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The cyanobiont in an *Azolla* fern is neither *Anabaena* nor *Nostoc*

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Abstract

The cyanobacterial symbionts in the fern *Azolla* have generally been ascribed to either the *Anabaena* or *Nostoc* genera. By using comparisons of the sequences of the phycocyanin intergenic spacer and a fragment of the 16S rRNA, we found that the cyanobiont from an *Azolla* belongs to neither of these genera.

Keywords: *Azolla* fern; Cyanobacterial symbiont; Phylogenetic relationship

1. Introduction

The symbiotic relationship between the floating aquatic fern *Azolla* and nitrogen-fixing cyanobacteria has been exploited for many years as a source of nitrogen for agriculture [1,2]. The endosymbiotic cyanobacteria have usually been identified as *Anabaena azollae* [1–3]. However, using DNA probes, Plazinski et al. [1] showed genetic variation in cyanobacterial symbionts of *Azolla* spp. and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. Moreover, on the basis of morphological assessments and allozyme analyses, Gebhardt and Nierzwicki-Bauer [3] classified the cyanobacteria isolated from *Azolla pinnata* as a species of *Anabaena*, whereas the isolate from *Azolla mexicana* was classified as a species of *Nostoc*.

We investigated the phylogenetic relationships between an *Azolla* endosymbiont and selected representatives of cyanobacteria by using two separate fragments of genomic sequence. The 16S ribosomal RNA (16S rRNA) gene has been used extensively to elucidate the phylogeny of organisms [4]. It has been shown [5] that DNA sequence polymorphisms in the 16S rRNA variable regions V6–V8 (*Escherichia coli* 16S rRNA nucleotides 334–939) can be utilised to classify cyanobacteria and prochlorophytes into major phyletic groups. The intergenic spacer between the β and α subunits of the phycocyanin genes of cyanobacteria has been shown to be highly conserved within a genus but differs significantly between genera [6].

2. Materials and methods

2.1. Isolation and morphology of the cyanobiont

Samples of the floating fern, *Azolla filiculoides* Lam. var. *rubra*, were collected from Lake Madgwick, Armidale, NSW, Australia. To release the cyanobiont from the leaf cavities of the *Azolla*, a 'wash–squash' method was devised. The *Azolla* was washed, placed between two glass slides with sterile distilled water and gently squashed to release the cyanobacteria from the fern tissue. A sample of the cyanobiont suspension, examined microscopically, appeared similar in morphology to free-living *Anabaena* and *Nostoc* types, with solitary trichomes and intercalary heterocysts. The suspension of cyanobacterial cells was collected and concentrated by centrifugation to about 1×10^6 cells ml^{-1}.

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2.2. Polymerase chain reaction (PCR) amplification and sequencing

PCR amplification of the phycocyanin intergenic spacer (PC-IGS) was performed as previously described [6,7], using approximately 1000 cells of the prepared cyanobacterial suspension as DNA template. The PCR primers used are highly specific for the phycocyanin genes found only in cyanobacteria, cryptophytes and red algae [7]. Amplification of a fragment of the V6–V8 region of the 16S rRNA gene was performed with PCR reagents as used for the PC-IGS [6,7], using approximately 1000 cells of the cyanobacterial suspension, with the universal forward primer, 27F1, 5′-AGAGTTTGATCCTGGCTCAG-3′, and a reverse primer specific to cyanobacteria, 5′-GCTTCGGCACGGCTCGGGTCGATA-3′. Thus, the employment of these primers will not amplify DNA template from bacteria which would invariably be present in the leaf cavity and, in all cases, only one clear band was observed. Thermal cycling consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 10 s, primer annealing at 55°C for 20 s, strand extension at 72°C for 1 min, and a final extension step at 72°C for 2 min.

The DNA from PCR amplifications was purified using a PCR purification kit (QIAquick, Qiagen) and sequenced using the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems) and an ABI Prism 3700 DNA Analyzer (PE Applied Biosystems).

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY181211, AY181213 (respectively, *Azolla* cyanobiont and *Anabaena solitaria* strain NIES 80 PC-IGS sequences) and AY181212 (*Azolla* cyanobiont 16S rRNA fragment).

