Common evolutionary origin of planktonic and benthic nitrogen-fixing oscillatoriacean cyanobacteria from tropical oceans

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Abstract

The filamentous cyanobacteria belonging to the genus *Hydrocoleum* (*Blennothrix*) are among the most common mat-forming cyanobacteria in tropical oceans. We present here the evidence that these benthic cyanobacteria are morphologically and phylogenetically very close to the planktonic species of *Trichodesmium*. Genetic relationship was established independently with regard to sequences of the 16S rRNA gene, *nifH* gene, and phycocyanin and phycoerythrin intergenic spacers. The species of both genera formed a common distinct branch in phylogenetically reconstructed cyanobacterial trees, suggesting that the main constituents of cyanobacterial benthos and plankton have an early common origin and both represent major contributors to nitrogen budget of tropical oceans today as in the distant geological past.

Introduction

The evolutionary history of cyanobacteria is today studied by two independent but complementary lines of inquiry: by exploration of the fossil record and by reconstruction of phylogenetic relationships among modern organisms by molecular sequencing (Knoll & Butterfield, 1989). The fossil record showed convincingly that oceans of the early Earth were dominated by ancient cyanobacteria as principal primary producers responsible for major impacts on planetary atmosphere and climate (Holland, 1994). While cyanobacteria left ample benthic fossil record in form of stromatolites and silicified microbial fossils (Schopf & Klein, 1992; Golubic & Seong-Joo, 1999), the fossil record of planktonic cyanobacteria is poor (Sergeev *et al.*, 2002), possibly because of their small size and/or delicate cellular structures (Golubic, 1980). Exploring phylogenetic interrelations among modern benthic and planktonic cyanobacteria bears relevance toward illuminating their historic origins.

Plankton in modern oceans maintains a substantial proportion of unicellular cyanobacteria (Urbach *et al.*, 1998), including those that form monophyletic grouping indicating their common origin at some time after the initial diversification of cyanobacteria. Some picoplanktonic phototrophs are known to fix nitrogen (Zehr *et al.*, 2001; Montoya *et al.*, 2004), a property of considerable selective advantage in a nitrogen-limited ocean. However, the bloom-forming non-heterocystous filamentous cyanobacteria of the genus *Trichodesmium* Ehrenberg (Carpenter *et al.*, 1992) are considered the most important nitrogen fixers in tropical oceans (Capone *et al.*, 1997, 2005). In contrast, benthic cyanobacteria and their potential to fix nitrogen are poorly known.

In the course of our studies of microbial mats in tropical lagoons of Tikehau Atoll, French Polynesia and around New Caledonia, we have frequently encountered rapidly expanding benthic blooms of cyanobacteria (Abed *et al.*, 2003a, b) that were macroscopically distinguishable by their intense coloration. Microscopic examination performed immediately following collection established that each of these blooms was a population of a single sheathed cyanobacterium identified as *Hydrocoleum* Kützing. Preliminary sequencing of these populations showed a close phylogenetic relationship with the planktonic species of *Trichodesmium* Ehrenberg (Abed *et al.*, 2003b). To verify this relationship, we performed here detailed morphological and molecular characterizations, including multigene sequencing of 16S rRNA and *nifH* gene fragments as well as phycocyanin and

phycoerythrin intergenic spacers, on natural populations of *Hydrocoleum* from two geographically distant locations (French Polynesia and New Caledonia). Our results demonstrate that the most common benthic mat-forming cyanobacteria in tropical oceans have the potential to fix nitrogen and show phylogenetic proximity to the planktonic *Trichodesmium*, indicating a common origin.

Materials and methods

Sampling, microscopy and identification of field material

Hydrocoleum populations from Tikehau Atoll and New Caledonia were collected by SCUBA dives. Microscopy was carried out using Zeiss universal light and fluorescence microscope equipped with transmitted light, phase contrast and Nomarski interference contrast illumination (DIC), immediately following collection. Scanning electron microscopy (SEM) was performed on samples that were fixed in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) using a Hitachi S-450 scanning electron microscope. Morphometric analysis was carried out on scanned photomicrographs and in-scale camera lucida projections of cell dimensions using Sigma Scan Image software (Jandel Scientific, Sausalito, CA) with its statistics package. The variations in dimensions are provided as: mean ± standard deviation ($n = 100$) projected as cell width to length relation. Samples for molecular analysis were preserved in guanidine thiocyanate solution (5 M guanidine thiocyanate, 100 mM EDTA pH 8.0 and 3.4 mM *N*-lauroyl sarkosine). Morphological identification was carried out in accordance with traditional phycological (Gomont, 1892; Komárek & Anagnostidis, 2005) and bacteriological (Castenholz *et al.*, 2001) systems, while awaiting further confirmation by molecular sequencing.

