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Molecular Phylogeny of *Anabaena circinalis* and Its Identification in Environmental Samples by PCR

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Although the cyanobacterium *Anabaena circinalis* occurs worldwide, Australian isolates are believed to exclusively possess the saxitoxin group neurotoxins (paralytic shellfish poisons). Identification of *A. circinalis* in a mixed population is complicated due to limited morphological differences between *Anabaena* species. Sequence analysis of the DNA-dependent RNA polymerase (*rpoC1*) gene from 24 *Anabaena* isolates, including 12 designated *A. circinalis*, permitted a phylogenetic analysis to be performed. In addition, an *A. circinalis*-specific PCR was developed and tested successfully on environmental samples.

Anabaena circinalis produces neurotoxins, anatoxin-a and paralytic shellfish poisons. While *A. circinalis* is distributed worldwide, the production of PSPs is believed to be exclusive to Australian strains (5). Paralytic shellfish poisons, including saxitoxin, neosaxitoxin, and gonyautoxin, are alkaloids that are potent sodium channel blockers in nerve axons which cause progressive paralysis and death from respiratory failure (3, 12). In a study of the cyanobacterial component of the surface waters of the Murray-Darling Basin in Australia, Baker and Humpage (1) identified the cyanobacterial genus *Anabaena* as most abundant in natural-bloom samples, with up to seven coiled *Anabaena* morphotypes coexisting at any one time. *A. circinalis* was found in 41% of samples, with 55% of *A. circinalis* blooms shown to produce neurotoxins. Differentiation of *A. circinalis* from other coiled *Anabaena* species using microscopic methods is difficult and time consuming. In view of the potential toxicity of *A. circinalis*, it is paramount that this species be correctly identified in environmental samples. A combination of genotypic and microscopic identification techniques is of potential benefit.

The 16S rRNA gene has been used extensively for cyanobacterial identification (7, 8, 10); however, the DNA-dependent RNA polymerase (*rpoC1*) gene has been described as a more discriminatory marker (9). A phylogenetic examination of cyanobacterial *rpoC1* gene sequence data is presented, focusing on the genus *Anabaena* and in particular the species *A. circinalis*. We report here for the first time an *A. circinalis*-specific PCR assay targeting the *rpoC1* gene to detect this organism directly in environmental water samples.

Molecular techniques. The cyanobacterial strains used in this study (Table 1) were grown under constant light intensity for up to 14 days at 25°C in ASM-1 medium (4). Genomic DNA was extracted from reference strains as previously described (15) and from environmental samples using the InstaGene matrix (Bio-Rad) and phenol-chloroform treatment. Briefly, 10-ml environmental samples were pelleted by centrifugation and resuspended in 90% InstaGene matrix and 10% Triton X-100 to 200 μl. Following incubation at 55°C for 30 min, the cells were vortexed for 1 min, heated to 95°C for 10 min, and then centrifuged. DNA was extracted once with an equal volume of phenol-chloroform and then once with chloroform. The DNA was precipitated, resuspended in 50 μl of water, and used directly in PCRs.

PCR amplification using cyanobacterium-specific primers targeted to the *rpoC1* gene (rpoC1-1 [5′-GAGCTCYAWNAC CATCCAYTCNGG] and rpoC1-T [5′-GGTACCNAAYGGN SARRTNGTTGG]) has been previously described (9). Primers used for the *A. circinalis*-specific PCR assay detailed in this study were Ana2 (5′-GATAGCATCCTCAATTTCTAGCC ATTGG), Ana4 (5′-CTCTGAAGCCAGAAATGGACGGC), and Ana-ICF (5′-TAGCCATTGGCATATCCAAGAGAATA GC) and were constructed from the sequence determined in this study. Each 50-μl PCR mixture contained 1 to 10 ng of genomic DNA, 20 pmol of Ana2, 20 pmol of Ana4, 200 μM deoxynucleoside triphosphates, 2.5 mM magnesium chloride, 1× PCR buffer II, 2.5 U of Ampli *Taq* Gold, and 2 pg of an internal control fragment (ICF). The following protocol was used: 94°C for 10 min for 1 cycle; 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles; 72°C for 15 min for 1 cycle; and holding at 4°C. Nucleotide data was analyzed with GeneJockeyII (Biosoft, Cambridge, United Kingdom), sequence alignments were done with ClustalX (13), and phylogenetic trees were constructed with the MEGA analysis platform (6). The Jukes-Cantor method was used to calculate pairwise distances, and a tree was constructed with the neighbor-joining algorithm. Bootstrap analyses were performed with 500 replicates.