Table 1
Database entries used for PC-IGS and 16S rRNA sequence comparisons with *A. filiculoides* endosymbiont cyanobacterial sequences in this study

Organism	Strain	Origin	Database acc. no.
PC-IGS sequences:			
Azolla cyanobiont		Australia	AY181211
A. circinalis	AWQC 118C	Australia	AF426004
A. solitaria	NIES 80	Japan	AY181213
Anabaena affinis	NIES 40	Japan	AF427973
Aphanizomenon sp.		USA	AJ243968
Aphanizomenon sp.		USA	AJ243969
Aphanizomenon sp.		Sweden	AJ243970
Aphanizomenon flos-aquae		Ireland	AJ243971
Arthrospira sp. Maxima			AJ401168
Arthrospira sp. Paracas 98			AJ401175
Arthrospira sp.	PCC 7345		AJ401178
Cylindrosperrmopsis raciborskii		Brazil	AF426793
C. raciborskii		Germany	AF426798
C. raciborskii		USA	AY078437
Fischerella sp. Cohn			M75599
Lyngbya sp.	PCC 7419		AJ401187
Nodularia harveyana		Baltic Sea	AF364342
Nodularia sphaerocarpa		Baltic Sea	AF367150
Nodularia spumigena		USA	AF101453
Nostoc sp.	PCC 7120		AP003582
Planktothrix rubescens		Switzerland	AJ131820
P. rubescens		Switzerland	AJ132279
Spirulina sp.	PCC 6313		AJ401188
16S rRNA sequences:			
Azolla cyanobiont		Australia	AY181212
A. circinalis	AWQC 118C	Australia	AF247571
A. solitaria	NIES 80	Japan	AF247594
Anabaena flos-aquae	NRC 525-17		AF247597
Anabaena cf. *cylindrica*	133		AJ293110
Aphanizomenon flos-aquae			AY038035
Aphanizomenon gracile	NIVA-CYA 1-03	Norway	Z82806
C. raciborskii		Australia	AF092504
Nostoc sp. (PCC 7120)	NIVA-CYA 246	USA	Z82803
Nostoc sp. (Lichen cyanobiont)		China	AF506239
Nostoc flagelliforme			Y12688
M. aeruginosa	PCC 7806	The Netherlands	AF139299
P. rubescens			Y12680
Sponge (*Mycale* sp.) cyanobiont			AJ292192

2.3. Phylogenetic analysis of the 16S rRNA and PC-IGS sequences

The PC-IGS and 16S rRNA nucleotide sequences obtained were compared to entries deposited in the GenBank and EMBL databases (Table 1), using 'BlastN' [8]. The sequences were aligned and analysed using programmes of the Wisconsin Package, version 8.1 [9], available through the Australian National Genetic Information Service. 'Pileup' was used for sequence alignment and unrooted phylogenetic trees were constructed using the neighbour-joining method of Feng and Doolittle [10] on Jukes and Cantor distances. Bootstrap analyses of 1000 resamplings were performed for the consensus trees.

3. Results and discussion

All PCR amplicons from 16S rRNA and phycocyanin templates gave single clean bands when subjected to agarose gel electrophoresis, and produced clear, unambiguous sequences, demonstrating that a single type of cyanobiont was dominant in the *Azolla* fern. Alignment of a 358-bp fragment in the V6–V8 region of the 16S rRNA gene of

