Curr. Issues Mol. Biol. (2002) 4: 1-11.

The Molecular Evolution and DNA Profiling of Toxic Cyanobacteria

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Abstract

Rapid and sensitive methods for the detection and genetic characterization of cyanobacteria have been developed based on DNA amplification techniques. This article describes the molecular methods that have been used to characterize cyanobacteria and their use as tools to identify toxin-producing strains. Different species and strains were compared using restriction fragment length polymorphism (RFLP) of amplified fragments of the phycocyanin gene and the 16S-23S rRNA internal transcribed spacer.

Introduction

Current protocols for cyanobacterial classification rely on the observation of morphological parameters. Many of the biochemical (e.g. range of carbon substrates for heterotrophic growth) and physiological properties used in prokaryotic systematics are not appropriate markers for the study of cyanobacterial taxonomy. Therefore strain discrimination based on relatively simple and often minor morphological differences has been used to describe this immensely diverse bacterial phylum. Expression of phenotypes in cyanobacteria, as with other organisms, is often affected by environmental conditions. Typical manifestations of this are seen during laboratory selective culturing of cyanobacterial isolates, exemplified by the loss of gas vacuoles and colonial habit in planktonic strains (1-3). Hence classifications of cyanobacteria, and certain other prokaryotes, based solely on morphology has been described as artificial and arbitrary.

For more than 150 years cyanobacteria were considered to be eukaryotic algae prior to the efforts of Gibbons and Murray (4) and Stanier *et al.* (5). The inclusion of cyanobacteria under the bacteriological code resulted in the collection of strains as pure cultures instead of herbarium samples, as required for botanical systematics. More importantly, this recognition of cyanobacteria as prokaryotes allowed the postulation of cyanobacterial evolution for taxa within the radiation, with regards to other bacteria, and to other photosynthetic organisms (6). The ubiquity and relative conservation of the 16S rRNA gene across life forms makes it an appropriate molecule for inferring phylogeny. Comparisons of 16S rRNA gene sequences has supported the previously described affiliations between cyanobacteria and related organisms. Specifically, members of the cyanobacterial cluster are distantly related to the Archaea (*Methanococcoides*) and show a shallow branching from the Gram-positive bacteria (*Bacillus subtilis*) lineage. The cyanobacteria, as shown in other studies (7, 8), are not the most ancestral of bacterial groups even though this may be indicated by available microfossil evidence.

The relationships between cyanobacteria and photosynthetic organelles have also gained further confirmation by molecular systematics. Both the chloroplast of *Euglena gracilis* and the cyanelle of *Cyanophora paradoxa* form a distinct cluster closely related to the cyanobacteria. It appears that there was a common progenitor to both the photosynthetic organelle and cyanobacterial groups (9). It has been shown that green chloroplast 16S rRNA sequences form a monophyletic lineage and that these origins are well placed within the cyanobacterial radiation (8, 10). Alternatively, oligonucleotide cataloguing revealed divergence of the chloroplast and cyanelle lineages prior to establishment of the diversity shown for extant specimens of cyanobacteria (11). In fact, a primary bifurcation of the derived 16S rRNA gene phylogeny (disregarding outgroups) shows a definite clustering of bacterial oxygenic phototrophs external to that of the organelles, whether they be more physiologically related to cyanobacteria or prochlorophytes. Woese (7) has also identified sequence signatures based on the 16S rRNA primary structure which are shared by the cyanobacteria, *Prochloron*, and chloroplasts.

It appears that the prochlorales (*Prochloron* sp.) originated entirely from within the cyanobacterial radiation. Upon closer examination, a single ancestral taxon was the progenitor for filamentous, and heterocystous cyanobacteria (*Nostoc* PCC7120) and a cluster containing the prochlorophyte and *Synechocystis* lineages. Phylogenetic inferences by Wilmotte (10), based on 16S rRNA sequences, confirm this clustering of *Prochloron* sp. with strains of *Synechocystis*. However, studies on the molecular diversity of this group of bacteria, which contain both chlorophyll *a* and *b*, has revealed it to be polyphyletic with comparatively large genetic distances between representative strains (12-14).

Apart from 16S rRNA sequencing (8-10) few systems for classification, based on molecular genetics, have been proposed for cyanobacterial genera. This fact is particularly pertinent for those genera of toxin-producing cyanobacteria (15). Morphological differences between the proposed orders of cyanobacteria can be quite marked (2), although within these sections there are a number of specific taxonomic problems. These include the delineation of the genus *Microcystis* from *Synechocystis*, *Anabaena* from *Nostoc*, as well as *Anabaena* from the genera *Aphanizomenon, Cylindrospermopsis,* and *Nodularia*. In addition, the benthic and planktonic cyanobacteria of the

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Table 1. Primers for the amplification of the cyanobacterial 16S-23S rRNA ITS and phycocyanin operon

Name	Sequence[a]	Locus	Tm(°C)[b]
rRNA ITS			
23SRITS	TAGCAGGAAACAGCTATGAC-CCTCTGTGTGCCTAGGTATCC	26-45 (23S rRNA)	58
16SCITS	TGTAAAACGACGGCCAGTGAA-GTCGTAACAAGG	1475-1505 (16S rRNA)	94
Phycocyanin gene			
PCβF	GGCTGCTTGTTTACGCGACA	*cpcB* gene	57
PCαR	CCAGTACCACCAGCAACTAA	*cpcA* gene	55

