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.
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; Wilmotte, 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 nifI 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-11 + NaHCO3 (5 mM) and BG-11 + 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′-GATGGCGATGTGCTGCTAAC-3′ (corresponding to nucleotide positions 474–499 of nifH) and 5′-GACGGAAGTAGAACGGCAACCTTG-3′ (corresponding to nucleotide positions 277–302 of nifD). 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 DYEEnamic 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 ≥5% from the target score were rejected; branch-length optimization equalled one-dimensional Newton Raphson with pass, where the limit = 20 and δ = 1e-06; starting trees were obtained via stepwise addition; sequence addition was random; one
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 $\leq 10^{-2}$; 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 Scytosoma 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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