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Genetic Diversity and Phylogeny of Toxic Cyanobacteria Determined by DNA Polymorphisms within the Phycocyanin Locus

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Cyanobacteria are a highly diverse group in relation to form, function, and habitat. Current cyanobacterial systematics relies on the observation of minor and plastic morphological characters. Accurate and reliable delineation of toxic and bloom-forming strains of cyanobacteria has not been possible by traditional methods. We have designed general primers to the phycocyanin operon (*cpc* gene) and developed a PCR which allows the amplification of a region of this gene, including a variable intergenic spacer sequence. Because of the specificity of this PCR for cyanobacterial isolates, the assay is appropriate for the rapid and reliable identification of strains in freshwater samples. Successive restriction endonuclease digestion of this amplification product, with a total of nine enzymes, yielded many identifying DNA profiles specific to the various taxonomic levels of cyanobacteria. The restriction enzyme profiles for *Msp*I, *Rsa*I, and *Taq*I were conserved for strains within each of the eight genera (40 strains) studied and clearly discriminated among these genera. Intrageneric delineation of strains was revealed by the enzymes *Alu*I, *Cfo*I, and *Hae*III for members of the genus *Microcystis*, while strains of genus *Anabaena* were differentiated by the digestion patterns provided by *Alu*I, *Cfo*I, and *Scr*FI. Phenetic and cladistic analyses of the data were used to infer the genetic relatedness and evolution of toxic and bloom-forming cyanobacteria.

Broadly classified as oxygenic phototrophs containing chlorophyll *a* and accessory pigments, cyanobacteria inhabit most of Earth's environments. The phylum Cyanobacteria is large and diverse, containing over 1,000 species of oxyphototrophs, and its members are classified by using both botanical and bacteriological taxonomic codes (10, 27–29, 38, 39). The current status of cyanobacterial systematics is such that the identification of particular species based on morphology is not possible (47, 48). Species of toxic cyanobacteria are common members of the microscopic populations found in eutrophic water systems. The extensive growth of cyanobacteria, into what is known as a blue-green algal bloom, presents a considerable threat to the health and welfare of humans and other animals (8). Recently, a single bloom event in Australia caused the noncontinuous contamination of over 1,000 km of a river system. Blooms of cyanobacteria are also frequently encountered in the reservoirs and dams which supply water to major cities. This study focuses on the nuisance members of this group which have deleterious effects on freshwater systems and pose a significant health risk to humans.

Apart from having limited characters as the basis for this system of nomenclature, sampling and culturing procedures inevitably lead to underestimations of the diversity in the cyanobacterial bloom population. To overcome the observed plastic morphotypes, several macromolecular analyses have been developed for the classification of cyanobacteria to the strain level (5, 11, 43, 45). However, variable environmental conditions may also affect the recording of phenotypes based on expressed proteins, such as photosynthetic pigments and isozymes, or plasmid content. Similarly, the toxicity of a strain may alter as a result of varying culture conditions (15, 47). Genotypic characters, including DNA base composition, DNA and RNA hybridizations, and gene sequences, directly indicate the genetic relatedness of a group of organisms and can therefore be used as taxonomic markers and to infer phylogeny (26, 30, 49). Fossil records are also available to attach a timescale to the evolution and support the cyanobacterial phylogeny (42).

The photosynthetic apparatus of a cyanobacterium contains chlorophyll *a* and specific accessory pigments, including allophycocyanin, phycocyanin (PC), and phycoerythrin (24). PC and the other biliprotein pigments of the phycobilisome are the major light-harvesting antennae in photosystem II of cyanobacteria, rhodophytes, and cryptophytes (17, 25). Of these organisms cyanobacteria are the most often encountered and predominant members of the freshwater environment (7, 18). This distribution of PC in aquatic microorganisms makes the study of PC gene sequence heterogeneity ideal for the classification of freshwater cyanobacteria. The entire PC operon contains genes coding for two bilin subunits and three linker polypeptides (4). The intergenic spacer (IGS) between the two bilin subunit genes, designated β (*cpcB*) and α (*cpcA*), of the PC operon was chosen as a potentially highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level.

Amplification of this PC-IGS sequence, via PCR (31), from extracted DNA and crude lysates of nonaxenic environmental isolates of cyanobacteria was possible. The assay system developed here selectively amplifies only the cyanobacterial genetic material present in a sample. The restriction fragment length polymorphisms (RFLPs) detected by digestion of the PCR products with a range of 4-bp-recognizing restriction endonucleases provided signature profiles specific to the genus, species, and population classifications of cyanobacteria. This relatively simple and rapid method was applied to the major genera of toxigenic, freshwater cyanobacteria, including *Anabaena* and *Microcystis*. Alternative cyanobacterial systematics and the inferred molecular phylogeny based on amplified PC-

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TABLE 1. Cyanobacterial strains, sources, and PC RFLP profile types

Strain	Origin	Restriction enzyme type[a]								
		A	C	Ha	Hi	M	R	T	Sc	Sa
Anabaena spp.										
A. affinis NIES40	Japan	c	g	a	c	b	a	b	a	a
A. circinalis AWT001	Australia	b	c	c	a	a	c	b	a	c
A. circinalis AWT002	Australia	b	c	c	a	a	c	b	a	c
A. circinalis AWT006	Australia	e	h	c	a	a	c	b	b	c
A. circinalis NIES41	Japan	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND
Species cluster 2 PCC7108	United States	d	c	c	a	a	c	a	b	b
A. cylindrica NIES19 (PCC73105)	England	c	g	c	a	c	d	a	e	c
A. flos-aquae NIES73	Japan	c	h	c	a	a	a	b	b	a
A. solitaria NIES80	Japan	e	h	c	a	a	c	b	b	c
A. spiroides NIES76	Japan	e	h	c	a	a	c	b	b	c
A. spiroides NIES78	Japan	c	c	c	a	a	c	b	a	c
Aphanizomenon spp.										
Aphanizomenon flos-aquae PCC7905	The Netherlands	b	b	c	a	a	c	a	d	b
Aphanizomenon flos-aquae NIES81	Japan	b	c	c	a	a	c	a	d	b
Cylindrospermopsis spp.										
C. raciborskii AWT021	Australia	c	h	c	a	c	a	c	b	c
C. raciborskii AWT205	Australia	c	h	c	e	c	a	c	c	c
Microcystis spp.										
M. aeruginosa AWT101	Australia	g	d	b	c	b	a	a	b	a
M. aeruginosa AWT104	Australia	d	e	c	c	d	a	a	b	a
M. aeruginosa AWT105	Australia	e	b	a	c	e	a	a	b	a
M. aeruginosa AWT107	Australia	b	a	c	c	d	a	a	b	a
M. aeruginosa AWT108	Australia	b	f	b	c	b	a	c	b	a
M. aeruginosa AWT114	Australia	c	e	c	d	a	c	b	a	c
M. aeruginosa AWT139	Australia	g	f	b	c	b	a	a	b	a
M. aeruginosa NIES44	Japan	c	d	a	c	b	a	a	c	a
M. aeruginosa NIES87	Japan	f	d	c	c	b	a	a	b	a
M. aeruginosa NIES89	Japan	e	g	c	b	b	a	a	b	a
M. aeruginosa NIES98	Japan	c	g	c	b	b	a	a	b	a
M. aeruginosa NIES298	Japan	g	f	b	c	b	a	a	b	a
M. aeruginosa PCC7005	United States	c	d	b	c	b	a	b	b	a
M. aeruginosa PCC7806	The Netherlands	c	d	b	c	b	a	a	b	a
M. aeruginosa PCC7820	Scotland	c	d	b	c	b	a	b	b	a
M. aeruginosa PCC7941	Canada	e	d	b	c	b	a	c	b	a
M. elabens NIES42	Japan	ND	ND	ND	ND	ND	ND	ND	ND	ND
M. holsatica NIES43	Japan	b	b	a	c	b	a	a	b	a
M. viridis NIES102	Japan	c	d	b	c	b	a	a	b	a
M. wesenbergii NIES107	Japan	b	g	b	c	b	a	a	b	a
M. wesenbergii NIES111	Japan	a	d	a	c	b	a	a	c	a
M. wesenbergii NIES112	Japan	c	d	a	c	b	a	a	c	a
Nodularia spumigena PCC73104	Canada	d	h	c	a	a	b	d	b	b
Nostoc spp.										
N. commune NIES24	Japan	NA[c]	NA	NA	NA	NA	NA	NA	NA	NA
N. punctiforme PCC73102	Australia	NA	NA	NA	NA	NA	NA	NA	NA	NA
Oscillatoria spp.										
O. agardhii NIES204	Japan	c	c	c	b	b	c	b	b	a
O. raciborskii NIES207	Japan	c	c	b	c	b	c	b	a	a
Other genera										
Pseudanabaena sp.	Australia	h	c	c	d	a	c	b	b	c
Synechococcus sp.	Australia	c	e	a	c	d	a	a	b	a