[a] Oligonucleotide sequences written in 5' to 3' orientation. Underlined sequences are those of the linked universal sequencing primers which were attached to assist in successive sequencing of PCR products.
[b] Theoretical primer disassociation temperatures.

order *Oscillatoriales* present difficulties for accurate identification and classification. Amplification and restriction enzyme digestion or sequencing of PCR products has provided a specific method for the delineation of cyanobacterial genera (16-20). In particular, the genetic polymorphisms detected in the phycocyanin (PC) operon and the 16S-23S ribosomal RNA internal transcribed spacer (rRNA ITS) readily define genus boundaries and show varying degrees of concordance with traditional classification schemes. Molecular approaches to cyanobacterial identification are not influenced by ecological variables and in many instances do not require axenic or unicyanobacterial cultures for analysis. Indeed, due to the sensitivity afforded by DNA amplification technology, minute sample sizes, containing little cyanobacterial biomass, are required for the generation of genetic profiles.

Specific procedures involving DNA transfer and probe hybridisation have been previously employed, and allow the delineation of closely related and potentially toxic cyanobacteria (3, 21-24). However, most analyses of nucleic acids as molecular markers for cyanobacteria have focused on the relatively amorphous, unicellular genus *Synechococcus* and the symbiotic filamentous genera *Anabaena* and *Nostoc*. These experiments were based on heterologous gene probe hybridisation to RFLPs of genomic DNA. RFLP data, corroborated by 16S rRNA sequences (8, 11, 25), show that diversity between marine and freshwater strains of *Synechococcus*, representing separate serogroups, was as great as that seen in the entire cyanobacterial radiation (21, 23, 24, 26). Similarly, RFLPs detected in the genomes of filamentous cyanobacteria revealed the presence of multiple lineages of symbiotic cyanobacteria associated with the *Azolla* fern (27), cycads (28) and angiosperms (29). These protocols, and hence their utility for cyanobacterial classification are limited by the complex experimental procedure, including a necessity for large quantities of DNA, and the lack of informative genotypes observed.

DNA amplification technology, combined with sequences of cyanobacterial genes, has provided an opportunity to design and develop rapid and sensitive protocols for detection and delineation of cyanobacterial strains. This article describes molecular methods which provide descriptive DNA profiles, composed of phylogenetic characters, which were appropriate for the inference of

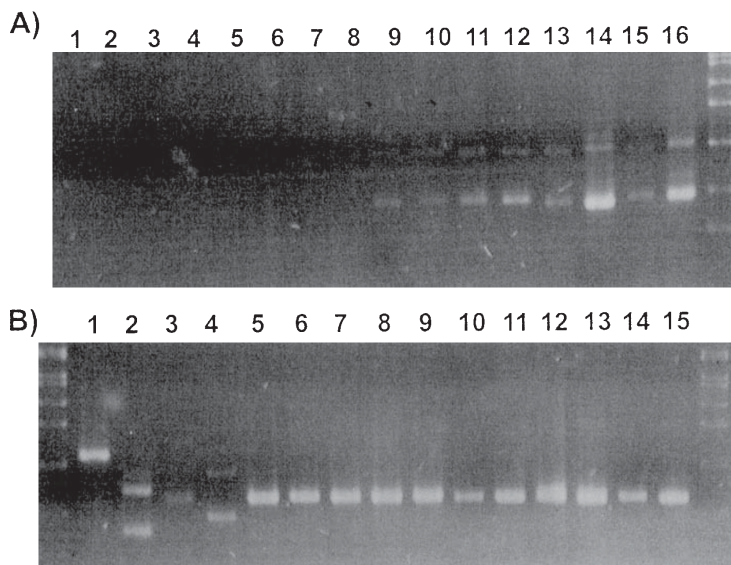

Figure 1. Specific amplification fragment length polymorphisms of the 16-23S ITS for the genera of cyanobacteria studied. Agarose gels (1% normal/1.5% low melt) were electrophoresed in TAE buffer and stained with ethidium bromide before photography using UV transillumination. A. Lanes 1 to 16 are the PCR products from *Vibrio* S14, *Campylobacter jejuni*, *Helicobacter pylori*, *Proteus vulgaris*, *Klebsiella pneumonii*, *Lactobacillus fermentum*, *Rhizobium* sp. NU259, *Aeromonas* sp. A234, *A. circinalis* NIES41, *A. circinalis* AWT001, *A. circinalis* AWT006, *A. affinis* NIES40, *A. solitaria* NIES80, *A. flos aqua* NIES73, *A. spiroides* NIES76, and *Aphanizomenon flos aqua* NIES81, respectively. Lane 17 is the molecular weight marker SPP-1/*Eco*RI (marker bands of 720 and 480 bp approximately comigrate with the major and minor ITS bands of *Anabaena*). B. Lanes 1 and 17 are SPP-1/*Eco*RI. Lanes 2 to 16 correspond to PCR products from *Pseudanabaena* sp. (AWT), *Cylindrospermopsis raciborskii* AWT205, *Nodularia* PCC73104, *Plectonema* sp. (UNSW), *Microcystis aeruginosa* strains AWT139, PCC7806, PCC7820, PCC7941, PCC7005, NIES44, NIES298, NIES98, *Microcystis wesenbergii* strains NIES107, NIES111, and *Microcystis viridis* NIES102, respectively. Figure reproduced with permission (9).