Extraction and analysis of carotenoids and phycobiliproteins

Two milliliters of *Hydrocoleum* suspensions preserved in guanidine thiocyanate solution were filtrated through Whatman GF/F glass fiber filters. The frozen filters were homogenized with ice-cold 90% acetone, incubated at 4 °C for 2 h, and finally centrifuged at 4500 *g* for 20 min at 5 °C to remove cellular particle debris. All steps were carried out under dim light. Pigments were separated using the RP-HPLC technique as described in Stoń & Kosakowska (2002). Identification was performed by cochromatography with commercially available standards. Phycobiliproteins were isolated according to Grossman *et al.* (1993). Cells were broken using lysozyme and the supernatant was then centrifuged overnight on sucrose gradients. *In vivo* absorption spectra and spectra of isolated phycobilisomes were

measured at room temperature with a Hitachi 3000 spectrophotometer.

DNA extraction and 16S rRNA gene-PCR amplification

DNA was extracted from 1 mL of the *Hydrocoleum* suspensions in guanidine thiocyanate as described before (Abed *et al.*, 2003b). 16S rRNA gene fragments were amplified using two sets of cyanobacteria-specific primers described in Nübel *et al.* (1997) and Nadeau *et al.* (2001), producing 700 and 1200 bp fragments, respectively. The PCR reaction of 50 µL contained 1× RED Taq PCR Buffer, 200 µM of each deoxynucleotide, 100 µg BSA, 250 ng of each oligonucleotide primer, 2.5 U of RED Taq DNA polymerase (Sigma-Aldrich) and 5 µL of DNA extract. Thirty-five PCR cycles were performed; each consisted of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. Purified PCR products (QIAquick PCR Purification Kit, Qiagen, Germany) were commercially sequenced in both directions.

Amplification and cloning of *nifH* genes

The *nifH* gene fragment was amplified from three representative *Hydrocoleum* populations (RD3, RD8 and GV58) using degenerate oligonucleotides described by Zehr & McReynolds (1989). The amplified *nifH* fragments (359 bp including primers) were purified by gel electrophoresis and cloned in competent *Escherichia coli* cells using the TOPO TA Cloning Kit, Invitrogen. The recombinant clones carrying the correct-sized insert were sequenced on both strands. A phylogenetic tree was constructed using all cyanobacterial *nifH* fragments published to date.

Amplification of the PC-IGS region and phycoerythrin β and α subunit gene fragments

The amplification of parts of the phycobilin operons was carried out using primers for phycocyanin (Neilan *et al.*, 1995) and phycoerythrin (Zehr & McReynolds, 1989). All reactions were subjected to an initial denaturation step of 94 °C for 4 min and a final extension step of 72 °C for 5 min. Four incubation cycles followed, each consisting of 45 s at 94 °C, 1 min at 50 °C and 1 min 72 °C; and finally 25 incubation cycles followed: (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min). Both operon fragments were commercially sequenced on both sides.

Sequence analysis and phylogenetic affiliation

Sequence alignment and phylogeny of 16S rRNA and *nifH* gene fragments were carried out using the ARB software (Ludwig *et al.*, 1998). Cyanobacterial gene sequences available from GenBank were imported and aligned in the database of the ARB software. To evaluate the consistency