[a] The different restriction types detected with each enzyme among the 42 strains analyzed are designated by lowercase letters. Abbreviations for restriction enzymes: A, *Alu*I; C, *Cfo*I; Ha, *Hae*III; Hi, *Hin*FI; M, *Msp*I; R, *Rsa*I; T, *Taq*I; Sc, *Scr*FI; Sa, *Sal*I.
[b] ND, not digested.
[c] NA, not amplified.

IGS RFLPs are presented and compared with the expanding information available from 16S rRNA sequences.

MATERIALS AND METHODS

Bacterial and algal cultures. Cyanobacterial strains were either obtained from international culture collections or supplied by local Australian culture collections. The strains listed in Table 1 with the prefixes PCC, NIES, and AWT were obtained from the Pasteur Culture Collection (Paris, France) (40), the National Institute for Environmental Studies (Tsukuba, Japan) (32), and Australian Water Technologies (Sydney, Australia), respectively. The combined collection of strains described in this paper represents the majority of toxigenic and bloom-forming cyanobacterial populations encountered in freshwater habitats. In this study, we investigated 44 strains of cyanobacteria from the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Pseudanabaena*, and *Synechococcus*. Once received, all strains were grown in JM media (9). In general, the purity of the culture used was not critical as long as the sample was unicyanobacterial. Exponential-phase culture volumes of 2 ml were sufficient to perform the range of experiments described. Other cultures used as controls for this work included isolates of bacterial species possibly encountered in aquatic (*Vibrio*, *Alteromonas*, *Aeromonas*, *Escherichia coli*, *Lactobacillus*, and

Campylobacter isolates) and terrestrial (*Rhizobium* and *Rhodococcus* isolates) habitats. Green (*Scenedesmus*) and red (*Delisea*) eukaryotic algal species (University of New South Wales Culture Collection, Sydney, Australia) and members of the family *Cryptophyceae* (Commonwealth Scientific and Industrial Research Organisation Culture Collection, Hobart, Australia) were also used as controls to test PCR primer specificity.

Extraction of PCR template. Samples of type cultures were harvested immediately upon receipt, and PCR template was prepared by one of two methods. In the first method, total genomic DNA was extracted by a modification of a technique for purification of DNA from gram-negative bacteria (34). Briefly, a 1-ml aliquot of mid- to late-exponential-phase culture was pelleted by centrifugation, the medium was decanted, and the pellet was resuspended in 500 μl of 50 mM Tris-HCl (pH 8.0)–5 mM EDTA (pH 8.0)–50 mM NaCl. Lysozyme was added to give a final concentration of 1 mg/ml, and the solution was incubated at 55°C for 30 min. After the addition of 10 μl of proteinase K (10 mg/ml) and 20 μl of 10% sodium dodecyl sulfate, the mixture was incubated at 55°C for 10 min or until the solution cleared (complete cell lysis). The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The organic extraction was repeated, and the supernatant was added to an equal volume of 4 M ammonium acetate. Total genomic DNA was precipitated by the addition of 2 volumes of isopropanol and centrifugation for 10 min at room temperature. The integrity and concentration of the extracted genomic DNA were determined by spectrophotometry at wavelengths of 260 and 280 nm.

Alternatively, PCR template was prepared by the rapid lysis of cyanobacterial cells and liberation of DNA into the supernatant. In this procedure, approximately 10^5 to 10^7 cells were centrifuged to a pellet and the culture medium was removed (36). Cells were resuspended in 200 μl of Instagene matrix (Bio-Rad, Hercules, Calif.) supplemented with 20 μl of 1% Nonidet P-40 or 1% Triton X-100. Cells were incubated at 55°C for 30 min, vortexed for 1 min, and heated to 95°C for 10 min. The solution was vortexed again for 1 min, and the cell debris was pelleted by a brief centrifugation. Between 1 and 5 μl of the supernatant contained sufficient genetic material for successful PCR amplification. The supernatant of this cell lysate has been stored at 4°C for over 2 years and found to retain sufficient DNA to act as a template for PCR amplification.

Amplification of the PC-IGS. Oligonucleotide primers were designed to be suitable for amplification of the PC-IGS from a diverse range of cyanobacteria. Published peptide and DNA sequences for the PC operon from the GenBank and EMBL databases were aligned by using the multiple sequence alignment program of the Clustal W package supplied by the Australian National Genome Information Service (Sydney, Australia). The aligned PC sequences were from the cyanobacteria *Anabaena* sp. strain PCC7120 (4), *Calothrix* sp. strain PCC7601 (44), *Pseudanabaena* sp. strain PCC7409 (17), *Synechococcus* sp. strain WH8020 (14), *Synechocystis* sp. strain PCC6701, and *Agmenellum* (*Synechococcus*) *quadruplicatum* PCC7002 (13), with GenBank accession numbers X05239, M36276, M99426, M95288, D13173, and K02660, respectively. PCR priming sites were chosen from completely conserved regions within the β (forward primer) and α (reverse primer) subunits of the PC peptide sequence. The codon bias for the photosynthetic-apparatus genes of several cyanobacterial species was used to select the final DNA sequences of the PCR primers (19). The primer sequences were checked for homology to any other known sequences deposited in the available databases by using the BLAST (2) option (Australian National Genome Information Service). Peptide sequences in the regions chosen for these primers were also checked against red algae (*Algaothamnion*, *Cyanidium*, and *Porphyridium* spp.), cryptomonads (*Chroomonas* spp.), and cyanelle sequences in the PIR protein database. Oligonucleotides were synthesized on the Oligo 1000 DNA synthesizer (Beckman, Fullerton, Calif.).