Table 2. Diagnostic RFLP types for cyanobacterial genera

Genus	Restriction profile[a] of amplified PC-IGS genes digested with:								
	***Alu*I**	***Cfo*I**	***Hae*III**	***Hin*FI**	***Msp*I**	***Rsa*I**	***Taq*I**	***Scr*FI**	***Sau*3AI**
Anabaena	*	*	c	a	a	c	c	*	b
Aphanizomenon	b	*	c	a	a	c	b	d	a
Cylindrospermopsis	c	h	c	*	c	a	c	*	c
Microcystis	*	*	*	c	b	a	a	*	*
Nodularia	d	h	c	a	a	b	b	b	d
Oscillatoria	c	c	*	*	b	c	a	*	b
Pseudanabaena	h	c	c	d	a	c	c	b	b
Synechococcus	c	e	a	c	d	a	a	b	a

[a] Multiple RFLP profiles designated by the asterisk (*) exist for strains within these genera using the stated restriction endonuclease. The different patterns detected with each enzyme from 42 strains analysed are designated by lowercase letters (52).

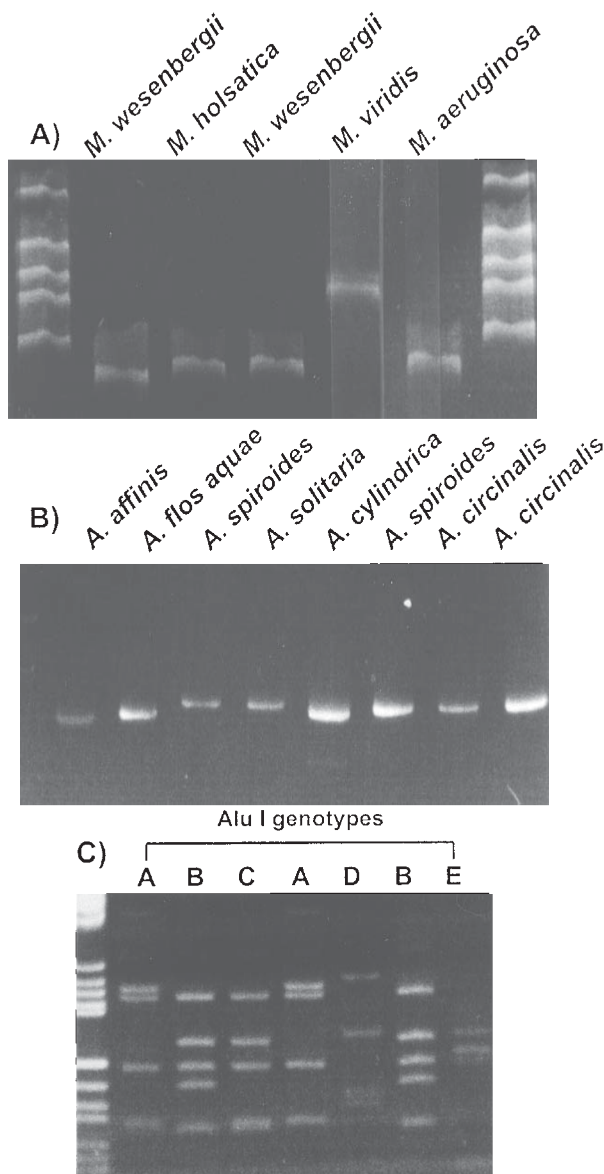

Figure 2. Ethidium bromide stained gels showing the amplification products (undigested) for strains of cyanobacteria. A. Amplification products for strains of *Microcystis* electrophoresed on a 6% polyacrylamide (19:1 crosslinked) gel in TBE buffer. M=molecular weight marker pBR322/*Hae*III. B. Amplification products (AFLPs) of the PC-IGS for strains of the genus *Anabaena* run on a 6% polyacrylamide gel (19:1 crosslinked) gel in TBE buffer. Molecular weight standard (M) is SPP1/*Eco*RI. C. Restriction digests of the PC-IGS PCR products of cyanobacteria electrophoresed on 6% polyacrylamide (19:1 crosslinked) gel in TBE buffer and stained with ethidium bromide. Illustration of the highly discriminatory restriction endonuclease *Alu*I used in the present study. Five of the eight potential genotypes generated by this enzyme for the 40 strains studied are shown. *Microcystis aeruginosa* strains for the profiles shown are, from left to right; NIES111, AWT001, NIES112, NIES111, AWT104, AWT107, and AWT105. DNA molecular weight standard (M) is pUCBM21/*Hpa*II-pUCBM21/*Dra*I/*Hind*III. Figure reproduced with permission (18).

relatedness and evolution of cyanobacterial taxa. These phylogenetic characters provided differing levels of strain discrimination. The sensitivity of the methods described here are a reflection of the number of restriction endonuclease recognition sites in cyanobacterial- specific gene amplification products, in particular, the 16S-23S rRNA ITS and the phycocyanin operon. The DNA profiles generated depict genomic polymorphisms and allow the unambiguous identification of toxic cyanobacteria.