***rpoC1* sequence and phylogeny.** A 612-bp fragment of the *rpoC1* gene from 24 *Anabaena* strains and 2 *Aphanizomenon* strains was amplified and sequenced (Table 1). An alignment of the sequences showed that in 4 out of 12 *A. circinalis* strains examined, identical nucleotide changes were observed at 18 of 540 positions. This suggests that genotypic groups of *A. circinalis* exist (termed types I and II). It is interesting that at position 57, three out of eight type I strains (ANA019A, ANA209F, and ANA301A) had the same nucleotide change, which coincided with the same change in type II isolates. Subsequent analysis failed to associate the observed differences in *rpoC1* sequence to strain origin or toxin production. Importantly, the *rpoC1* gene provided sufficient sequence variation to differentiate the genus *Anabaena* to the species level and *A. circinalis* to the strain level.

A phylogenetic analysis of a range of cyanobacteria based on partial *rpoC1* sequences has previously been presented (15) and is combined here with the additional *rpoC1* gene se-

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TABLE 1. Characterization of cyanobacterial strains used in the present study

Strain	Location[a]	*rpoC1* sequence	*A. circinalis* PCR	Saxitoxin production[d]	*A. circinalis* type
Anabaena circinalis					
ANA019A	Chaffey Dam, NSW	Y[b]	+	+	I
ANA059C	Renmark, SA	Y	+	NA	I
ANA118A	Booligal, NSW	Y	+	+	I
ANA118C	Booligal, NSW	Y	+	+	I
ANA125A	Yarrawonga, VIC	ND[c]	+	NA	ND
ANA150A	Burrinjuck Reservoir, NSW	Y	+	+	I
ANA175A	Collarenebri, NSW	Y	+	NA	I
ANA209F	Myponga Reservoir, SA	Y	+	NA	I
ANA301A	Riverslea, QLD	Y	+	NA	I
ANA306A	Palm Island, QLD	ND	+	−	ND
ANA311E	Geelong, VIC	Y	+	NA	II
ANA323B	Perth, WA	ND	+	+	ND
ANA332H	Yarraman, QLD	Y	+	−	II
ANA349H	Hope Valley Reservoir, SA	Y	+	NA	II
ANA350C	Craigbourne Dam, TAS	Y	+	NA	II
Anabaena flos-aquae					
ANA051A	Murtho Reserve, SA	ND	−	NA	
ANA076C	Iraak Creek, VIC	ND	−	NA	
ANA264A	Darling Anabranch, NSW	Y	−	−	
ANA354A	River Murray, SA	Y	−	NA	
Anabaena spiroides f. *spiroides*					
ANA139A	Torrumbarry Weir, VIC	Y	+	NA	II
ANA281A	Windamere Dam, NSW	ND	−	NA	
ANA292C	Lake Ballyrogan, NSW	Y	−	NA	
Anabaena spiroides f. *minima*					
ANA044A	Lake Albert, SA	ND	−	NA	
ANA084E	Lake Alexandrina, SA	ND	−	NA	
ANA234B	Menindee Lake, NSW	ND	−	NA	
Anabaena sp.					
ANA062C	Moolooroo, SA	ND	−	NA	
ANA098C	Lagoon, NSW	ND	−	NA	
ANA193E	Binghi, NSW	ND	−	NA	
ANA238A	Darling Anabranch, NSW	ND	−	NA	
ANA238C	Darling Anabranch, NSW	Y	−	NA	
ANA255C	Darling Anabranch, NSW	Y	−	NA	
Anabaena aphanizomenoides					
ANA023C	Murtho Park, SA	ND	−	NA	
ANA214B	Nildottie Lagoon, SA	ND	−	NA	
ANA217A	Nildottie Lagoon, SA	ND	−	NA	
ANA259C	Darling Anabranch, NSW	ND	−	NA	
ANA280A	Chaffey Dam, NSW	Y	−	−	
ANA303C	Riverslea, QLD	ND	−	NA	
Anabaena pertubarta f. *tumida*					
ANA112D	Cobram, VIC	ND	−	NA	
ANA146C	Fish Creek Farm Dam, VIC	ND	−	NA	
ANA187A	Mungindi, NSW	ND	−	NA	
ANA221E	Yarramundi, SA	Y	−	NA	
ANA297B	Deniliquin, NSW	Y	−	NA	
ANA313C	Bundaleer Reservoir, SA	ND	−	NA	
ANA313D	Bundaleer Reservoir, SA	ND	−	NA	
Anabaena solitaria					
ANA060B	Renmark, SA	ND	−	NA	
ANA075C	Iraak Creek, VIC	ND	−	NA	
ANA207A	Bridgewater Farm Dam, SA	Y	−	NA	
ANA282B	Windamere Dam, NSW	Y	−	NA	
ANA337A	Goulbourn, NSW	Y	−	NA	
Aphanizomenon issatschenkoi					
APH016B	Torrumbarry Weir, VIC	ND	−	NA	
APH025A	Wongulla Lagoon, SA	ND	−	−	
APH027A	Rockhampton, QLD	ND	−	−	
Aphanizomenon gracile					
APH015B	Bundaleer Reservoir, SA	ND	−	NA	
APH026E	Joan Powling, VIC	Y	−	NA	
Aphanizomenon ovalisporum					
APH028A	Palm Lakes, QLD	Y	−	NA	