Small-volume (capillary) PCR was performed to study variability in the lengths of amplification products for the PC-IGS (36). The reaction volume was 20 μl and contained 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 mg of gelatin per ml, 3 mM $MgCl_2$, deoxynucleotide triphosphates (200 μM each), 5 pmol of each oligonucleotide primer, 0.5 U of *Tth* Plus thermostable DNA polymerase (Biotech International, Perth, Australia), and either 1 μl of cell lysate supernatant or 50 ng of genomic DNA. Reactions were carried out in heat-sealed positive-displacement pipette tips. The initial denaturation of template DNA was achieved by incubation at 94°C for 2 min. Reaction mixtures were then subjected to 40 cycles of 94°C for 5 s, 47°C for 10 s, and 72°C for 30 s in the FTS-1S capillary thermocycler (Corbett Research, Sydney, Australia). Large-scale DNA amplifications were required for successive restriction fragment analyses of the PCR products. One hundred-microliter reaction volumes contained 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 mg of gelatin per ml, 2.5 mM $MgCl_2$, deoxynucleotide triphosphates (200 μM each), 20 pmol of each primer, 2 U of *Tth* Plus thermostable DNA polymerase (Biotech International), and 2 μl of cell lysate or 100 ng of genomic DNA. Reaction mixtures were overlayed with mineral oil and incubated at 94°C for 5 min to denature the PCR template. The reaction tube was immediately subjected to 40 cycles of 20 s at 94°C, 30 s at 50°C, and 60 s at 72°C. All PCR products were analyzed by electrophoresis in 2.5% agarose in Tris-acetate-EDTA buffer or 6% polyacrylamide (acrylamide-bisacrylamide [19:1]) in Tris-borate-EDTA buffer.

Restriction endonuclease digestion of PC-IGS PCR product. Mineral oil was removed from reaction mixtures by chloroform-isoamyl alcohol (23:1) extraction. The aqueous phase was purified by using the Wizard PCR purification system (Promega, Madison, Wis.) to remove amplification reaction components, including unincorporated primers and nucleotides. PC-IGS amplification fragments were then digested separately with nine restriction endonucleases which recognise and cleave tetranucleotide motifs. Approximately 200 ng of PCR product was combined with 1.5 μl of the supplied corresponding enzyme buffer and 5 U of restriction enzyme in a 15-μl digest. Reaction mixtures were incubated overnight at the temperature suggested by the enzyme supplier. The DNA restriction enzymes *Alu*I, *Cfo*I, *Hae*III, *Hin*FI, *Msp*I, *Rsa*I, *Sau*3AI, *Scr*FI, and *Taq*I (Boehringer, Mannheim, Germany) were used to generate RFLP patterns specific to the cyanobacterial strains studied. The profile of a particular digest was used as a genotypic marker and formed the basis for RFLP typing to group cyanobacterial strains on the basis of the degree of common RFLP profiles.

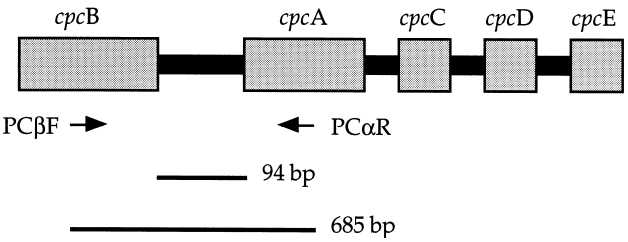

FIG. 1. Schematic representation of the PC operon. The entire operon is shown, but it is not drawn to scale. Black regions indicate IGS sequences; the open reading frames (PC subunits) are shown as shaded boxes. From *Anabaena* sp. strain PCC7120 (4), the length of the PC-IGS PCR product (with the primers and reaction conditions described in the text) is 685 bp, with an IGS of 94 bp. Arrows indicate the relative positions of PCR primers at highly conserved loci of the β and α subunits of the operon.

Phylogenetic analysis of RFLP data. The RFLP patterns generated were based on the primary structure of the PC operon and treated as observed phenotypes for the purpose of inferring relatedness among cyanobacteria. Phenetic analysis of these data was performed by calculating pairwise genetic distances from a binary matrix. Each strain or operational taxonomic unit (OTU) was identified by the presence or absence of an RFLP profile for each of the nine restriction digests. Distances were calculated by using the 18 algorithms supplied by the RAPDistance package developed by Armstrong et al. (3). All available metrics were used, and the resulting trees were constructed by using the neighbor-joining method and the NJTREE program (41). The RFLP profiles were also used for cladistic analysis in which character changes along branches of the derived tree were minimized. The rules of parsimony were applied to the profile data by using the Dollo parsimony method (20) supplied by the DOLLOP program in the PHYLIP package (version 3.53c) (22). Relatedness between strains was inferred on the basis of the number of shared homologous characters, i.e., RFLP profiles for this study. A single, strict consensus cladogram was reconstructed by the majority rule method from the equally parsimonious trees found (22). Outgroups were not available for this study; therefore, the phylogenetic trees presented should be considered unrooted.

RESULTS

Cyanobacterium-specific PCR and amplification fragment length polymorphisms (AFLPs) of the PC amplicon. Alignments of the available PC peptide and gene sequences were the basis for the design of two PCR primers which were nondegenerate for the current database sequences. This lack of primer redundancy was possible because of the presence of completely conserved regions within both the α and β subunits of the PC operon. The primers situated within these functional subunits and flanking the variable IGS were designated PCβF (5′-GGCTGCTTGTTTACGCGACA-3′), the forward primer, and PCαR (5′-CCAGTACCACCAGCAACTAA-3′), the reverse primer (Fig. 1). The primers contained no redundancies and had theoretical disassociation temperatures of 62 and 60°C, respectively. Non-PC sequences, either homologous or with up to 30% mismatch with the sequences of these oligonucleotides, were not detected in the current DNA sequence databases. On the basis of published DNA sequence information, *Anabaena* sp. strain PCC7120 possesses a PC-IGS of 94 bp and a total amplification fragment of 685 bp with the described PCR primers (4).

Optimal PCR conditions were found at a primer-template

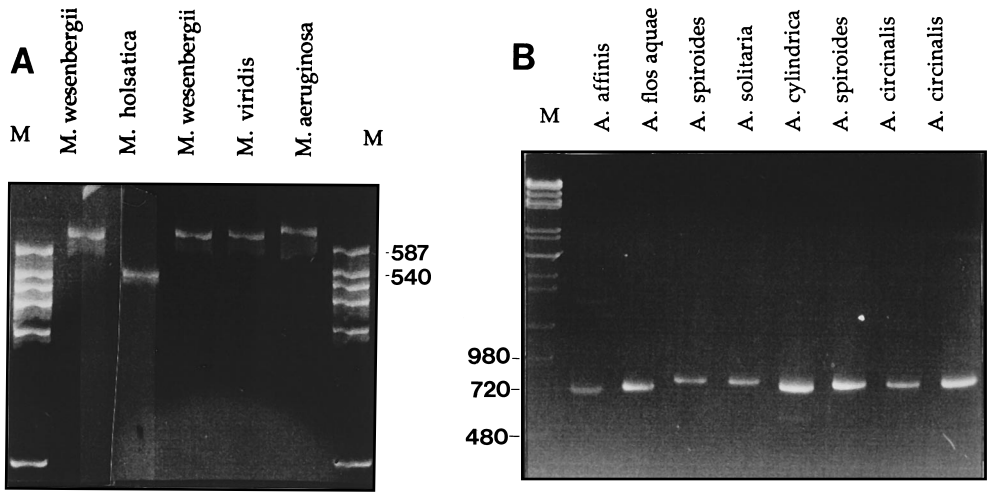

FIG. 2. Photographs of ethidium bromide-stained gels showing the amplification products (undigested) of strains of cyanobacteria. (A) Amplification products of *Microcystis* strains electrophoresed on a 6% polyacrylamide (19:1 cross-linked) gel in Tris-borate-EDTA buffer. Lanes M, molecular weight marker (in base pairs) pBR322 digested with *Hae*III. (B) AFLPs of the PC-IGS of *Anabaena* strains run on a 6% polyacrylamide (19:1 cross-linked) gel in Tris-borate-EDTA buffer. Lane M, molecular weight standard (in base pairs) SPP-1 digested with *Eco*RI.