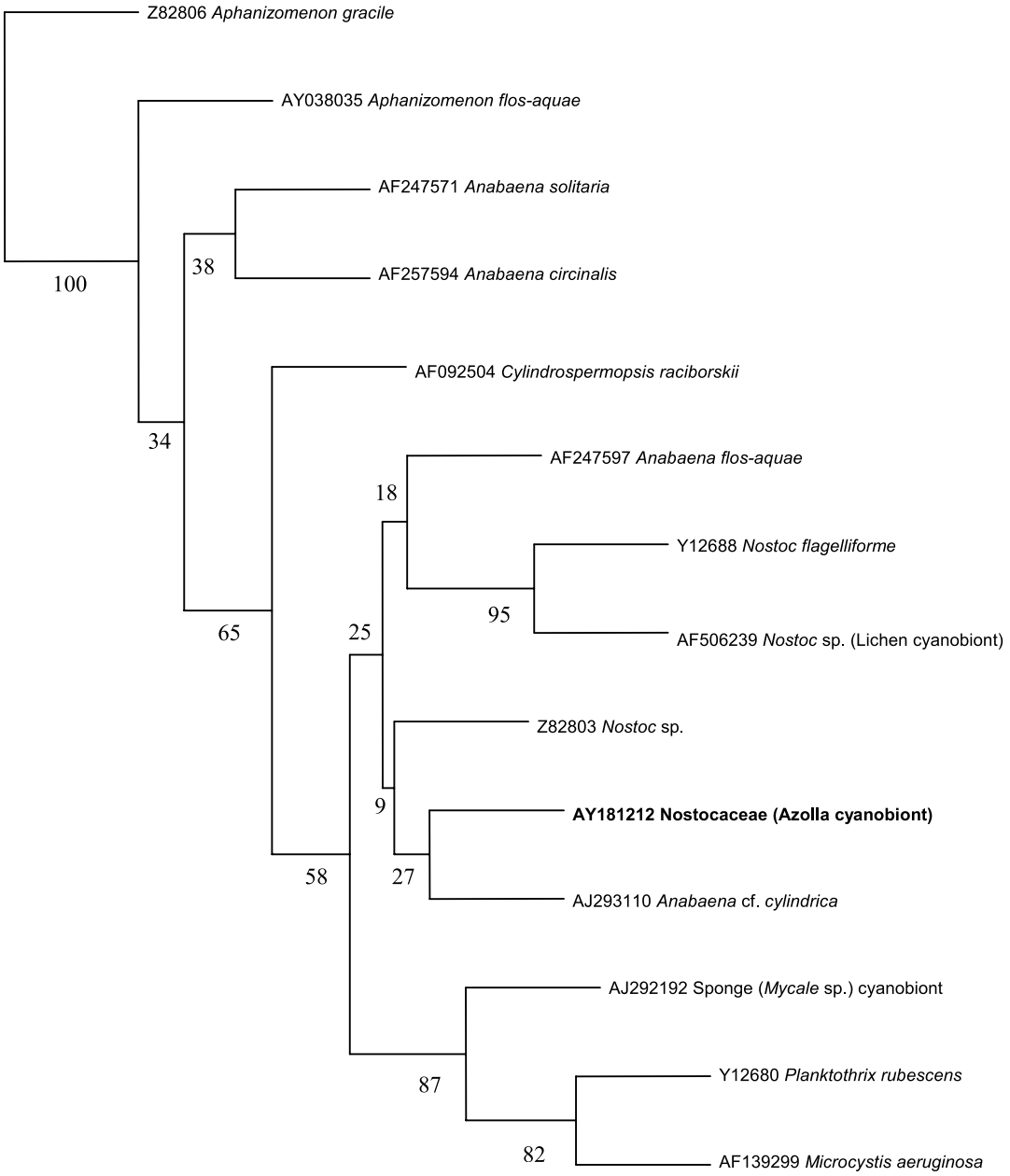

Fig. 1. Unrooted phylogenetic tree, based on the V6–V8 region (358-bp fragment) of 16S rRNA gene sequences, showing the relationships between a Nostocaceae cyanobiont from *Azolla* fern, some other cyanobionts and some planktonic cyanobacteria (see Table 1). The sequences were aligned and a consensus tree derived from maximum parsimony was constructed using the neighbour-joining method. The sequence of the fragment of 16S rRNA of *Microcystis aeruginosa* was used as the outgroup. Bootstrap values are based on 1000 resampled sets of data.