[a] NSW, New South Wales; SA, South Australia; VIC, Victoria; QLD, Queensland; WA, Western Australia; TAS, Tasmania.
[b] Y, yes.
[c] ND, not determined.
[d] According to reference 14. NA, not analyzed.

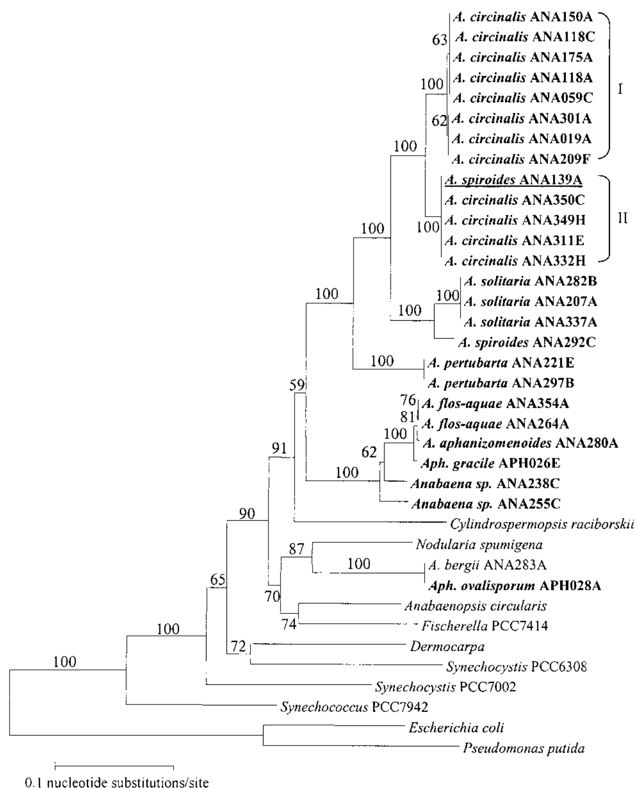

FIG. 1. Phylogenetic tree constructed from *rpoC1* nucleotide sequence alignments. Strains sequenced in this study are in bold type (GenBank accession no. AF199423 to AF199433). The *A. spiroides* ANA139A isolate reclassified as *A. circinalis* ANA139A type II is underlined. Bootstrap values derived from 500 replicates of the sequence data are shown.

quences obtained for a range of *Anabaena* and *Aphanizomenon* species (Fig. 1). The two types of *A. circinalis* are evident, with the *Anabaena* genus fitting into the previously identified heterocyst-forming cluster (15). Strain ANA139A was originally identified as *A. spiroides* on the basis of morphological criteria (Table 1). However, analysis of its *rpoC1* sequence showed 100% identity to *A. circinalis* type II and as shown in Fig. 1, it is within the *A. circinalis* grouping. This result prompted its identification to be changed and highlighted the importance of *rpoC1* typing for *Anabaena*, as clearly species assignment based on morphological criteria alone can lead to misidentification. Due to the association of *A. circinalis* with toxicity, misidentification of this species is of considerable concern.

