Molecular phylogeny of the heterocystous cyanobacteria (subsections IV and V) based on *nifD*

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The heterocystous cyanobacteria are currently placed in subsections IV and V, which are distinguished by cellular division in one plane (false branching) and in more than one plane (true branching), respectively. Published phylogenies of 16S rRNA gene sequence data support the monophyly of the heterocystous cyanobacteria, with members of subsection V embedded within subsection IV. It has been postulated that members of subsection V arose from within subsection IV. Therefore, phylogenetic analysis of nucleotide sequences of the nitrogen-fixation gene *nifD* from representatives of subsections IV and V was performed by using maximum-likelihood criteria. The heterocystous cyanobacteria are supported as being monophyletic, with the non-heterocystous cyanobacteria as their closest relative. However, neither subsection IV nor subsection V is monophyletic, with representatives of both subsections intermixed in two sister clades. Analysis of *nifD* does not support recognition of two distinct subsections.

Cyanobacteria are oxygenic, photosynthetic prokaryotes that can be found in almost every aquatic and terrestrial environment (Castenholz & Waterbury, 1989) and are ancient organisms that date back 3.5 billion years in the fossil record (Castenholz, 1992). It has been proposed that cyanobacteria were responsible for converting the ancient Earth's anaerobic atmosphere to an aerobic one (Hayes, 1983; Schopf *et al.*, 1983). Many cyanobacteria fix nitrogen (diazotrophy) and they comprise one of the largest global suppliers of fixed nitrogen in the environment (Sprent & Sprent, 1990). In addition, some species of nitrogen-fixing cyanobacteria are involved in symbiotic relationships with many plant species and supply the host directly with a source of reduced nitrogen.

The taxonomy of the cyanobacteria has been debated vigorously and revised many times (Golubic, 1976; Turner, 1997). The cyanobacteria have been reclassified by using bacteriological instead of botanical criteria, based on morphological and developmental features, but most species, including those used in this study, have not yet been given validly published names under the Bacteriological Code. Cyanobacteria are currently divided into five subsections (Rippka

et al., 1979; Rippka, 1988; Castenholz & Waterbury, 1989; Castenholz, 1992, 2001; Rippka & Herdman, 1992). Subsection I strains are unicellular and divide by binary fission or budding (Rippka *et al.*, 1979). Subsection II strains are also unicellular but divide by multiple fission, resulting in the formation of baeocytes (Rippka *et al.*, 1979; Rippka, 1988; Castenholz & Waterbury, 1989; Castenholz, 1992, 2001; Rippka & Herdman, 1992). Subsection III strains are filamentous, non-heterocystous cyanobacteria that reproduce by trichome breakage (Rippka *et al.*, 1979; Rippka, 1988; Castenholz & Waterbury, 1989; Castenholz, 1992, 2001; Rippka & Herdman, 1992). Subsections IV and V are composed exclusively of heterocystous cyanobacteria, which are filamentous strains that reproduce by hormogonia formation and have the ability to develop heterocysts and akinetes (Rippka *et al.*, 1979; Rippka, 1988; Castenholz & Waterbury, 1989; Castenholz, 1992, 2001; Rippka & Herdman, 1992; Hoffman & Castenholz, 2001). The heterocystous cyanobacteria are placed into subsections IV and V, based on their ability to divide in one (false branching) or more than one (true branching) plane, respectively (Rippka *et al.*, 1979; Rippka, 1988; Castenholz & Waterbury, 1989; Castenholz, 1992, 2001; Rippka & Herdman, 1992; Hoffman & Castenholz, 2001). Subsection IV is divided further into two subgroups: subsection IV.I, which is composed of strains that do not show basal–apical polarity of their trichomes, and subsection IV.II, which is composed of strains that show distinct basal–apical polarity of their trichomes, with the trichomes tapering from the apex to the base (Castenholz, 2001; Rippka *et al.*, 2001). A table

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The GenBank/EMBL/DBJ accession numbers for *nifD* sequences determined in this study are AY196951–AY196958.

A table showing the current taxonomic placement of the heterocystous cyanobacteria and an alignment of *nifD* gene sequences are available as supplementary material in IJSEM Online.

showing the current taxonomic placement of the heterocystous cyanobacteria into subsections IV.I, IV.II and V is available as supplementary material in IJSEM Online.