Cyanobacterial Specific Gene Amplification and RFLP Analysis

A cyanobacterial specific forward PCR primer was designed by targeting the 3' region of cyanobacterial 16S rRNA gene sequences (Table 1). In combination with a bacterial consensus reverse 23S rRNA gene primer the selective amplification of the 16S-23S rRNA ITS from cyanobacteria within a consortium of other microorganisms was possible (9). Amplification of this region resulted in the detection of heterogeneity, with respect to the size of the PCR product, across several cyanobacterial genera. The genus *Anabaena*, for example, possessed two distinct PCR products, indicating the presence of multiple, heterologous rRNA operons. On the other hand, the genus *Microcystis*

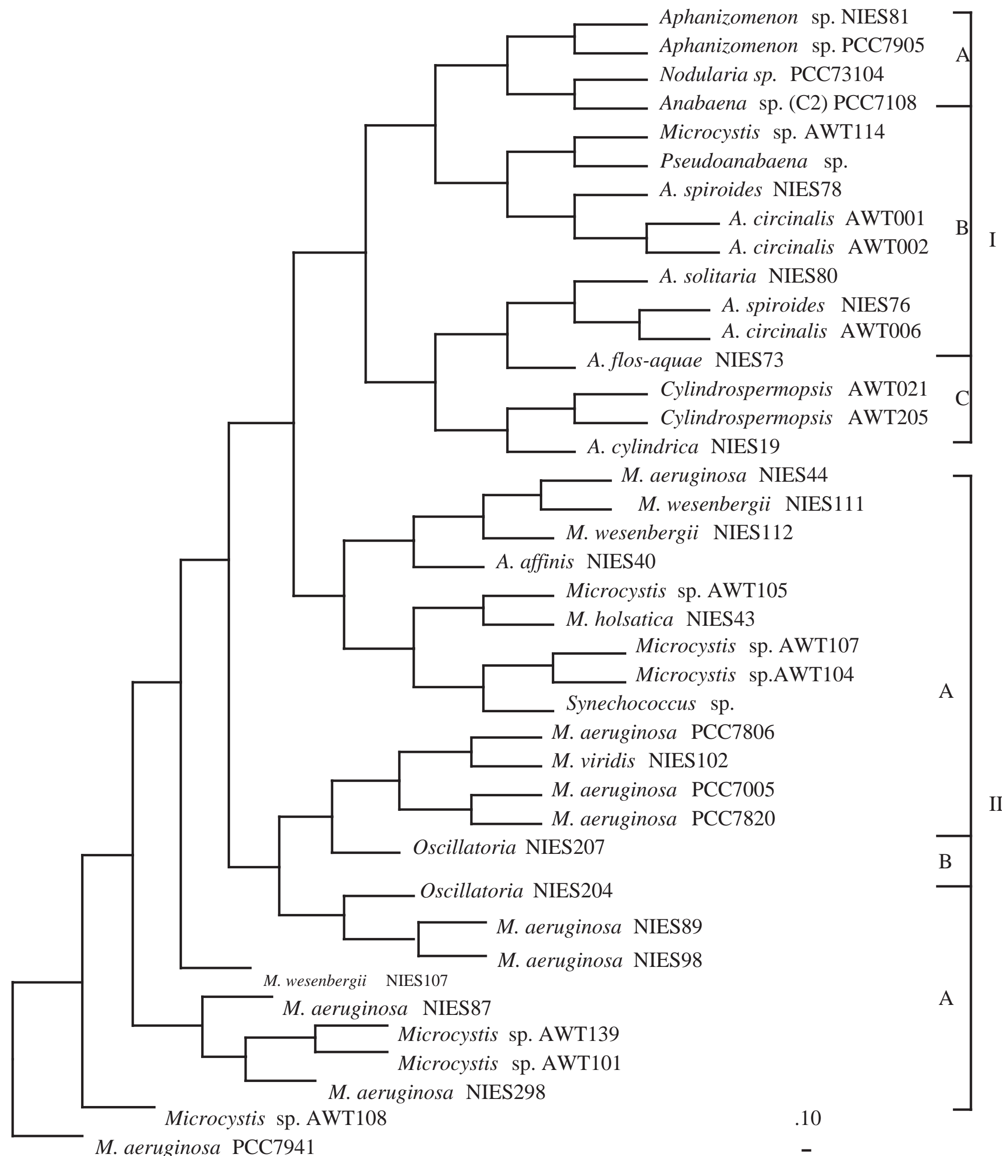

Figure 3. Strict consensus cladogram constructed from 45 binary characters applied to the rules of Dollo parsimony. The presence or absence of PC-IGS restriction digest patterns for each of the 40 strains was used as the basis for inferring cyanobacterial evolution. Twenty eight equally parsimonious trees were obtained using the DOLLOP program from the PHYLIP package and used to produce the presented tree. Relative genetic divergence is indicated by the scale. Figure reproduced with permission (18).

revealed a single amplification product for this region. Specific and discriminating amplification length fragments from the 16S-23S ITS were also observed for the genera *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Pseudanabaena*, *Plectonema*, and *Synechococcus* (Figure 1).

In freshwater environments the phycocyanin operon is found almost exclusively in cyanobacteria. Amplification of the region spanning the intergenic spacer (IGS) between the two bilin subunit genes *cpc*B and *cpc*A allowed the rapid detection of small quantities of non-axenic cyanobacterial isolates (18). RFLP analysis of these amplification products, using up to 9 different four base pair recognition endonucleases, revealed an array of DNA profiles specific for toxin-producing cyanobacteria (Figure 2). Table 2 describes the restriction enzymes and genotypes which differentiate between certain cyanobacterial genera. Phylogenetic analysis of this data clustered the non-heterocystous, filamentous genera *Oscillatoria* with the unicellular genera *Microcystis* and *Synechococcus* as well as placing the strains *M. elabens*, *M. holsatica*, and numerous Australian isolates of *Microcystis*, on the same lineage as *Synechococcus* (Figure 3). All heterocyst-forming cyanobacteria studied appeared to share a common ancestor although species of *Anabaena* formed a polyphyletic lineage occupying