Interestingly, *Anabaena bergii* ANA283A clusters with *Aphanizomenon ovalisporum* APH028A (11), separately from the *Anabaena* genus. In addition, this cluster is distant from the position of *Aphanizomenon gracile* APH026E (Fig. 1). Both *Aphanizomenon ovalisporum* and *A. bergii* are also known to produce the cyanobacterial toxin cylindrospermopsin. *rpoC1* sequence analysis revealed 100% identity between the two isolates, and this is supported by 99% 16S rRNA gene sequence identity (M. A. Schembri, unpublished data). Despite morphological data initially indicating that strain ANA283A belongs to the *Anabaena* genus, sequence analysis demonstrated that this is not the case and that this isolate and *Aphanizomenon ovalisporum* are morphological variants of the same cyanobacterium. A more detailed genetic and morphological analysis of the taxonomy of these two organisms must now be performed.

Morphological differences observed between strains of *Anabaena flos-aquae* II led Cronberg and Komárek (2) to define a new cyanobacterial species, *Anabaena pertubarta* f. *tumida*. A number of strains from the culture collection of the Australian Water Quality Centre were reclassified accordingly. While previously based on morphological observations, definition of the species *A. pertubarta* f. *tumida* is now also supported by genetic data, with the *rpoC1* sequence analysis presented here clearly indicating distinct phylogenetic separation between *A. flos-aquae* and *A. pertubarta* strains (Fig. 1).

***A. circinalis* PCR assay.** A PCR assay to specifically detect *A. circinalis* in reference and environmental samples was developed using primers designed on the basis of conserved regions of the *A. circinalis rpoC1* gene. An ICF was constructed using a previously described method (15) employing primers