annealing temperature of 50°C. This reaction provided reliable amplifications of the PC-IGS from cyanobacteria with no PCR products detected for noncyanobacterial templates. Sequencing of this PCR product from several strains confirmed, by genetic sequence homology, that the described PCR amplified the PC-IGS region of cyanobacteria (data not shown). Of the 44 cyanobacterial strains used in this study, all but 2 cultures gave consistent DNA amplification results. The PC gene fragments from *Nostoc commune* NIES24 and *Nostoc punctiforme* PCC73102 were unable to be amplified by a variety of protocols for PCR and template DNA extraction. These *Nostoc* strains have been used for successful amplifications of other genes, including the ca. 1,500-bp 16S rRNA gene (34a). No amplification product within the correct size range was seen for any noncyanobacterial environmental microorganism tested. Similarly, lysates of a green algae *Scenedesmus* sp., which also does not possess PC, gave no PCR product. The PC-containing rhodophyte strain did not act as a suitable template for this optimized PCR. The two species of cryptophytes used in this study produced similar amplification patterns, with multiple bands, by the described PCR. Changing the PCR stringency by decreasing or increasing the annealing temperature did not result in altered amplification of PC from red algae or the cryptophyte strains. The remaining 42 strains of cyanobacteria listed in Table 1 were suitable for reproducible amplification reactions.

Cyanobacterial strains produced amplification fragments that ranged between approximately 740 and 500 bp, with the majority of strains providing ca. 700-bp products. The varied sizes of PCR products, known as AFLPs, were analyzed for specificity to cyanobacterial systematics (Fig. 2). At the intergeneric level, there was no relationship between PCR product size and taxonomic placement. The range of AFLP sizes for members of the genera *Microcystis* and *Anabaena* overlapped and varied intragenerically by approximately 5% (Fig. 2). The PCR product of *Microcystis holsatica* NIES43 was markedly smaller, approximately 500 bp, than that of any other strain tested (Fig. 2A). Neither was the distribution of PCR fragment lengths specific to particular species within any of the genera studied. The observed variability of amplification fragments between strains was the only taxonomically applicable result observed after simple electrophoretic analysis of the PC-IGS amplification product. Intraspecific differences in PCR product size, combined with microscopic examination, were suitable for identification to the species level of classification. Because of the specificity of the PCR for amplifying cyanobacterial DNA, water and sediment samples were able to be analyzed directly for the presence of this microorganism among many other bacteria, protozoa, and algae (data not shown).

Cyanobacterial diversity measured by RFLPs of the PC amplicons. Large-scale PCR (100 μl) was performed with 42 strains, and the amplification products were purified. Strains *Anabaena circinalis* NIES41 and *Microcystis elabens* NIES42 consistently revealed multiple PCR products and were not included in RFLP analyses. All other cultures of cyanobacteria resulted in a single product after DNA amplification of the PC-IGS. Nine restriction endonucleases were used separately to generate restriction profiles of these PCR fragments, and all of these enzymes produced polymorphic patterns for the strains studied (Fig. 3). The enzymes *Hae*III and *Taq*I gave three patterns (a to c), while digests with *Rsa*I, *Sau*3AI, and *Scr*FI produced four patterns (a to d). For the cyanobacterial populations studied, *Hin*FI and *Msp*I gave five profiles and the enzymes *Alu*I (Fig. 3) and *Cfo*I produced eight different profiles. In total, the restriction cleavage of PCR products from 40 strains of cyanobacteria resulted in the production of 45 distinguishable profiles. Each of these profiles contributed to the cyanobacterial classification based on these nine restriction enzymes and collectively resulted in a cyanobacterial RFLP type.

From Table 1, it can be seen that the *Hae*III digest grouped members of the filamentous genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, *Oscillatoria*, and *Pseudanabaena* as a single profile (type c), whereas *Microcystis* species possessed varied patterns (type a, b, or c). Conversely, *Taq*I grouped *Microcystis* and *Oscillatoria* strains (type a) but delineated *Nodularia* and *Aphanizomenon* (type b) and *Cylindrospermopsis* (type c) strains. Other digests which characterized members of the genus *Microcystis* as a distinct taxonomic group were catalyzed by the enzymes *Hin*FI (type c), *Msp*I (type b), and *Rsa*I (type a). Although showing more heterogeneity, members of the genus *Anabaena* were also characterized

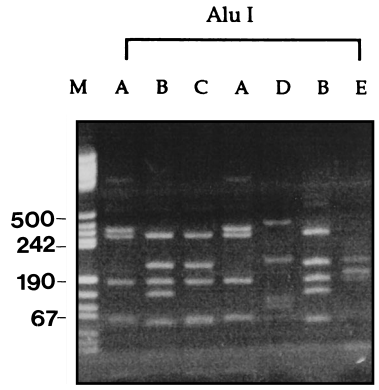

FIG. 3. Restriction digests of the PC-IGS PCR products of cyanobacteria electrophoresed on a 6% polyacrylamide (19:1 cross-linked) gel in Tris-borate-EDTA buffer and stained with ethidium bromide. This is an illustration of the highly discriminatory restriction endonuclease *Alu*I. Five of the eight potential genotypes (A to E) generated by this enzyme for the 40 strains studied are shown. The strains for the profiles shown are as follows, from left to right: NIES111, AWT001, NIES112, NIES111, AWT104, AWT107, and AWT105. Lane M, DNA molecular weight standard (in base pairs) pUCBM21 digested with *Hpa*II combined with pUCBM21 digested with *Dra*I and *Hin*dIII.

by these digests. The enzymes *Alu*I and *Cfo*I were highly discriminatory for members of the genera *Microcystis* and *Anabaena*, with strains distributed over a set of 15 possible profiles. *Hae*III was also able to discriminate between *Microcystis* strains on the basis of these genetic polymorphisms. At the species level, the current, morphology-based taxonomy was not supported by the RFLP data. For instance, the strains *Anabaena spiroides* NIES76 and *Anabaena solitaria* NIES80 had identical RFLP types for all nine enzymes, while *A. spiroides* NIES78 exhibited three RFLP profile differences compared with *A. spiroides* NIES76. The species classifications of *Microcystis aeruginosa* and *Microcystis wesenbergii* were also composed of several nonspecific RFLP typings. Similarly, strains designated *M. aeruginosa* were not revealed to be a homogenous group with respect to this molecular taxonomy. The specificity of this method was highlighted by the fact that for only three pairs of strains, previously classified to the same species level, was an identical RFLP grouping achieved. The delineation of strains was also possible for *Microcystis* isolates with respect to the geographical origin of each isolate. However, this RFLP genotyping did not discriminate between strains of *M. aeruginosa* (PCC7820 and PCC7005) on the basis of their levels of toxin production (Fig. 4 and 5). Duplicate amplifications and digestions of purified DNA and cell lysates for the same sample were done for several strains, with identical results on each occasion.