It has been postulated that some of the morphological characters that are used to delineate the cyanobacteria may not reflect true evolutionary relationships within the lineage (Mollenhauer, 1988; Rippka, 1988; Rippka & Herdman, 1992). Molecular phylogenies have been employed to resolve evolutionary relationships within the cyanobacteria. In general, molecular phylogenies support the monophyly of subsections II, IV and V, whereas subsections I and III are not supported as being monophyletic (Giovannoni *et al.*, 1988; Wilmette, 1994; Turner, 1997; Zehr *et al.*, 1998, 2000; Turner *et al.*, 1999). 16S rRNA phylogenies support the monophyly of the heterocystous cyanobacteria and place subsection V within subsection IV, suggesting that the former arose from within the latter. Analysis of partial *nifH* sequences also supports the monophyly of the heterocystous cyanobacteria as a whole, but with members of subsections IV and V intermixed (Zehr *et al.*, 1998, 2000).

Our objective was to examine whether or not phylogenetic analysis of *nifD* supports the division of the heterocystous cyanobacteria into subsections IV and V. We examined phylogenetic relationships within the heterocystous cyanobacteria by analysing *nifD* nucleic acid sequences from representatives of subsections IV and V. *nifD* was chosen as previous studies have indicated that its divergence is intermediate between that of *nifH* and *nifK* (Mathur & Tuli, 1990) and should thus provide sufficient phylogenetic signal to resolve relationships within and among subsections IV and V. Furthermore, previous studies in our laboratory have shown that *nifD* is a good marker for resolving relationships within the heterocystous lineage (Henson *et al.*, 2002). It has been questioned whether *nif* genes have evolved by lateral transfer or vertical descent, which could affect our analyses, but recent evidence suggests that *nifD* evolved by vertical descent (Henson *et al.*, 2004). We analysed orthologous *nifD* genes that belong to the *nif1* operon, excluding the alternative nitrogenases *anfD*, *vnfD* and *nifD2*. To ensure that the heterocystous cyanobacterial sequences were *nifD1* sequences and not *nifD2* sequences, we included the *nifD2* sequence from *Anabaena variabilis* (Thiel, 1993) in some of our analyses. All analyses that included the *nifD2* sequence placed it outside the heterocystous cyanobacteria, with the non-heterocystous cyanobacteria (data not shown).

Heterocystous cyanobacteria examined were obtained from the Pasteur Culture Collection (PCC) and the University of Texas culture collection (UTEX). Cultures were grown in illuminated shaking incubators at 27 °C, in BG-11₀ + NaHCO₃ (5 mM) and BG-11₀ + NaNO₃ (2 mM) + NaHCO₃ (10 mM) media (Rippka *et al.*, 1979; Rippka & Herdman, 1992). Cultures were checked regularly for bacterial contamination by plating on Luria broth (LB) solidified medium and incubating overnight at 37 °C.

Genomic DNA was extracted by using a Purgene DNA

Isolation kit (Gentra Systems), with slight modification. Amplification of *nifD* was accomplished by using the Expand Long Template PCR system (Roche). The PCR primers used to amplify *nifD* were 5'-GATGGCGATGTATGCTGCTAAC-AAC-3' (corresponding to nucleotide positions 474–499 of *nifH*) and 5'-GtACGGAAGTAAGCAACGCAACCTTG-3' (corresponding to nucleotide positions 277–302 of *nifK*). PCR products were verified by agarose gel electrophoresis and purified with a QIAquick Gel Extraction kit (Qiagen). PCR products were cloned by using a TOPO TA Cloning kit for sequencing (Invitrogen). Sequencing was accomplished with a BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) and a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences), using capillary electrophoresis on ABI 310 and ABI 3100 genetic analysers (Applied Biosystems). Both the forward and reverse strands of *nifD* were sequenced completely from one clone; the forward strand from an additional clone from each strain was sequenced to ensure that no PCR artefacts were present. Sequences were deposited in GenBank (accession numbers are listed in Fig. 1).