of computed tree topologies subsets of data were analyzed using various algorithms as follows. A variety of single and multiple outgroup sequences representing phylogenetically diverse organisms were included in the analysis. To assess the influence of the most variable nucleotide positions they were excluded from some calculations by applying filters based on character frequency (ARB manual; Ludwig *et al.*, 1998). *NifH* gene sequences were edited and aligned with the BioEdit Sequence Alignment Editor (Hall, 1999). The amino acid sequences of our *nifH* gene sequences were predicted in BioEdit. For the purpose of phylogenetic reconstruction, the entire *nifH* database was downloaded from SwissProt and aligned using ARB software package. All *nifH* sequences obtained in this study were added and aligned to the database. The alignment was corrected manually. The phylogenetic trees were constructed by applying the three different methods integrated in the ARB software namely maximum likelihood, maximum parsimony and neighbor joining. The latter calculation was based on a matrix of evolutionary distances determined using the Jukes–Cantor or Felsenstein equations and subject to bootstrap analysis (1000 replicates). The 16S rRNA gene sequence of *E. coli* was used as outgroup.

Results and discussion

Extensive areas of the sea floor in the tropical lagoons around Nouméa, New Caledonia and Tikehau atoll, Tuamotu archipelago, French Polynesia are covered by microbial mats dominated by a large, sheathed non-heterocystous filamentous cyanobacterium (Sprachta *et al.*, 2001; Abed *et al.*, 2003b), which was identified, according to the phycological system, as *Hydrocoleum* (*Blennothrix*) *cantharidosmum* (Gomont, 1892; Komárek & Anagnostidis, 2005).

This organism is not recorded in Bergey's Manual of Systematic Bacteriology (Castenholz *et al.*, 2001). Such mats were also identified on several locations around Gran Canaria Island (own observations) and reported from tropical sea grass beds on Fiji Island, Papua New Guinea and Great Barrier Reef, Australia (Iizumi, 1994; Iizumi & Yamamuro, 2000). The mats consisted of films of interwoven filaments on various substrates (mud, sand, gravels and corals) and were observed at depths up to 50 m, but optimum growth at depths less than 10 m. The studied populations formed local benthic blooms characterized by intense coloration. Each population proved to be unicyanobacterial upon microscopic inspection. The filaments contained one to several cellular trichomes inside a firm, externally rough gelatinous sheath (Figs 1a and b). The trichomes have gliding motility and frequently abandon their sheaths (Figs 1c and d). The populations differed from one another in pigmentation and sheath consistency. Cell dimensions showed narrow normal distribution within each population, with significant overlap among populations (Fig. 2).

Trichomes of *Hydrocoleum* populations had many morphotypic properties in common with *Trichodesmium* species: slight constriction at the cross-walls, abruptly narrowed trichome tips with a distinct, calyptrate end cell and a tendency to keratomize (i.e. vacuolize by separation of thylakoids) (Komárek & Anagnostidis, 2005) along the distal parts of the trichome (comp. Figs 1d and e). Both contain similar set of pigments, including β-carotene, zeaxanthine, echinenone, and myxoxanthophyll. Further similarities between the representatives of the genera *Hydrocoleum* and *Trichodesmium* were found by analysis of phycoerythrin, which is the dominant phycobilin protein antenna pigment in species of both genera. In most
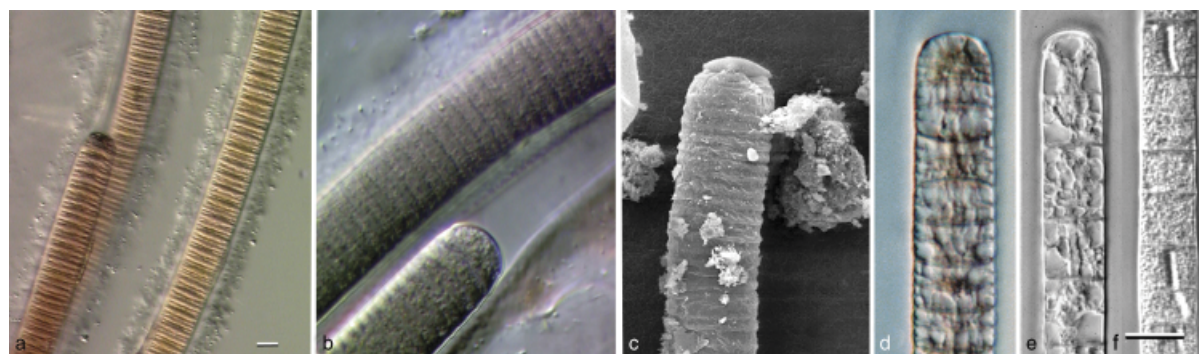