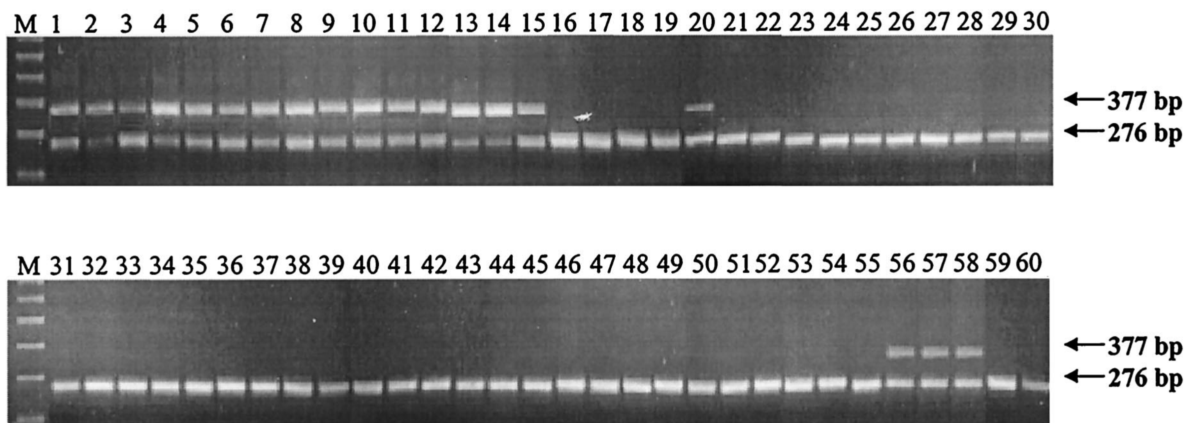

FIG. 2. *A. circinalis*-specific PCR assay. The diagnostic product (377 bp) and the ICF (276 bp) are indicated. Lanes 1 to 15 are *A. circinalis* isolates (019A, 059C, 118A, 118C, 125A, 150A, 175A, 209F, 301A, 306A, 311E, 323B, 332H, 349H, and 350C), lanes 16 to 19 are *A. flos-aquae* isolates (051A, 076C, 264A, and 354A), lanes 20 to 22 are *A. spiroides* f. *spiroides* isolates (139A, 281A, and 292C), lanes 23 to 25 are *A. spiroides* f. *minima* isolates (044A, 084E, and 234E), lanes 26 to 31 are *Anabaena* sp. isolates (062C, 098C, 193E, 238A, 238C, and 255C), lanes 32 to 37 are *A. aphanizomenoides* isolates (023C, 214B, 217A, 259C, 280A, and 303C), lanes 38 to 44 are *A. pertubarta* f. *tumida* isolates (112D, 146C, 187A, 221E, 297B, 313C, and 313D), lanes 45 to 49 are *A. solitaria* isolates (060B, 075C, 207A, 282B, and 337A), lanes 50 to 52 are *Aphanizomenon issatschenkoi* isolates (016B, 025A, and 027A), lanes 53 and 54 are *Aphanizomenon gracile* isolates (015B and 026E), lane 55 is an *Aphanizomenon ovalisporum* isolate (028A), lane 56 is an environmental sample containing *A. circinalis* (Geelong, Victoria, Australia), lane 57 is an environmental sample containing *A. circinalis* (Gawler, South Australia, Australia), lane 58 is an environmental sample containing *A. circinalis* (Bahia Blanca, Argentina), lane 59 is an environmental sample containing *C. raciborskii* (Broken Hill, New South Wales, Australia), lane 60 is the ICF only, and lane M contains molecular size markers (700, 600, 500, 400, 300, and 200 bp).

Ana2, Ana4, and Ana-ICF. Ana-ICF recognized a sequence internal to the region bounded by Ana2 and Ana4. A PCR with Ana2 and Ana-ICF yielded a fragment which was used in a subsequent PCR with Ana2 and Ana4, which recognizes a terminal decameric sequence incorporated using Ana-ICF. The final product was 276 bp in length and contained 3′ and 5′ sequences which exactly matched Ana2 and Ana4. Two picograms of this internal control was spiked into each PCR mixture. Amplification of the ICF yielded a 276-bp fragment, with the presence of *A. circinalis* chromosomal DNA resulting in an additional 377-bp product. The ICF indicates if failure to amplify the diagnostic product is due to genuine absence of the target sequence or because the PCR failed due to sample inhibition. In this case, the detection limit is approximately 2,000 cells but lower levels should be detectable with careful optimization of PCR conditions.

Reference cultures of *Anabaena* and *Aphanizomenon* (Table 1) and four environmental samples were screened with the assay, including an environmental sample from Bahia Blanca, Argentina (Fig. 2). This sample was known to contain *A. circinalis* and *Microcystis* and *Ceratium* spp. The 377-bp diagnostic band was amplified in all *A. circinalis* reference cultures (lanes 1 to 15) and in three of the environmental samples known to contain *A. circinalis* following microscopic analysis (lanes 56 to 58). Sequence analysis of the three diagnostic PCR products confirmed the identification of *A. circinalis* in the environmental samples. In addition, the specific PCR also supported the definition of *A. spiroides* f. *spiroides* ANA139A as *A. circinalis* (lane 20). This is the first report of a PCR assay that is specific for *A. circinalis* and able to identify this species in an environmental water sample. A PCR test which targets toxin-encoding genes in *A. circinalis* would be ideal. However, in the absence of DNA sequence information regarding these genes, but the strong association of this species with toxicity, the rapid and specific identification of *A. circinalis* is a useful tool in the management of toxic algal blooms.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this work have been deposited with the GenBank database under the following accession numbers: *A. circinalis* type I *rpoC1*, AF199423; *A. circinalis* type IA (representing strains ANA019A, ANA209F, and ANA301A), AF199424; *A. circinalis* type II *rpoC1*, AF199425; *Anabaena solitaria rpoC1*, AF199426; *A. spiroides* ANA292C *rpoC1*, AF199427; *A. pertubarta rpoC1*, AF199428; *A. flos-aquae rpoC1*, AF199429; *Anabaena aphanizomenoides rpoC1*, AF199430; *Aphanizomenon gracile rpoC1*, AF199431; *Anabaena* sp. strain ANA238C *rpoC1*, AF199432; *Anabaena* sp. strain ANA255C *rpoC1*, AF199433.

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