In addition to the *nifD* sequences generated in this study, other sequences were obtained from GenBank (accession numbers are listed in Fig. 1). Inferred amino acid sequences were aligned initially by using CLUSTAL W (Thompson *et al.*, 1994), with gaps inserted for optimal alignment. The amino acid alignment was then adjusted visually by using MacClade 4.0 (Maddison & Maddison, 2000). We compared our alignment visually to the PFAM alignment of *nifD* (<http://pfam.wustl.edu/cgi-bin/getalignment>), to ensure correct alignment. The nucleotide alignment was generated from the amino acid alignment by using Codon Align 1.0 (Barry Hall, University of Rochester). The alignment produced by Codon Align was inspected visually by using MacClade 4.0 (Maddison & Maddison, 2000). A supplementary figure showing our nucleotide sequence alignment is available as supplementary material in IJSEM Online.

Analysis of the nucleic acid data matrix was performed by using maximum-likelihood criteria with PAUP* 4.0b10 (Swofford, 2002). Prior to analysis, we determined the evolutionary model that best described our data by using MODELTEST 3.06 (Posada & Crandall, 1998); the GTR + G model was determined to be the best fit for our data (likelihood score, 15973.05941). Trees were rooted with outgroup analysis by using *nifD* sequences from four non-heterocystous cyanobacteria, six proteobacterial representatives and one actinobacterium (*Frankia*). Maximum-likelihood analysis was conducted by using the heuristic search option, with the likelihood settings corresponding to the GTR + G model as follows: no molecular clock was enforced; starting branch-lengths were determined via the Rogers–Swofford approximation method; trees with likelihoods that were $\geq 5\%$ from the target score were rejected; branch-length optimization equalled one-dimensional Newton Raphson with pass, where the limit = 20 and $\delta = 1e^{-06}$; starting trees were obtained via stepwise addition; sequence addition was random; one

Fig. 1. Maximum-likelihood tree of *nifD* nucleic acid sequences with a $-\ln L$ score of 8850-58935. Cyanobacterial subgroups are shown in square brackets. Numbers above branches are bootstrap values. GenBank accession numbers are given on the right.

tree was held at each step during stepwise addition; the tree bisection-reconnection (TBR) branch-swapping algorithm was used; steepest descent option was not in effect; maxtrees was set to 50 000; branches were collapsed if the branch-lengths were $\leq 1e^{-08}$; the multrees option was in effect; and topological constraints were not enforced. Maximum-likelihood analysis was performed with the third codon position excluded and without. Bootstrap values were calculated for 1000 replicates to evaluate branch support by using parsimony criteria (Felsenstein, 1985; Bremer, 1994; Huelsenbeck *et al.*, 1995). Parsimony and distance analyses of the data matrix were also performed.

Maximum-likelihood analysis generated a single tree with

a $-\ln L$ score of 8850-58935 (Fig. 1). The heterocystous cyanobacteria are monophyletic, with the non-heterocystous cyanobacteria, proteobacteria and *Frankia* as sisters. Support for their monophyly is weak, with a bootstrap value of 55 % (Fig. 1); however, this is consistent with analyses of *nifH* (Zehr *et al.*, 1997, 1998, 2000). *nifD* does not support the monophyly of either subsection IV or subsection V. The heterocystous cyanobacteria occur in two major clades (1 and 2), with members of subsections IV and V intermixed.

Clade 1 is composed of two smaller branches, clades 1a and 1b. Clade 1a is composed of representatives of *Calothrix* (IV.II), *Scytonema* (IV.I), *Fischerella* (V) and *Chlorogloeopsis* (V) and clade 1b contains representatives of *Nostoc* (IV.I),

Cylindrospermum (IV.I) and *Chlorogloeopsis* (V). In clade 1a, *Fischerella* strains PCC 7603, PCC 7414 and UTEX 1931 occur with *Scytonema* strains PCC 7814 and PCC 7110 and *Calothrix* strain PCC 7102 in an unresolved polytomy, with *Fischerella* strain PCC 1903 and *Chlorogloeopsis* strain PCC 6718 as sister. Clade 1b contains *Nostoc* strains PCC 7120, PCC 6720 and PCC 7423, *Cylindrospermum* strain PCC 7604 and *Chlorogloeopsis* strain PCC 6912.

