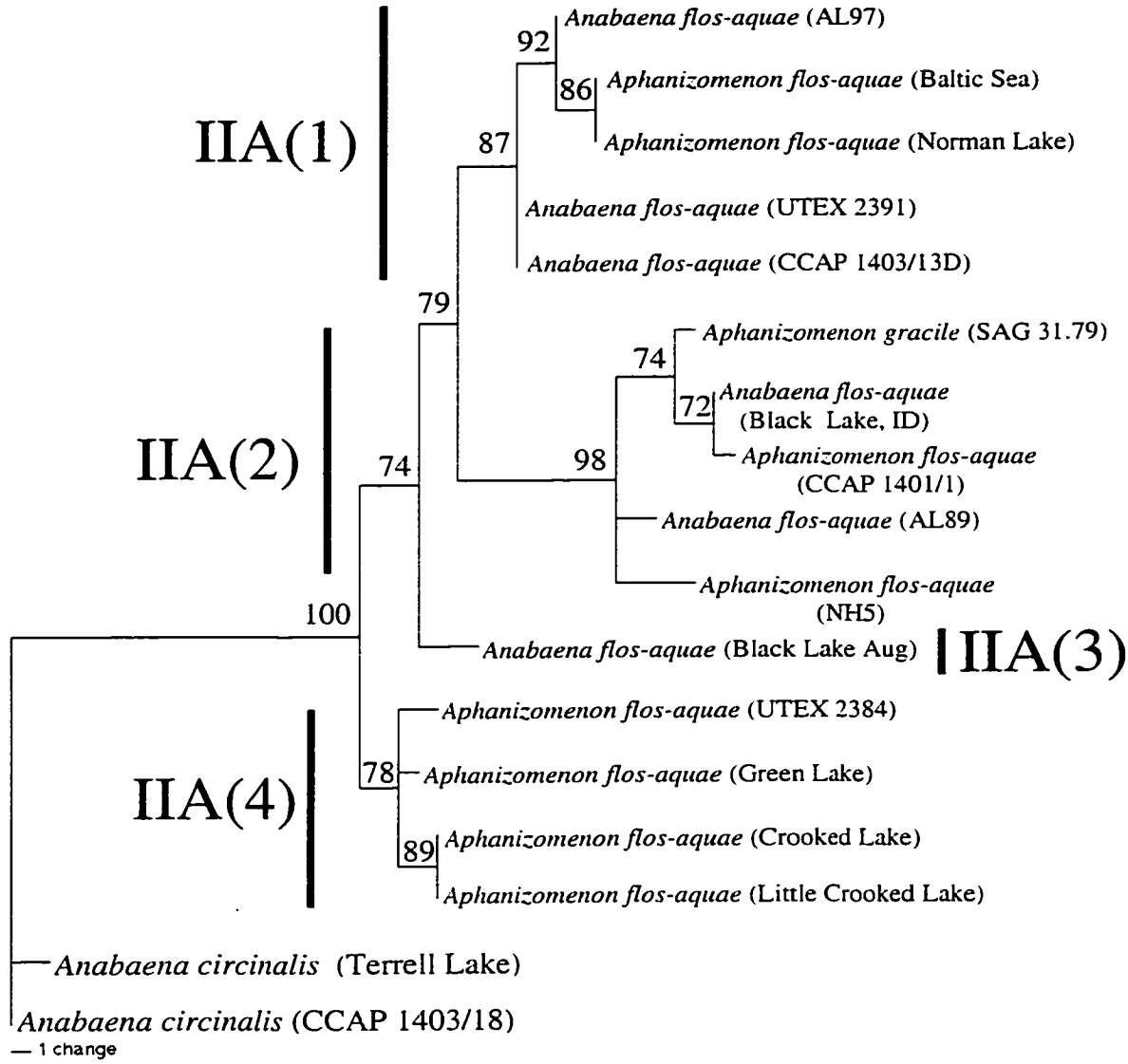

Figure 35. Phylogram based on maximum parsimony analysis of *Anabaena* and *Aphanizomenon cpc*BA-IGS sequences with *Anabaena circinalis* as the outgroup. Tree shown is 1 of 2 most parsimonious trees recovered in a heuristic search. Numbers above the nodes are labeled with bootstrap values for parsimony (1000 replicate samples) and nodes with less than 50% bootstrap values are not labeled. Tree length = 63 steps; CI = 0.841; RI = 0.905; RC = 0.761; HI = 0.159. Scale bar for 1 nucleotide changes is shown at bottom. All clades except for IIA(4) are found in Europe and North America.