Phylogenetic analysis of PC PCR RFLPs. Molecular evolutionary distances were inferred by using 18 algorithms supplied by the RAPDistance package (3), and phenograms were constructed by the neighbor-joining method (41). The topologies of the trees by these clustering methods were identical, as were the relative distances between terminal OTUs which determined the tree branch lengths. One of the phenograms derived by using the algorithm of Nei and Li (33) is presented in Fig. 4. In this analysis, it was not possible to include outgroup taxa because of the specificity of the method; therefore, the trees presented are essentially unrooted. The data generated here were used as phenotypes, whereas RFLP data are typically used to infer nucleotide substitution rates, which manifest the presence or absence of defined restriction fragments, instead of restriction digest profiles. Characters, or RFLP profiles, for each strain and each restriction endonuclease were either present or absent. Thus, 45 binary characters were produced for the cyanobacteria studied. The phenetic relationships inferred revealed an essentially bifurcating phylogeny, with one cluster dominated by filamentous, heterocyst-forming strains and the other consisting mainly of unicellular cyanobacteria. At this level of morphology, the derived phylogeny is extremely robust. These clusters, named I and II, were further divisible into taxonomically relevant groups. Cluster I contained three branchings, designated IA, IB, and IC, which represented the populations of the filamentous, heterocyst-forming cyanobacterial genera *Anabaena* (IA), *Aphanizomenon* together with *Nodularia* (IB), and *Cylindrospermopsis* (IC). Cluster II was divided into two branches, with members of the unicellular genera *Synechococcus* and *Microcystis* (IIA) in one branch and members of the filamentous, non-heterocyst-forming genus *Oscillatoria* (IIB) in the other. Within *Microcystis* cluster IIA, there was no definite clustering for the morphological speciation of *M. aeruginosa*, *M. wesenbergii*, *M. viridis*, and *M. holsatica*. *Microcystis* strains AWT104, AWT105, and AWT107 could be reassigned as freshwater *Synechococcus* strains on the basis of this PC-IGS RFLP phylogeny. More importantly, no genetic distance separated *M. aeruginosa* PCC7820 and *M. aeruginosa* PCC7005, which synthesize significantly different levels of heptapeptide hepatotoxin (11a). Unicellular *M. aeruginosa* AWT114 spuriously outgroups within the filamentous group, and studies are being performed to further characterize this strain.

In addition, these data were applied to cladistic analysis; the resulting phylogenetic trees are presented in Fig. 5. The relationships among cyanobacteria, as indicated by tree topology, were essentially identical by the genetic distance and parsimony methods for tree construction. The Dollo parsimony cladogram supported the expected finding that filamentous, heterocyst-forming strains reside on a separate and distinct phyletic line from that of unicellular, non-heterocyst-forming cyanobacteria. Terminal branching of the heterocyst-forming lineage revealed discrimination among members of the genera *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, and *Pseudanabaena*. Strains of *Anabaena* were dispersed throughout cluster I, as seen by the phenetic approach. Similarly, with the *Microcystis* strains of cluster II, the relative position of OTUs at terminal nodes was constant in both phylogenetic analyses. Members of the genus *Oscillatoria* were also retained within the cluster of unicellular cyanobacteria.

DISCUSSION

The development of a molecular method for the identification of cyanobacteria is essential for the rapid and accurate analysis of members of a bloom population and for the elucidation of cyanobacterial systematics. The method described in this paper made it possible to amplify a fragment of the PC gene containing the IGS from cyanobacteria commonly associated with toxic bloom events. Successive restriction enzyme digestions provided signature arrays of RFLP profiles, which achieved the identification of cyanobacterial isolates and inferences of cyanobacterial phylogeny. Since the PC gene is found in freshwater environments almost exclusively in cyanobacteria (7, 18, 25), it provides a means of rapid and direct identification of cyanobacterial strains in samples containing complex microbial communities (6). Additionally, the PC gene contains a relatively large IGS, compared with those of other photosynthetic pigment genes of cyanobacteria, which has the potential of being highly polymorphic.

For the detection and identification of microorganisms,

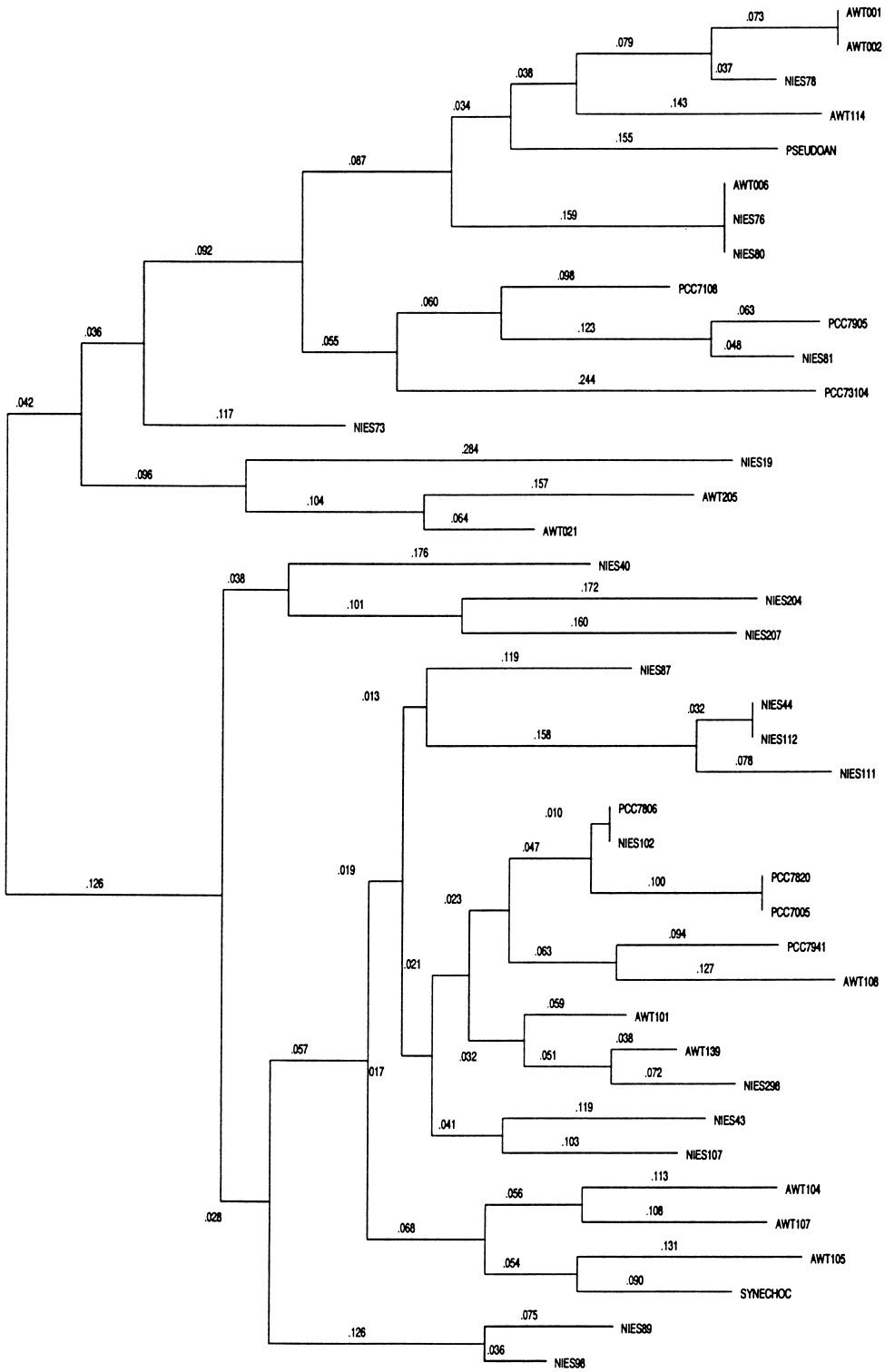

FIG. 4. Phenogram obtained by using the algorithm of Nei and Li (33) to calculate the genetic distances among cyanobacterial strains (3). The genetic distance from the node, based on the 45 possible RFLP profiles, is indicated above each branch. The phenetic distance tree was constructed by the neighbor-joining method (41). The culture collection numbers used in this tree are abbreviated from the strain designations shown in Table 1.