Fig. 1. Photomicrographs of the benthic cyanobacterium *Hydrocoleum cantharidosmum* (a–d) as compared with the planktonic *Trichodesmium thiebautii* (e–f). (a) Filaments of *Hydrocoleum* with bundled and single trichomes within thick, externally uneven sheaths; (b) close-up view of *Hydrocoleum* trichomes in common gelatinous sheath; note the dark phycoerythrin-rich pigmentation and calyptrate end cell; (c) scanning electron micrograph of critical point-dried *Hydrocoleum* trichome with calyptra-covered end cell; (d) keratomized trichome tip in *Hydrocoleum*; end cell with calyptra; (e) similarly keratomized trichome end in *Trichodesmium*, also with calyptra-covered end cell; (f) *Trichodesmium* cells with elongated packets of gas vesicles. Scale bar is 10 μm for all pictures (scale bar in f is also for d and e).
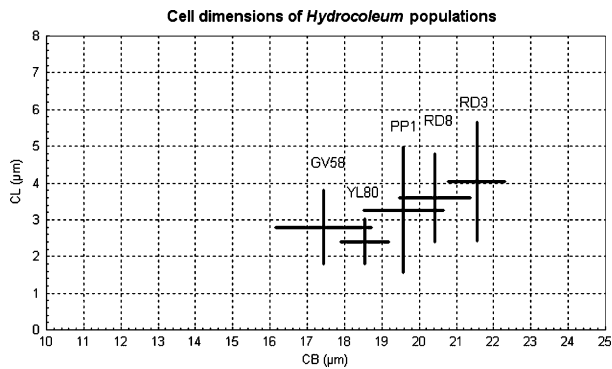
Fig. 2. Cell dimensions (breath vs. length) of five selected natural populations of *Hydrocoleum* (RD3, RD8, PP1, YL80 and GV58). The means plot at the intersection of cross diagrams with bars of one standard deviation on each side. Cell dimensions show narrow normal distribution within each population, with significant overlap among populations.

Fig. 3. Spectrum of isolated phycobilisomes of the *Hydrocoleum* RD3; three absorption peaks at 495, 547 and 562 nm corresponding to phycoerythrin and one at 617 corresponds to phycocyanin. The same pattern was obtained for all investigated *Hydrocoleum* populations.

cyanobacteria, the absorption spectrum of phycoerythrin showed a single peak at 565 nm (Glazer, 1988), whereas the phycoerythrin in all studied *Hydrocoleum* populations exhibited three absorption bands at 495, 547 and 562 nm (Fig. 3), a pattern which is similar to that of the phycoerythrin in red algae (Carpenter, 1983). This pattern was also reported for *Trichodesmium thiebautii* (Fujita & Shimura, 1974; Carpenter, 1983). Triple peaks might give an advantage to marine phototrophs in that the strong absorption band at the shortest wavelength extends the effective light absorbing region to the predominant shorter wavelengths in the sea, particularly to organisms occupying deeper euphotic ranges.