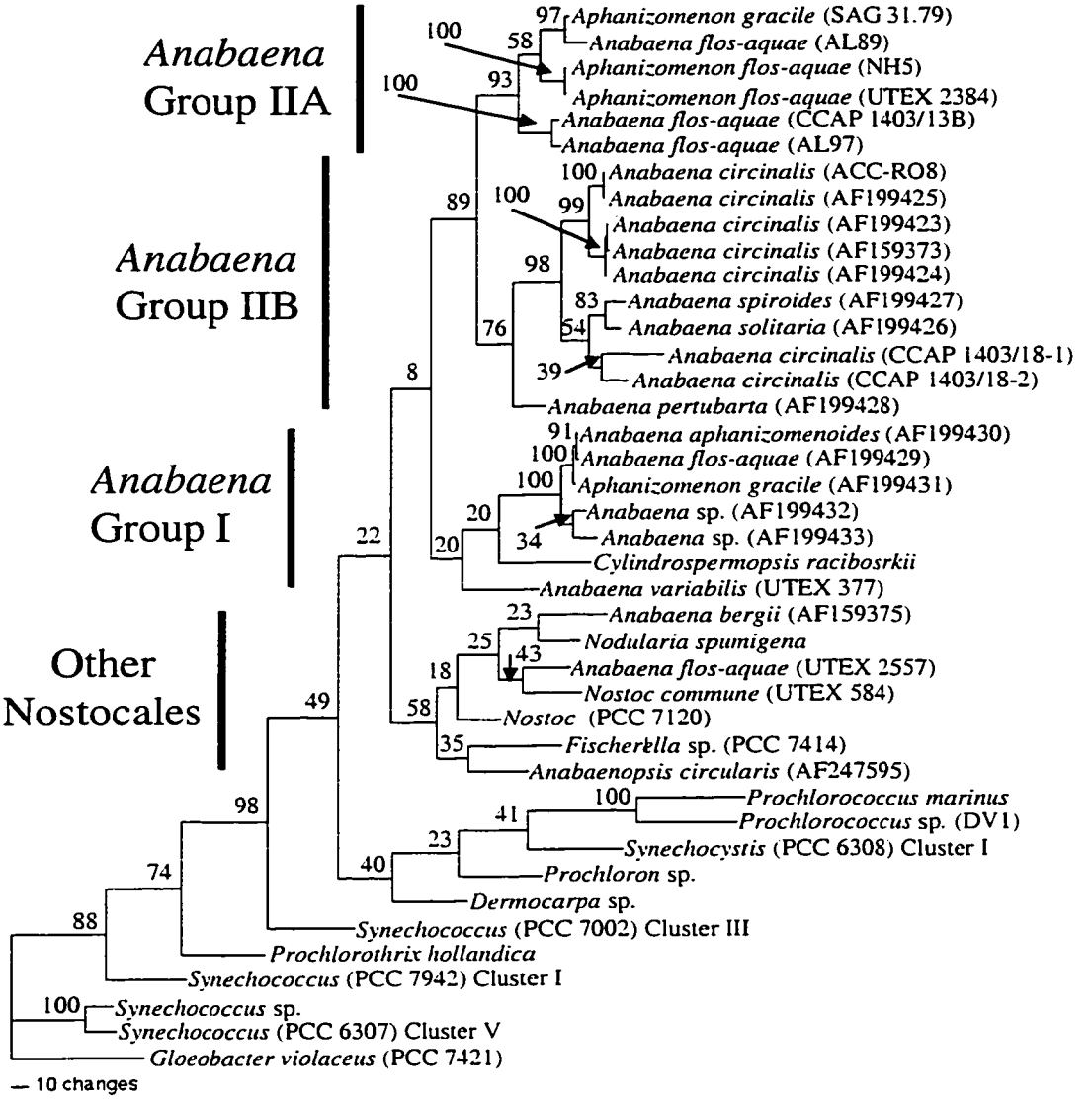

Figure 36. Phylogram based on maximum parsimony analysis of cyanobacteria *rpo*C1 sequences. Tree shown is the most parsimonious tree recovered in a heuristic search. Numbers above the nodes are labeled with bootstrap values for parsimony (1000 replicate samples). Tree length = 2060 steps; CI = 0.315; RI = 0.582 RC = 0.183; HI = 0.685. Scale bar for 10 nucleotide changes is shown at bottom. This tree confirms the results found with the *cpc*BA-IGS marker. A distinct Group IIA and IIB are identified within an *Anabaena* clade that includes all *Aphanizomenon* strains sequenced in this study. The correct topology where *Anabaena* and *Nostoc/Nodularia* meet is unclear.