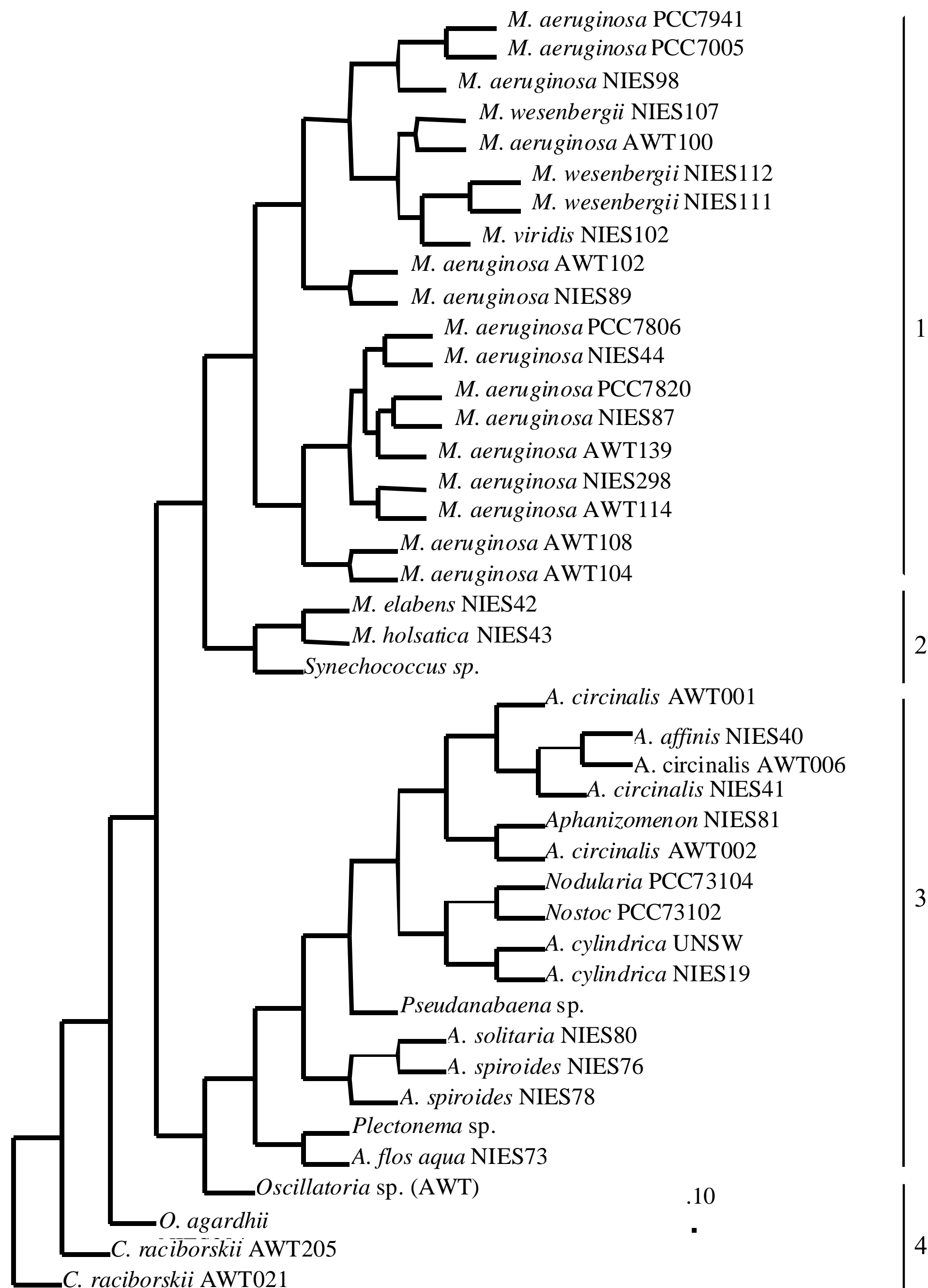

Figure 4. Relatedness of toxic and bloom-forming cyanobacteria based on RFLPs detected in the 16S-23S rRNA internal transcribed spacer region. The phylogeny was reconstructed using the Dollo parsimony program of the Phylip package. The strict consensus tree is shown with relative genetic distances for each branch indicated by the scale. Numbers 1 to 4 refer to the grouping of cyanobacterial clusters designated as *Microcystis*, *Synechococcus*, *Anabaena*, and *Cylindrospermopsis/Oscillatoria*, respectively. Figure reproduced with permission (9).

terminal branches with the genera *Cylindrospermopsis*, *Aphanizomenon*, and *Nodularia*. The genus *Cylindrospermopsis*, which has been recently isolated from Australia and produces hepatotoxic alkaloids (30), exhibited an early divergence from the rest of the heterocystous cyanobacteria. Interestingly, *Nostoc* strains in this study were not amplified using the described phycocyanin primer set, thus readily differentiating them from the *Anabaena* strains tested (18).

RFLPs of the amplified phycocyanin IGS and the 16S-23S rRNA ITS revealed genotypes which were specific to the strain classification of cyanobacteria (18, 19). The diversity of genotypes detected by the 16S-23S rRNA ITS, however, was not as extensive as those revealed by the

sequence polymorphisms in the cyanobacterial phycocyanin operon. Inferred phylogenies, using these RFLP genotypes as phylogenetic characters, are presented in Figures 3 and 4. Within the unicellular domains, *Microcystis* species generally occupied multiple lineages and exhibited no relation to their traditional taxonomic designations. Strains of *M. aeruginosa* from geographically distinct sources, however, did form a single cluster consistently across phylogenetic methods. Similarly, the presented trees did not delineate the *Microcystis* based on levels of hepatotoxin production. Based on mouse bioassay and protein phosphatase inhibition toxicity data there did not appear to be any correlation between the molecular evolution of phycocyanin genotypes and toxigenicity within the genus *Microcystis*. On the other hand, strains of *Microcystis* were clustered by 16S-23S rRNA ITS RFLPs with respect to their ability to produce microcystins, with all strains in the toxic cluster giving positive results for hepatotoxicity in the mouse bioassay (Figure 4). Bolch *et al.* (16) have investigated genetic polymorphisms within the phycocyanin operon and shown that considerably higher levels of intra-specific variation exists between strains of *M. aeruginosa* and *Nodularia spumigena* compared to strains of *A. circinalis*.