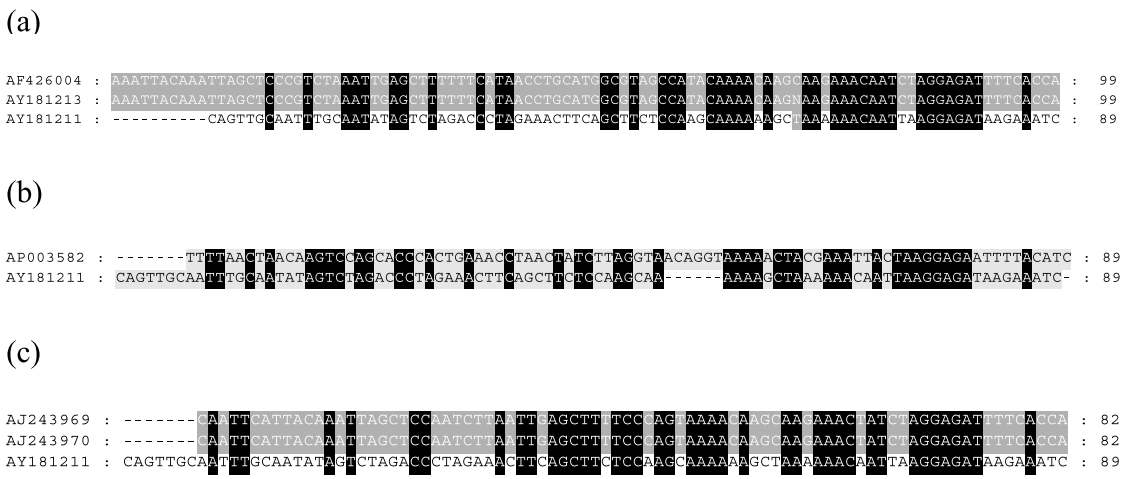

Fig. 2. Comparison of the PC-IGS sequence of the *Azolla* endosymbiont cyanobacteria (AY181211) isolated from Lake Madgwick with (a) *Anabaena circinalis* AWQC 118C (AF426004) and *A. solitaria* NIES 80 (AY181213); with (b) *Nostoc* sp. PCC 7120 (AP003582) (only database entry of the phycocyanin region of a *Nostoc* strain); and with (c) *Aphanizomenon* sp. (AJ243969) and *Aphanizomenon* sp. (AJ243970).

the *Azolla* cyanobiont with corresponding sequences belonging to various cyanobionts and planktonic cyanobacterial genera showed greater sequence similarity to members of the order *Nostocales* than to members of the orders *Chroococcales* and *Oscillatoriales* and other cyanobionts (results not shown). A phylogenetic tree, based on this alignment, clearly places the *Azolla* symbiont in the order *Nostocales*, which includes the genera *Anabaena*, *Nostoc*, and *Aphanizomenon* (Fig. 1). However, the 16S rRNA gene data did not provide enough information to indicate the position of the *Azolla* symbiont in relation to the genera in the order *Nostocales*.

Examples of genera from the *Nostocales* with similar cell morphology, including intercalary heterocysts were then compared with the *Azolla* endosymbiont by use of the PC-IGS sequence. In each comparison by sequence alignment there was $<50\%$ sequence similarity (Fig. 2). As the PC-IGS sequences of members of a cyanobacterial genus have $>90\%$ similarity in sequence and $>95\%$ similarity in length [6], we concluded that the *Azolla* symbiont does not belong to any of these genera. An unrooted phylogenetic tree, based on the alignment of the PC-IGS sequences of cyanobacteria available in databases, showed that the *Azolla* cyanobiont was not closely related to either the *Anabaena* or *Nostoc* genus (Fig. 3). Based on morphology, Komárek and Anagnostidis [11] placed *Azolla* endosymbionts in a revised genus named *Trichormus*. The results in this communication are consistent with this conclusion. However, a comprehensive study of many *Azolla* symbionts, using the methods described in this paper, would have to be conducted to support this contention. Nevertheless, our results support the proposal that *Azolla* endosymbionts are a separate group of cyanobacteria within the *Nostocales*. The molecular approach demonstrated here could be used to analyse and resolve the classification of an extensive range of *Azolla* and other cyanobionts.