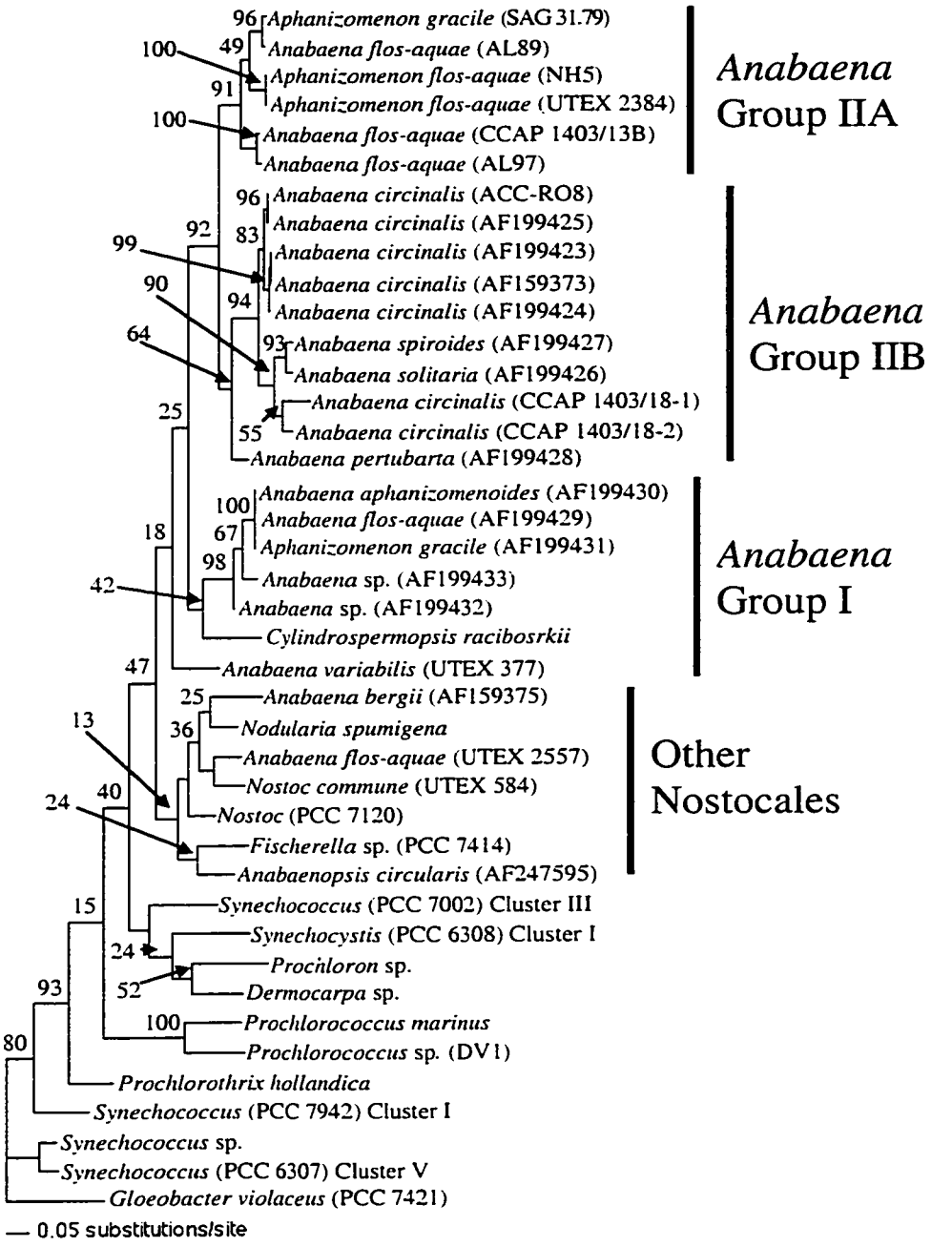

Figure 37. Phylogenetic tree inferred from a heuristic search using maximum likelihood from cyanobacteria *rpo*C1 sequence data (-ln = 7476.10474). Horizontal branch lengths are proportional to the mean number of nucleotide substitutions per site. Numbers above the nodes indicate bootstrap values using maximum likelihood (100 replicate samples). ML analysis confirms *Anabaena* Group II includes *Aphanizomenon*.