Both PCR-RFLP approaches showed strains of *Oscillatoria* to be monophyletic, although this was not supported by partial 16S rRNA gene sequencing data, which depicted *Oscillatoria* to be a polyphyletic lineage with members closely related to different species of other filamentous and unicellular cyanobacteria (8). Strains of the genera *Aphanizomenon* and *Nostoc* were also shown to be monophyletic. Strains of *Nostoc* and *Nodularia* were also differentiated from the genus *Anabaena* by 16S-23S rRNA ITS genotyping, sharing a terminal branch with the *A. cylindrica* subgroup. Strains of *A. circinalis*, *A. cylindrica*, *A. flos-aquae*, *A. solitaria*, and *A. spiroides* were placed within the heterocystous cluster with no relation to their species designation (Figure 4). As with the strains of *M. aeruginosa* and *M. wesenbergii*, the strains of *A. circinalis* and *A. spiroides* were as different from each other as they are from supposedly more distantly related strains. The *Cylindrospermopsis* strains isolated from Australian waters were monophyletic and exhibited quite an early divergence from the rest of the heterocyst-forming cyanobacteria.

Other DNA Amplification Procedures for Identification of Cyanobacteria

The gross topology of the phylogenies inferred from polymorphisms in the phycocyanin and rRNA operons was also supported by RAPD profiles of toxic cyanobacteria. Multiplexing 10 mer oligonucleotides in a single PCR was used for generating reproducible and informative DNA profiles (31). Clustering of *Anabaena* and *Microcystis* by genomic heterogeneity as separate lineages reflected the obvious morphological differences as well as 16S rRNA sequence divergence (9, 32). Other protocols involving nucleic acid content, including %mol G+C (33) and DNA/DNA hybridization (34), have successfully been applied to the classification of cyanobacteria, although non-axenic cultures and a continuum of genome content values and reassociation rates has made delineation of exact generic borders somewhat difficult.

Recently, the distribution of short tandem repeats in cyanobacterial genomes has been applied to the characterisation of toxic cyanobacteria. Rouhiainen *et al.* (3) has investigated many strains of toxin-producing cyanobacteria from several genera, including *Anabaena* and *Microcystis*, using the RFLPs detected by genomic transfer and hybridisation with a heptamer repeat of the nifJ gene. The results showed that readily distinguishable profiles were able to differentiate between filamentous, heterocystous cyanobacteria which were either neurotoxic (anatoxin-a) or hepatotoxic (microcystins). Cryptic short tandemly repeated sequences have also been described by Mazel *et al.* (22) and identified for use as molecular markers. However, caution should be adopted when applying these repeats to taxonomic studies, considering the potential mobility and amplification of such sequence elements. Cyanobacterial specific repeat sequences, termed highly iterative palindromes (HIP), have also revealed strain-specificity and can be applied to the discrimination of toxic and non-toxic cyanobacteria (35-37). The use of RNA polymerase gene sequences has also been shown to discriminate between strains of *Synechococcus*-like cyanobacteria and prochlorophytes isolated from natural bacterioplankton communities (38).

Characterisation of the 16S rRNA gene has been applied to many prokaryote lineages and may reveal sequence signatures which are useful for the *in situ* detection of specific bacterial strains within their natural population structure (39, 40). Several other procedures are being developed for the cyanobacteria and other bacteria which allow discrimination of strains based on stable genetic characters. These include denaturing gradient gel electrophoresis and single stranded conformational polymorphisms (DGGE and SSCP), analysis of genomic restriction fragment polymorphisms by selective restriction fragment amplification (SRFA), and amplification of microsatellite (dinucleotide repeat) and repetitive extragenic repeat (REP) sequences (41). In addition, the phylogenetic affiliations amongst the toxin-producing and bloom-forming cyanobacteria are currently being investigated using inferences from *rec*A (42) and 16S and 23S rRNA gene sequences (43, unpublished data).

Different Levels of Taxonomic Discrimination

The results of these studies show that genetic relatedness between the genera of bloom-forming cyanobacteria is supported by the described 16S-23S ITS and phycocyanin PCR-RFLP profiles. This system of molecular identification of cyanobacteria, when used in conjunction with traditional morphological evaluation, should continue to assist in the accurate delineation of novel isolates and the presentation of an amended system for cyanobacterial classification. The levels of sensitivity offered by molecular systematics are varied and afford a continuum of methods for strain discrimination. At the lowest taxonomic level, methods based on whole genome polymorphisms, such as RAPD PCR, readily differentiate between strains of cyanobacteria tested regardless of geographical origins (16, 31). The

limitation of this technique is the current need for strains to be maintained in axenic and unicyanobacterial culture and has been shown not to be suitable for direct analysis of environmental isolates (31, 44). Decreased discriminatory power was afforded by the PCR-RFLP analyses of the ribosomal spacer and the phycocyanin operon, based on the methods in this article. These procedures were, however, more robust and useful for the direct analysis of cyanobacteria isolated from complex natural populations. Within this category it is recommended that the size of the 16S-23S rRNA ITS PCR product be used for initial confirmation of genus identification and successive restriction digest of this locus and that of the phycocyanin gene be applied for delineation to the strain level. However, sequencing of complete genes, such as the 16S rRNA gene, is becoming more efficient and provides the most accurate method for determining relatedness and inferring evolution within this group of prokaryotes. These amplification procedures are of particular value when combined with organism-specific primers, allowing direct "cyanodiversity" assessment from environmental samples.