Clade 2 is composed of representatives of *Calothrix*, *Nostoc*, *Anabaena* (IV.I), *Nodularia* (IV.I), *Cylindrospermum* and *Tolypothrix* (IV.II), all of which are members of subsection IV. *Calothrix* strain PCC 7507 occurs with *Nostoc commune* UTEX 584; they are sister to *Anabaena* strains PCC 7122, PCC 7108 and ATCC 33047. Also occurring on clade 2 are *Nodularia* strains PCC 73104 and PCC 7804, *Cylindrospermum* strain PCC 7417 and *Tolypothrix* strain PCC 7101, which is located at the base of clade 2. Additional parsimony and distance analyses of the data matrix resulted in trees that were virtually identical to that in Fig. 1.

It has been suggested that classifications based on morphological and developmental features may not represent phylogenetic relationships within the heterocystous lineage, as some of the characters that have been used traditionally may be phenotypically plastic (Mollenhauer, 1988; Rippka, 1988; Rippka & Herdman, 1992). This is exemplified by *Nostoc* strain PCC 7120, which was formerly considered to be a species of *Anabaena* (Lachance, 1981; Rippka, 1988; Rippka & Herdman, 1992; Turner, 1997; Zehr *et al.*, 1997; Tamas *et al.*, 2000; Henson *et al.*, 2002), and by *Nostoc* strain PCC 6720, which was once described as *Anabaenaopsis* (Rippka *et al.*, 1979). The *nifD* phylogeny presented here supports the monophyly of the heterocystous cyanobacteria, which is congruent with 16S rRNA phylogenies (Giovannoni *et al.*, 1988; Wilmotte, 1994; Nelissen *et al.*, 1996; Turner, 1997; Turner *et al.*, 1999; Wilmotte & Herdman, 2001; K. C. Kenyon, L. E. Watson & S. R. Barnum, unpublished results), 23S rRNA gene sequences (K. C. Kenyon, L. E. Watson & S. R. Barnum, unpublished results) and partial *nifH* sequences (Zehr *et al.*, 1997, 1998, 2000). However, *nifD* does not support the monophyly of subsection V, which is congruent with *nifH* results (Zehr *et al.*, 1997, 1998, 2000), but incongruent with 16S rRNA phylogenies. Although 16S rRNA data support division of the heterocystous cyanobacteria into subsections IV and V, their division is not strongly supported as being monophyletic (Wilmotte & Herdman, 2001).

16S rRNA phylogenies support the monophyly of subsection V and its position as having arisen from within subsection IV (Giovannoni *et al.*, 1988; Wilmotte, 1994; Nelissen *et al.*, 1996; Turner, 1997; Turner *et al.*, 1999; Wilmotte & Herdman, 2001). Although subsections IV and V are still considered to be taxonomic divisions, Wilmotte & Herdman (2001) suggested that their distinction is not reflective of evolutionary relationships within the heterocystous lineage. *nifH* phylogenies do support the monophyly of the heterocystous cyanobacteria, but they do not support the monophyly of subsection V; rather, members of subsection V are

interspersed among members of subsection IV (Zehr *et al.*, 1997, 1998, 2000).

Although *nifD* does not differentiate the members of subsections IV and V, it does support the hypothesis that ability to undergo cellular division in more than one plane (the single characteristic that separates subsection V from subsection IV) may have arisen more than once (Turner, 1997). Interestingly, a *Nostoc* (subsection IV) symbiont of the moss *Blasia pusilla* has been observed to undergo cellular division in multiple planes in culture (Gorelova *et al.*, 1996), which raises the question of whether or not cellular division in one or more than one plane is a valid character for determining phylogenetic relationships.

nifD supports the monophyly of the heterocystous cyanobacteria. Our results failed to fully distinguish subsections IV and V, suggesting that their classification may not reflect phylogenetic relationships. Furthermore, the division of subsection IV into two distinct groups, IV.I and IV.II, based on whether or not their trichomes show basal-apical polarity, is not supported by *nifD*. Analysis of *nifD* is not congruent with morphological characters that are used to delineate the heterocystous cyanobacteria.

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