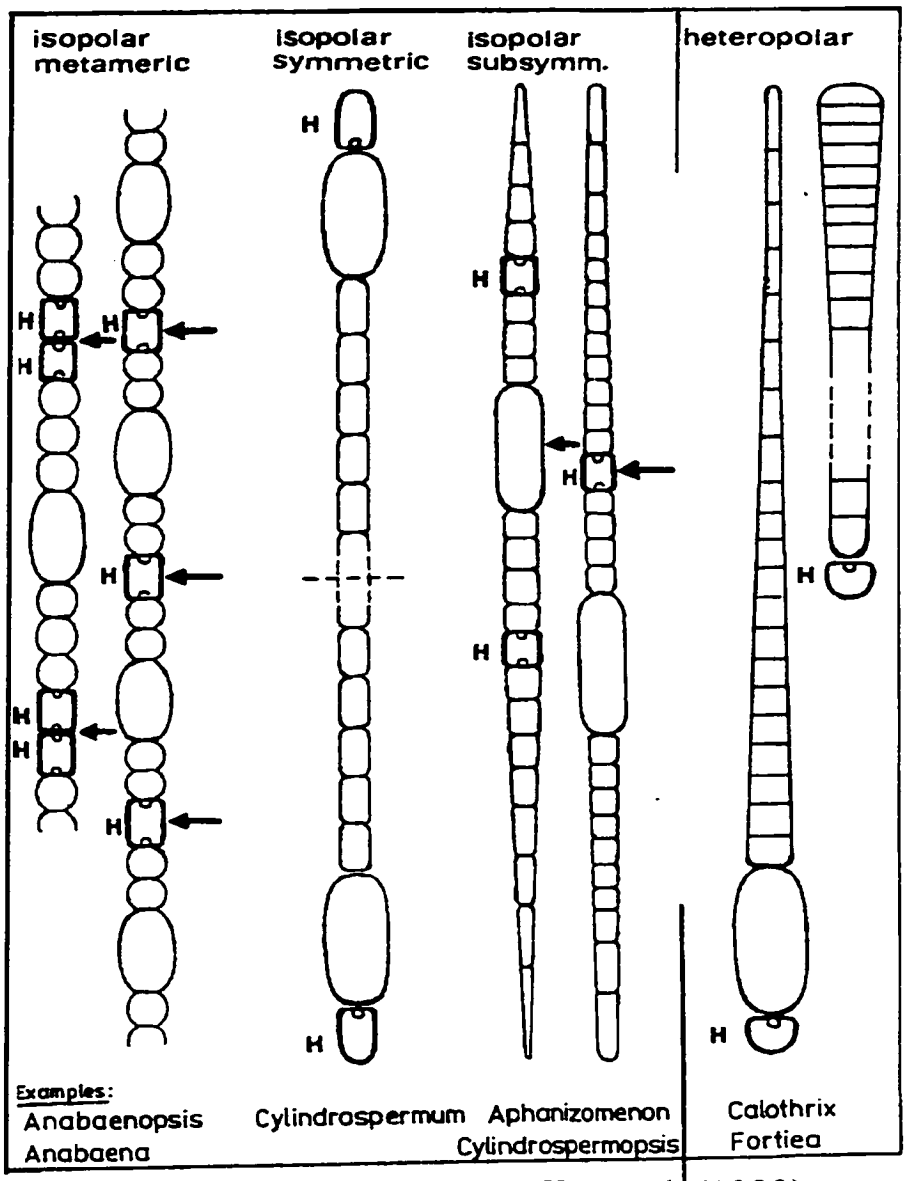

Figure 38. Diagram of trichome symmetry in Nostocaceae from Komárek (1989). Note that in Komárek's classification scheme, *Anabaena* has metameric trichomes and *Aphanizomenon* has subsymmetric trichomes. This character is not valid following the results of this study as metameric and subsymmetric strains are mixed in *Anabaena* Group IIA.

Table 3. Cyanobacteria strains included in the *Anabaena/Aphanizomenon* study.

Taxon	Strain	Toxicity	Source	Accession Number
Anabaena **Bory ex Bornet and Flahault**				
aphanizomenoides Forti	ANA280A	No	Australia	AF199430
bergii Komárek	ANA283A	No	Australia	AF159375
catenula (Kützing) Bornet et Flahault	UTEX 375	No	Netherlands	
circinalis Rabenhorst	ANA175A	?	Australia	AF199423
circinalis Rabenhorst	ANA019A	?	Australia	AF199424
circinalis Rabenhorst	ANA350C	?	Australia	AF199425
circinalis Rabenhorst	ACBU02	YES	Australia	
circinalis Rabenhorst	CCAP1403/18 #1	No	N. Ireland	
circinalis Rabenhorst	CCAP 1403/18 #2	No	N. Ireland	
circinalis Rabenhorst	GL-Sept	?	Green Lake, WA	
circinalis Rabenhorst	GL-Sept Linear	?	Green Lake, WA	
circinalis Rabenhorst	TL-Aug	?	Terrell Lake, WA	
cylindrica Lemmermann	UW	No	Unknown	
cylindrica Lemmermann	CCAP 1403/30	No	Saudi Arabia	