Traditional taxonomic approaches to classification are no longer adequate given the available technology, and a polyphasic approach to systematics is more suitable and accurate for the description of members from natural microbial populations. The combination of phenotypic and genotypic characters provides both a means for classification and a tool for determining biological evolution.

Protocols for DNA Extraction and Amplification

Samples of type cultures and environmental isolates were harvested immediately upon receipt and PCR template prepared according to two methods. In Protocol 1, total genomic DNA was extracted using a modification of a method for purification of DNA from Gram-negative bacteria (31). As an alternative, the PCR template was prepared by the rapid lysis of cyanobacterial cells and liberation of DNA into the supernatant using Protocol 2.

Primer Design and Amplification of the Phycocyanin IGS

Oligonucleotide primers (Table 1) 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 using the multiple sequence alignment program of the Clustal W package (45) supplied by the Australian National Genome Information Service (ANGIS, Sydney, Australia; morgan.angis.su.oz.au). The aligned PC sequences were from the cyanobacteria *Anabaena* sp. PCC7120, *Calothrix* sp. PCC7601, *Pseudanabaena* sp. PCC7409, *Synechococcus* sp. WH8020, *Synechocystis* sp. PCC6701, and *Agmenellum quadruplicatum* (*Synechococcus*) PCC7002 with GenBank accession numbers X05239, M36276, M99426, M95288, D13173, and K02660, respectively. PCR priming sites were chosen from completely conserved regions within the ((forward primer) and ((reverse primer) subunits of the PC peptide sequence. Codon bias for the photosynthetic apparatus genes of several cyanobacterial species was used to select the final DNA sequence of the PCR primers. The primer sequences were checked for homology to any other known sequences deposited in the available databases using the BLAST (46) option (ANGIS, Sydney, Australia). Peptide sequences in the regions chosen for these primers were also checked against those of red algae (*Algaothamnion*, *Cyanidium*, and *Porphyridium*), cryptomonads (*Chroomonas*) and cyanelle sequences in the PIR protein database. Oligonucleotides were synthesised using an Oligo 1000 DNA Synthesiser (Beckman, Fullerton, CA).

Amplification Reactions

Small volume (capillary) PCR was performed to study variability in the length of amplification products for the PC-IGS. Reactions contained (20µl reaction volume):

67 mM Tris-HCl, pH 8.8
16 mM $(NH_4)_2SO_4$
0.45% Triton X-100
200 mg/ml gelatin
3 mM $MgCl_2$
200 µM deoxynucleotide triphosphates
5 pmol of each oligonucleotide primer
0.5 units *Tth* Plus thermostable DNA polymerase (Biotech Int., Perth, Australia)
1 µl of cell lysate supernatant or 50 ng of genomic DNA

Protocol 1. Genomic DNA isolation-1

1. Pellet a 1 ml aliquot of mid to late log phase culture by centrifugation, decant the media, and resuspend the pellet in 500 µl of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 50 mM NaCl.
2. Add lysozyme to give a final concentration of 1 mg/ml and incubate at 55°C for 30 min.
3. Add 10 µl proteinase K (10 mg/ml) and 20 µl 10% SDS and incubate at 55°C for 10 min or until the solution clears (complete cell lysis).
4. Chill on ice and extract with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1).
5. Repeat the organic extraction and add an equal volume of 4 M ammonium carbonate to the supernatant.
6. Total genomic DNA is precipitated by addition of 2 volumes of isopropanol and centrifugation for 10 min at room temperature.

Protocol 2. Genomic DNA isolation-2

1. Approximately 10^5 to 10^7 cells are centrifuged to a pellet and the culture medium removed.
2. The cells are resuspended in 200 µl of Instagene matrix (Bio-Rad, Hercules, CA.) supplemented with 20 µl of 1% NP-40 or 1% Triton X-100.
3. Cells are incubated at 55°C for 30 min, vortexed for one minute and heated to 95ºC for 10 min.
4. The solution is vortexed again for one mine and the cell debris pelleted by a brief centrifugation.
5. Between 1 and 5 µl of the supernatant contained sufficient genetic material for a successful PCR amplification. Supernatant of this cell lysate has been stored at 4°C for over 2 years and found to retain sufficient DNA for a PCR template.

Reactions were carried out in heat sealed positive displacement pipette tips. Initial denaturation of template DNA was achieved by incubation at 94°C for 2 min. Reactions were then subjected to 40 cycles of 94°C, 5 sec; 47°C, 10 sec; and 72°C, 30 sec in an FTS-1S capillary thermocycler (Corbett Research, Sydney, Australia). Large-scale DNA amplifications were required for successive restriction fragment analysis of the PCR product. They contained 2.5 mM $MgCl_2$, 20 pmol of each primer, 2 units *Tth* Plus thermostable DNA polymerase (Biotech Int., Perth, Australia), and 2 µl of cell lysate or 100 ng of genomic DNA in a 100 µl volume. All other components were at the same concentration as 20 µl reactions. Reactions 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 sec at 94°C, 30 sec at 50°C, and 60 sec at 72°C. PCR products were analysed by electrophoresis in 1% agarose in TAE buffer.