methods which rely on the analysis of specific virulent, pathogenic, or metabolic processes of the group being studied have been developed (1, 12, 16). Our method is based on the conservation of the light-harvesting pigment PC in cyanobacteria to classify and differentiate this large and diverse family of bacteria. This study was confined to the medically, economically, and ecologically important cyanobacteria which are responsible for the formation of toxic blooms in freshwater and

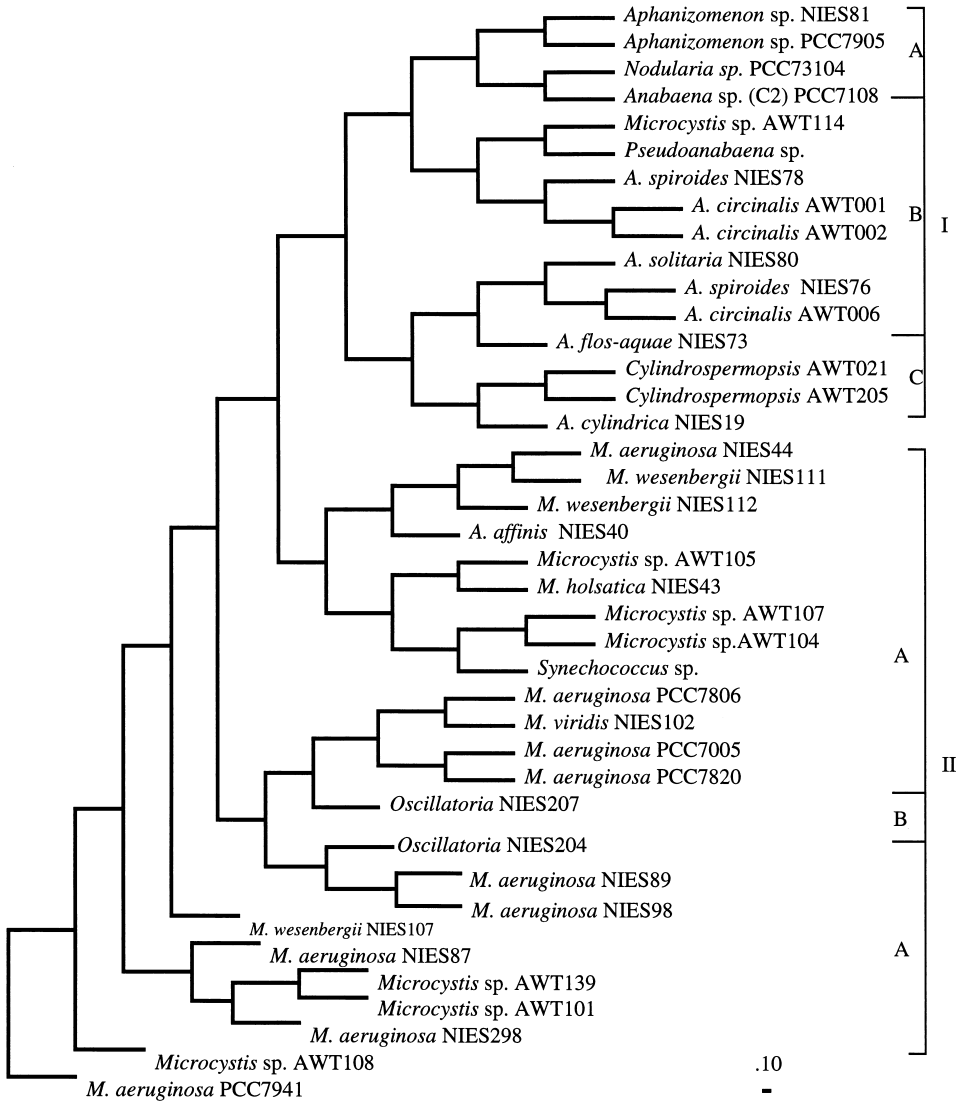

FIG. 5. Strict consensus cladogram constructed from 45 binary characters by applying the rules of Dollo parsimony. The presence or absence of PC-IGS restriction digest patterns for each of the 40 strains used was used as the basis for inferring cyanobacterial evolution. Twenty-eight equally parsimonious trees were obtained by using the DOLLOP program from the PHYLIP package (22) and were used to produce the tree presented. The relative genetic divergence is indicated by the scale. The culture collection numbers used in this tree are abbreviated from the strain designations shown in Table 1.

brackish water in Australia and other parts of the world. By either genomic DNA extraction or simple cell lysis, unicyanobacterial cultures were suitable as PCR templates for the amplification of a portion of the PC gene. Polymorphisms in the DNA sequence of this gene were readily detected by RFLP analysis. Strains of all of the genera tested, except *Nostoc* strains, were successfully amplified. Two strains from this genus were tested, and the lack of PCR products indicated an unexpectedly low level of homology between the *Nostoc* genome and the designed PC primer set. Strains of the genus *Nostoc* are closely related to and often confused with those of the genus *Anabaena* (11). DNAs from strains of green algae, several heterotrophic bacteria, and PC-containing marine red algae gave no PC gene amplification. PC-containing cryptophyte strains provided consistent amplification of multiple PCR products. Samples from recently isolated cyanobacteria and samples from strains which have been subjected to long-term culturing were equally applicable to the described method. Apart from being time-consuming, previous methods of analyzing RFLPs by genome digestion, Southern transfer, and probe hybridization were not used because of the restriction modifications, such as DNA methylation, of many cyanobacteria, including *Microcystis* spp. (data not shown) (37).

A total of 45 DNA profiles were obtained by digestions of the PC amplification products of 40 strains with nine restriction endonucleases. The combined genotypes for each strain formed the basis of a method for delineating cyanobacteria. The ability to discriminate between close and distantly related strains in this study was illustrated by the relative clustering of OTUs in various phylogenetic analyses. By utilizing the genotypic pattern produced by each enzyme as an identifying marker, 45 characters with binary states were used to infer relatedness among cyanobacteria. The presence or absence of any particular digestion pattern in a strain's overall PC genotypic profile was used to construct more than 19 phylograms. Both cladistic and phenetic approaches to the data were em-

ployed to reveal a single phylogeny for bloom-forming cyanobacteria. Calculations of genetic distances between taxa and the parsimonious tree derived from the number of shared homologous characters supported a common molecular evolution of these cyanobacteria based on mutational changes in the PC gene.