flos-aquae Brébisson ex Bornet et Flahault	ANA354A	No	Australia	AF199429
flos-aquae (Lyngbe) Brébisson	UTEX 2557	Yes	Hebgen Lake, MT	
flos-aquae (Lyngbe) Brébisson	UTEX2558	No	Hebgen Lake, MT	
flos-aquae Brébisson ex Bornet et Flahault	AL89	Yes	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	BLID	Yes	Black Lake, ID	
flos-aquae Brébisson ex Bornet et Flahault	AL97	No	American Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	BL	?	Black Lake, WA	
flos-aquae Brébisson ex Bornet et Flahault	CCAP 1403/13B	No	UK	
flos-aquae Brébisson ex Bornet et Flahault	CCAP 1403/13D	No	Wales, UK	
flos-aquae (Lyngbe) Brébisson	UTEX 1444	No	Mississippi	
flos-aquae Brébisson ex Bornet et Flahault	UTEX 2391	Yes	Alberta, Canada	
inaequalis (Kützing) Bornet et Flahault	UTEX 380	No	Netherlands	
inaequalis (Kützing) Bornet et Flahault	UTEX 381	No	Netherlands	
pertubarta Cronberg et Kómarek	ANA221E	No	Australia	AF199428
randhawae Venkatamaran	UTEX 1823	No	India	
soliataria Klebahn	ANA207A	No	Australia	AF199426
sphaerica Bornet et Flahault	UTEX 1616	No	India	
spiroides Klebahn	UTEX 1552	No	Fish pond, Israel	
spiroides Klebahn	ANA292C	No	Australia	AF199427
subcylindrica Borge	UTEX 1617	No	Nashville, TN	
variabilis Kützing	UTEX 377	No	Netherlands	