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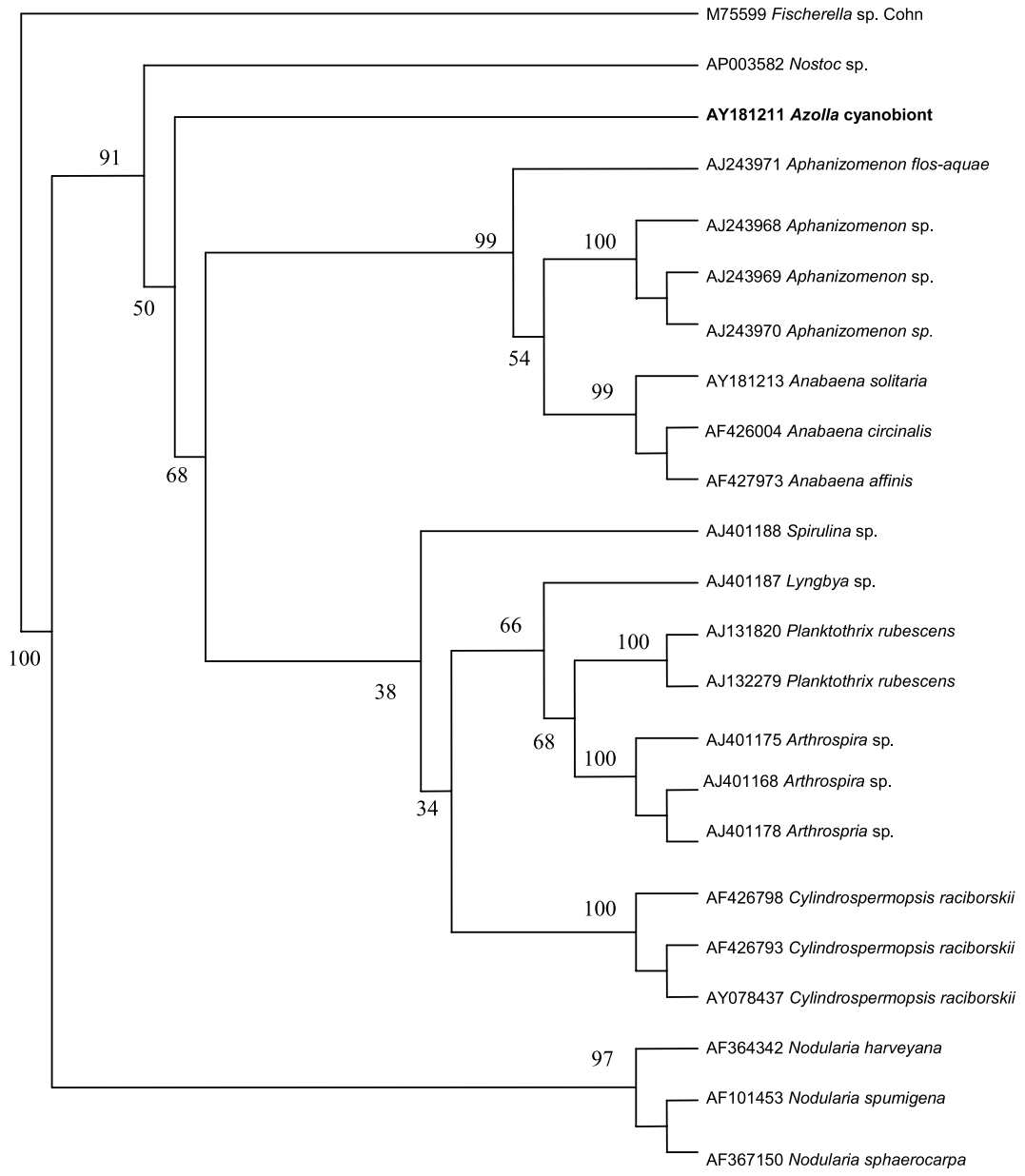

Fig. 3. Unrooted phylogenetic tree, based on the sequence of the PC-IGS regions of a range of cyanobacteria, showing the relationship between a Nostocaceae cyanobiont from *Azolla* fern and other cyanobacteria (see Table 1). The sequences were aligned and a consensus tree derived from maximum parsimony was constructed. The sequence of the PC-IGS region of *Fischerella* sp. Cohen was used as the outgroup. Bootstrap values are based on 1000 resampled sets of data.