Because the sizes of PC gene amplification products of different strains were variable, it was not possible to use the presence or absence of a restriction site as a shared character between any of the genera studied. It was determined that equivalent electrophoretic band migration and conservation of shared restriction endonuclease recognition sites would not be a valid assumption for this set of data. Rather, the observed DNA profiles were used as independent characters, and as with the Dollo parsimony method employed for tree construction, it was assumed that the loss of a character (or digestion pattern) was more likely than the gain of that character (21). This approach provides polarization of character states and hence allows ancestral estimation for a tree with minimal state changes along its branches. The similarity matrix algorithms used indicated the relative genetic or evolutionary distances among the OTUs in this phylogeny (Fig. 4).

All tree topologies by either phenetic or cladistic analyses were identical. At a gross level, the phylogeny presented supported the current classification of cyanobacteria to the genus level. The RFLP profiles and phylogenetic trees grouped cyanobacteria into distinct subgroups with relevance to obvious morphological features. These results represent the first analysis of genetic diversity within members of the genus *Microcystis*. The unicellular or non-heterocyst-forming strains tested were confined to the proposed cluster II. This group contained members of the genera *Microcystis*, *Synechococcus*, and *Oscillatoria*. Partial 16S rRNA gene sequencing data do not support this strict clustering, showing *Oscillatoria* strains to be a polyphyletic lineage closely related to different species of other filamentous and unicellular cyanobacteria (23). The clustering of *Microcystis* and *Synechococcus* strains in cluster IIA is supported both by previous sequences of genes coding for 16S rRNAs and by random amplified polymorphic DNA-inferred phylogenies (34, 35). Within this cluster, *Microcystis* strains occupied multiple genetic lineages, exhibiting no relation to their traditional taxonomic designations. *M. aeruginosa*, *M. wesenbergii*, *M. viridis*, and *M. holsatica* strains appeared to be arbitrarily positioned, with as much genetic divergence between clades as within each terminal node. These data did not support the current *Microcystis* taxonomy based on minor morphometric differences (27). The relative ordering of *Microcystis* strains was not similar to that found by random amplified polymorphic DNA analysis (34), which may have been due to method sensitivity or independent evolution of the PC gene. The heterogeneity observed may be explained by the fact that the taxonomic classifications of the strains studied are those of several different culture collections. Morphological plasticity, which occurs during sample isolation and under conditions of extended laboratory culturing, can also affect consistent and reliable classifications of cyanobacteria (15). Similarly, the trees presented in our study do not delineate *Microcystis* strains on the basis of hepatoxin production. It is unknown whether all *Microcystis* strains are toxigenic or exhibit varying levels of expression in response to environmental stimuli. Interestingly, many of the *Microcystis* isolates from Australian waters appeared to be most closely related to *Synechococcus* strains, which are found in both marine and freshwater environments. Previous reports show *Synechococcus* strains to be heterogeneous with respect to phylogeny and habitat, with marine strains as different from each other as they are from freshwater strains (49).

Cyanobacteria existing as trichomes and possessing differentiated cell types to enable anaerobic nitrogen fixation were contained within the proposed cluster I. *Anabaena* strains were dispersed throughout this cluster, once again indicating the inadequacies of the current taxonomy, which relies on the subjective observation of microscopic morphology. Strains of *A. circinalis*, *A. cylindrica*, *A. flos-aquae*, *A. solitaria*, and *A. spiroides* were placed within this group with no relation to their species designations. As described above for *M. aeruginosa* and *M. wesenbergii* strains, strains of *A. circinalis* and *A. spiroides* were as different from each other as they were from supposedly more distantly related strains. *A. affinis* NIES40 appears to have been misclassified, as it showed consistent placement outside the heterocyst-forming clade. The *Pseudanabaena* strain in this study appeared to be contained within a tentative *Anabaena* cluster but has previously been assigned to a separate and distinct branch on the basis of partial 16S rRNA gene sequences (34a). The positioning of a *Nodularia* strain in this clade is consistent with 16S rRNA gene sequence information. Members of the genera *Aphanizomenon* and *Cylindrospermopsis* have not previously been described in the context of molecular phylogeny. Both strains of *Aphanizomenon* resided on the same terminal branch, most closely related to both the *Nodularia* strain and a member of the *Anabaena* cluster 2 (PCC7108). The *Cylindrospermopsis* strains isolated from Australian waters were monophyletic and exhibited quite early divergence from the rest of cluster I. *Anabaena* strains NIES73 and NIES19 represented genetically distinct OTUs not contained in the main *Anabaena* clade and require further analysis to provide reliable taxonomic placement(s).

Ideally, the most suitable detection method for strains within cyanobacterial blooms would involve the direct analysis of the genes associated with hepatotoxin and neurotoxin production in these microorganisms. At present, information regarding the biosyntheses of these molecules in cyanobacteria is not available. The methods developed in this study were able to discriminate among strains from important toxic and bloom-forming genera of cyanobacteria. Additionally, it will be possible to apply this method of analyzing cyanobacterial populations to differentiate isolates from separate bodies of water in the same geographical region. Strains representing a single genus were readily delineated, although the inferred phylogeny did not support the currently used morphological taxonomy. The results indicate that *Anabaena* and *Microcystis* species, as they are currently classified, are polyphyletic and that a review of traditional classifications may be necessary. The trees obtained by using genetic distances and a similarity matrix to compare RFLP profile data did not infer the relative evolution of the two cyanobacterial clusters as they relate to each other, although they did indicate a more distantly related and common ancestor for both groups. Alternatively, the most parsimonious tree constructed from the data indicated that the filamentous cyanobacteria of cluster I were directly derived from an ancestral clade containing both unicellular and filamentous, non-heterocyst-forming morphotypes, deeply branched within cluster II. Phylogeny by this molecular technique correlates with the grouping of cyanobacteria at the intergeneric level (23, 46).

This proposed tool for analyzing cyanobacterial systematics is based on a stable genetic character, the PC gene locus. The sequence differences in this gene among cyanobacterial isolates were readily and rapidly assayable by PCR-RFLP to produce taxonomically significant cyanobacterial PC genotypes. The heterogeneity exhibited by cyanobacteria in this study and

detected by RFLPs of the PC gene is being investigated further by PC gene sequencing. The DNA sequence of this region will reveal the extents and locations of PC polymorphisms. Sequencing may also provide data for designing specific primers for the identification of cyanobacterial strains within mixed cyanobacterial populations in environmental samples.

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REFERENCES

1. **Akopyanz, N., N. O. Bukanov, T. U. Westblom, and D. E. Berg.** 1992. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen Helicobacter pylori. Nucleic Acids Res. **20:**6221–6225.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Armstrong, J., A. Gibbs, R. Peakall, and G. Weiller.** 1994. The RAPDistance package. ftp://life.anu.edu.au (150.203.38.74) pub/molecular_biology/rapdpack.exe.
4. **Belknap, W. R., and R. Hazelkorn.** 1987. Cloning and light regulation of expression of the phycocyanin operon of the cyanobacterium *Anabaena*. EMBO J. **6:**871–884.
5. **Ben-Amotz, A., T. G. Tornabene, and W. H. Thomas.** 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. **21:**72–81.
6. **Britschgi, T. B., and S. J. Giovannoni.** 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **57:**1707–1713.
7. **Bryant, D. A.** 1982. Phycoerythrocyanin and phycoerythrin: properties and occurrence in cyanobacteria. J. Gen. Microbiol. **128:**835–844.
8. **Carmichael, W. W.** 1992. Cyanobacteria secondary metabolites—the cyanotoxins. J. Appl. Bacteriol. **72:**445–459.
9. **Castenholz, R. W.** 1988. Culturing methods for cyanobacteria. Methods Enzymol. **167:**68–95.
10. **Castenholz, R. W., and J. B. Waterbury.** 1989. Oxygenic photosynthetic bacteria. Group I. Cyanobacteria, p. 1710–1728. *In* J. T. Staley, M. P. Bryant, N. Pfenning, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams and Wilkins Co., Baltimore.
11. **Caudales, R., and J. M. Wells.** 1992. Differentiation of free-living *Anabaena* and *Nostoc* cyanobacteria on the basis of fatty acid composition. Int. J. Syst. Bacteriol. **42:**246–251.