Table 3. Continued

Taxon	Strain	Toxicity	Source	Accession Number
Anabaena				
verrucosa Boye-Petersen	UTEX 1619	No	Austin, TX	
sp.	ANA238C	No	Australia	AF199432
sp.	ANA255C	No	Australia	AF199433
Anabaenopsis				
circularis (G. S. West) Woloszynska et Miller		No	Japan	AF159374
Aphanizomenon **Morren ex Bornet et Flahault**				
flos-aquae Ralfs ex Bornet et Flahault	UTEX 2384	No	Canada	
flos-aquae Ralfs ex Bornet et Flahault		?	Crroked Lake, IN	
flos-aquae Ralfs ex Bornet et Flahault		?	Little Crooked Lake, IN	
flos-aquae Ralfs ex Bornet et Flahault	GL	?	Green Lake, WA	
flos-aquae Ralfs ex Bornet et Flahault	TL	?	Lake Terrell, WA	
flos-aquae Ralfs ex Bornet et Flahault	BL	?	Black Lake, WA	
flos-aquae	CCAP 1401/1	Yes	N. Ireland	
flos-aquae Ralfs ex Bornet et Flahault		?	Baltic Sea	
flos-aquae Ralfs ex Bornet et Flahault		?	Norman Lake, IN	
flos-aquae	NH5	Yes	NH	
gracile Lemmermann	SAG 31.79	?	Holland	
gracile Lemmermann	APH026E	?	Australia	AF199431
Cylindrospermopsis				
raciborskii Woloszynska		Yes	Australia & Brazil	AF159371
Nodularia				
spumigena Mertens ex Bornet et Flahault	PCC 73104	?	Canada	AF159372
spumigena Mertens ex Bornet et Flahault	BC9427	?	Baltic Sea	AJ224915
spumigena Mertens ex Bornet et Flahault		?	Australia	AF101452
spumigena Mertens ex Bornet et Flahault		?	Australia	AF101450
Nostoc				
commune Vaucher	UTEX 584	No	Scotland	M29747
sp.	PCC 7120	No	North America	

Table 3. Continued

Taxon	Strain	Toxicity	Source	Accession Number
Calothrix				
sp.		?	--	
Fischerella				
ambigua (Nägeli) Gomont	UTEX 1903	?	--	
sp.	PCC 7414	?	--	Z11153
Outgroup Taxa				
Dermocarpa sp.		?	--	U522341
Gloeobacter violaceus Rippka, Waterbury et Cohen-Bazire		?	--	U52340
Prochlorococcus marinus Chisholm		?	--	Z11160
Prochlorococcus sp.	DV1	?	--	Z11159
Prochloron sp.		?	--	Z11161
Prochlorothrix hollandica Burger-Wiersma		?	--	Z11154
Spirulina sp.	PCC 7313	?	--	AJ401188
Synechococcus sp.	PCC 7002	?	--	
Synechococcus sp.	PCC 7942	?	--	
Synechococcus sp.	PCC 6307	?	--	
Synechococcus sp.		?	--	
Synechocystis sp.	PCC 6803	?	--	

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