Primer Design and Amplification of the 16S-23S rDNA ITS

Oligonucleotide primers (Table 1) were designed to be suitable for amplification of the ITS from a diverse range of cyanobacteria. Published DNA sequences for the 16S rRNA genes from cyanobacteria were aligned using the multiple sequence alignment program of the Pileup and Clustal W packages (45). The aligned 16S rRNA gene sequences were from the cyanobacteria *Anabaena* sp. PCC7120, *Synechococcus* sp. PCC6301, *Chlorogloeopsis* HTF PCC7518, *Microcystis aeruginosa* PCC7806, and *Microcystis aeruginosa* NIES89 with GenBank accession numbers X59559, X01296, X68780, Z28699, and Z28700, respectively. Potential cyanobacterial specific PCR priming sites were chosen from within the conserved sequences of the 3' region of these 16S rRNA molecules. Conserved sequences at the 5' region of bacterial 23S rRNA genes were used as the target for a downstream, reverse PCR primer (19). The forward primer (16CITS) sequence was checked for identity to 16S rDNA sequences or homology to any other sequences using the BLAST (46) search algorithm against sequences contained in the ribosomal database project and the GenBank and EMBL nucleotide sequence databases.
Large-scale DNA amplifications were performed as for the phycocyanin operon PCR except that the reaction tube was subjected to 30 cycles of 20 sec at 94°C, 30 sec at 48°C, and 60 sec at 72°C in a PE480 thermal cycler (Perkin-Elmer Cetus, Emeryville, CA). The cyanobacterial specific ITS PCR also required a hot-start protocol which entailed the addition of thermostable DNA polymerase to other PCR components which had been preheated to 96°C. PCR cycling was commenced directly after addition of the enzyme.

Restriction Endonuclease Digestion of 16S-23S rRNA ITS and PC-IGS PCR Products

PCR fragments were purified from reaction components and small by-products as described by Neilan *et al*. (18). Mineral oil was removed from PCR reactions by chloroform: isoamyl alcohol (23: 1) extraction. The aqueous phase was then purified using the Wizard PCR purification system (Promega, Madison, WI) to remove amplification reaction components including unincorporated primers and nucleotides. ITS amplification fragments were digested separately with eight restriction endonucleases which recognise and cleave tetra-nucleotide motifs. The DNA restriction enzymes *Alu*I, *Cfo*I, *Hae*III, *Hin*FI, *Msp*I, *Rsa*I, *Scr*FI, and *Taq*I (Boehringer Mannheim, Germany) were used to generate RFLP patterns specific to the cyanobacterial strains studied as previously described (18). Approximately 200 ng of PCR product was combined with 1.5 µl of the corresponding enzyme buffer supplied and 5 units of restriction enzyme in a 15 µl digest. Reactions were incubated overnight at the temperature recommended by the enzyme supplier. All PCR products were analysed by electrophoresis in 1% normal agarose / 1.5% low melt agarose in TAE buffer or 6% polyacrylamide (19: 1, acrylamide: bisacrylamide) in TBE buffer. DNA in agarose gels was stained with ethidium bromide or with silver nitrate when electrophoresed in an acrylamide matrix (47). The profile of a each digest was used as a genotypic marker and formed the basis for phylogenetic analyses.

Phylogenetic Analysis of RFLP Data

The RFLP patterns generated were based on the primary structure of the rRNA ITS and the PC operon. These DNA profiles were treated as observed phenotypes for the purpose of inferring relatedness between the

cyanobacteria. Phylogenetic analysis of the 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 a RFLP profile for each of the nine restriction digests. Distance calculations were performed using the eighteen algorithms supplied by the RAPDistance package developed by Armstrong *et al.* (48). All metrics available were used and the resulting trees constructed by the neighbour-joining method and the NJTREE program (49). The RFLP profiles were also used in a cladistic analysis where character changes were minimised along branches of the derived tree. The rules of parsimony, using the Dollo parsimony method supplied by the DOLLOP program in the PHYLIP package (version 3.53c), were applied to the profile data (50, 51). Relatedness between strains was inferred based on the number of shared homologous characters, being RFLP profiles for this study. A single, strict consensus cladogram was reconstructed, using the majority-rule method, from the equally parsimonious trees found. Outgroups were not available for this study and therefore the phylogenetic trees presented should be considered as unrooted.

Concluding Remarks

The application of nucleic-acid based and, more importantly, DNA amplification methods for the molecular typing of microorganisms in complex natural populations is making the study of cyanobacterial systematics and phylogenetics definitive rather than descriptive. The speed, economy, sensitivity, and reliability of the PCR-based methods described in this study make them ideal for accurately addressing basic questions in microbial ecology. The present work has provided DNA profiles, composed of phylogenetic characters, appropriate for the inference of relatedness and evolution of cyanobacterial taxa. These DNA profiles readily identify potential toxic species of bloom-forming or benthic cyanobacteria in drinking and recreational water supplies. In addition, most of these molecular taxonomic methods are, or will soon be, amenable to automation. For both environmentally and medically significant microbial populations this technology provides high-level discrimination for the detection of toxic or virulent strains. Apart from pathogenicity, the unambiguous identification of microorganisms is also crucial to other fields including, bioremediation, biotechnology, and biodiversity.

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