11a. **Chandresena, N.** Personal communication.

12. **Darrasse, A., S. Priou, A. Kotoujansky, and Y. Bertheau.** 1994. PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. Appl. Environ. Microbiol. **60:**1437–1443.
13. **de Lorimier, R., D. A. Bryant, R. D. Porter, W.-Y. Liu, E. Jay, and S. E. Stevens, Jr.** 1984. Genes for the α and β subunits of phycocyanin. Proc. Natl. Acad. Sci. USA **81:**7946–7950.
14. **de Lorimier, R., S. M. Wilbanks, and A. N. Glazer.** 1993. Genes of the R-phycocyanin II locus of marine *Synechococcus* spp., and comparison of protein-chromophore interactions in phycocyanins differing in bilin composition. Plant Mol. Biol. **21:**225–237.
15. **Doers, M. P., and D. L. Parker.** 1988. Properties of *Microcystis aeruginosa* and *M. flos-aquae* (Cyanophyta) in culture: taxonomic implications. J. Phycol. **24:**502–508.
16. **Dong, L., C. W. Sun, K. L. Thies, D. S. Luthe, and C. H. Graves, Jr.** 1992. Use of polymerase chain reaction to detect pathogenic strains of *Agrobacterium*. Phytopathology **82:**434–439.
17. **Dubbs, J. M., and D. A. Bryant.** 1991. Molecular cloning and transcriptional analysis of the *cpe* BA operon of the cyanobacterium *Pseudanabaena* sp. PCC7409. Mol. Microbiol. **5:**3073–3085.
18. **Ernst, A.** 1991. Cyanobacterial picoplankton from Lake Constance. I. Isolation by fluorescence characteristics. J. Plankton Res. **13:**1307–1312.
19. **Fay, P., and C. Van Baalen.** 1987. The cyanobacteria. Elsevier, Amsterdam.
20. **Felsenstein, J.** 1983. Parsimony in systematics: biological and statistical issues. Annu. Rev. Ecol. Syst. **14:**313.
21. **Felsenstein, J.** 1985. Confidence limits on phylogenies with a molecular clock. Syst. Zool. **34:**152–161.
22. **Felsenstein, J.** 1989. PHYLIP. Phylogeny inference package. Cladistics **5:**164–166.
23. **Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane, and N. R. Pace.** 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170:**3584–3592.
24. **Glazer, A. N.** 1984. Phycobilisomes—a macromolecular complex optimised for light energy transfer. Biochim. Biophys. Acta **748:**29–51.
25. **Glazer, A. N.** 1989. Light guides. Directional energy transfer in a photosynthetic antenna. J. Biol. Chem. **264:**1–4.
26. **Herdman, M., M. Janvier, J. B. Waterbury, R. Rippka, and R. Y. Stanier.** 1979. DNA base composition of cyanobacteria. J. Gen. Microbiol. **111:**63–71.
27. **Komarek, J.** 1991. A review of water-bloom forming Microcystis species, with regard to populations from Japan. Arch. Hydrobiol. Supplbd. **64:**115–127.
28. **Komarek, J., and K. Anagnostidis.** 1986. Modern approach to the classification system of cyanophytes. 2. Chroococcales. Arch. Hydrobiol. Supplbd. **73:**157–226. (Algol. Stud., vol. 43.)
29. **Komarek, J., and K. Anagnostidis.** 1989. Modern approach to the classification system of cyanophytes. 4-Nostocales. Arch. Hydrobiol. Supplbd. **82:**247–345. (Algol. Stud., vol. 56.)
30. **Lachance, M.-A.** 1981. Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. Int. J. Syst. Bacteriol. **31:**139–147.
31. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. Methods Enzymol. **155:**335–350.
32. **National Institute for Environmental Studies.** 1991. Microbial culture collection, list of strains, 3rd ed. Environmental Agency, Tsukuba, Japan.
33. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76:**5269–5273.
34. **Neilan, B. A.** 1995. Identification and phylogenetic analysis of toxigenic cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. Appl. Environ. Microbiol. **61:**2286–2291.

34a. **Neilan, B. A.** Unpublished data.

35. **Neilan, B. A., P. T. Cox, P. R. Hawkins, and A. E. Goodman.** 1994. 16S ribosomal RNA gene sequence and phylogeny of toxic *Microcystis* sp. (cyanobacteria). DNA Sequence **4:**333–337.
36. **Neilan, B. A., A. Gurvitz, D. Leigh, L. Y. C. Lai, and B. McDonald.** 1993. Rapid preparation of limited biological samples for capillary PCR. PCR Methods Applic. **2:**261–262.
37. **Padhy, R. N., F. G. Hottat, M. M. Coene, and P. P. Hoet.** 1988. Restriction analysis and quantitative estimation of methylated bases of filamentous and unicellular cyanobacterial DNAs. J. Bacteriol. **170:**1934–1939.
38. **Rippka, R.** 1988. Recognition and identification of cyanobacteria. Methods Enzymol. **167:**28–67.
39. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
40. **Rippka, R., and M. Herdman.** 1992. Pasteur culture collection (PCC) of cyanobacterial strains in axenic culture, vol. 1. Catalogue of strains. Institut Pasteur, Paris.
41. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
42. **Schopf, W., and B. Packer.** 1987. Early Archaen (3.3 billion-to 3.5 billion-years old) microfossils from the Warranoona Group, Australia. Science **237:**70–73.
43. **Stulp, B. K., and W. T. Stam.** 1984. Genotypic relationships between strains of *Anabaena* (Cyanophyceae) and their correlation with morphological affinities. Br. Phycol. J. **19:**287–301.
44. **Tandeau de Marsac, N., D. Mazel, T. Damerval, G. Guglielmi, V. Capuano, and J. Houmard.** 1988. Photoregulation of gene expression in the filamentous cyanobacterium *Calothrix* sp. PCC7601: light harvesting complexes and cell differentiation. Photosynth. Res. **18:**99–132.
45. **Tatsumi, K., M. F. Watanabe, and M. Watanabe.** 1991. Allozyme divergence in *Anabaena* (Cyanophyceae) and its taxonomic inference. Arch. Hydrobiol. Supplbd. **92:**129–140. (Algol. Stud., vol. 64.)
46. **Wilmotte, A.** 1994. Molecular evolution and taxonomy of the cyanobacteria, p. 1–25. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
47. **Wilmotte, A., and S. Golubic.** 1991. Morphological and genetic criteria in the taxonomy of Cyanophyta/Cyanobacteria. Arch. Hydrobiol. Supplbd. **92:**1–24. (Algol. Stud., vol. 64.)
48. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
49. **Wood, A. M., and D. Townsend.** 1990. DNA polymorphisms within the WH7803 serogroup of marine *Synechococcus* spp. (cyanobacteria). J. Phycol. **26:**576–585.