Distinctions between the compared planktonic and benthic taxa include the following: *Trichodesmium* species contain in most of their cells packets of gas vesicles (Fig. 1f) (aerotopes in Komárek & Anagnostidis, 2005). Gas-vesicles and buoyancy regulation in *Trichodesmium*, an important adaptation to planktonic way of life (Romans *et al.*, 1994), occurs in unrelated planktonic cyanobacteria and may have been acquired by lateral gene transfer as it was already suggested for planktonic *Nodularia* species (Lyra *et al.*, 2005). No gas vesicle packages exist in *Hydrocoleum* although a ring of low density vacuoles were observed around the terminal cell of some populations (Abed *et al.*, 2003b). *Trichodesmium* species form colonies, in which the trichomes are held together by a profuse exopolymeric gel (Romans *et al.*, 1994), whereas all species of the benthic *Hydrocoleum* are characterized by distinct sheaths that may vary in thickness and contain one to several trichomes (Figs 1a and b) as already described by Gomont (1892). It is noteworthy that the similarities between these genera refer to basic cell morphology, whereas the differences lie in adaptations to their respective planktonic and benthic habitats.
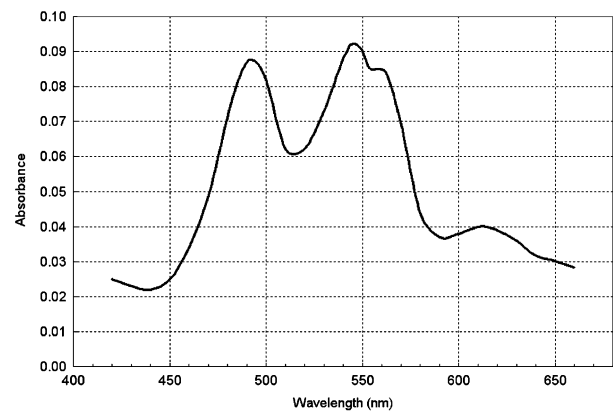
Phylogenetic reconstruction based on 16S rRNA gene sequences of five different natural populations of *Hydrocoleum* from New Caledonia (designated as PP1, RD3, RD8, GV58 and YL80) and three populations from Tikehau Atoll (designated as TK6, 15 and 21) (Fig. 4), confirmed their close relatedness to *Trichodesmium* species. The sequences of *Hydrocoleum* populations together with the published sequences of *Trichodesmium* formed a distinct cluster, well separated by more than 10% sequence divergence from other cyanobacteria. This divergence of 16S rRNA gene sequences corresponds to suprageneric taxonomic divisions in bacteriology (Wayne *et al.*, 1987). The phylogenetic trees obtained using the maximum likelihood, maximum parsimony and neighbor joining resulted in similar tree topologies and the *Hydrocoleum* populations and *Trichodesmium* species clustered always together, regardless of the applied treeing method. The *Hydrocoleum* populations shared more than 95% sequence similarity among themselves and between 96.0% and 97.1% to their closest relative *Trichodesmium* sp. NIBB 1067 (X70767). The similarity between *Hydrocoleum* sequences and those of other *Trichodesmium* species such as *Trichodesmium erythraeum* (AF013030) and *Trichodesmium thiebautii* (AF091321) varied between 95.61–96.74% and 95.44–96.92%, respectively. The compactness of the *Trichodesmium*/*Hydrocoleum* cluster would suggest generic level identity, however, because of morphological and ecological distinctions, we support their traditional taxonomic treatment as separate genera as it has been earlier decided for *Katagnymene* (Lundgren *et al.*, 2001).

The phylogenetic position of the analyzed *Hydrocoleum* populations and their relationship to *Trichodesmium* was further verified by cloning and sequencing of the *nifH* gene from three representative *Hydrocoleum* populations. Phylogenetic analyses (Fig. 5) supported the data obtained from

Fig. 4. Maximum likelihood phylogenetic tree showing affiliations of *Hydrocoleum* populations from two different locations, Tikehau Atoll, French Polynesia (asterisks) and New Caledonia with species of *Trichodesmium* based on publicly available cyanobacterial 16S rRNA gene sequences. *Escherichia coli* was used as outgroup. The 16S rRNA gene sequences were placed phylogenetically using parsimony criteria without changing the topology of the pre-established tree. GenBank accession numbers are indicated in parentheses. Bootstrap values from 1000 trees are included and indicated as percentage at relevant nodes. The bar indicates 10% sequence divergence.
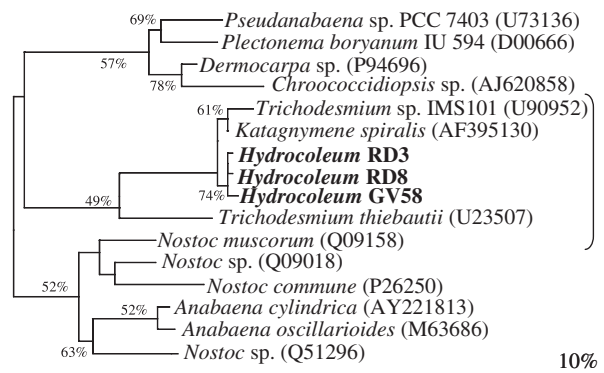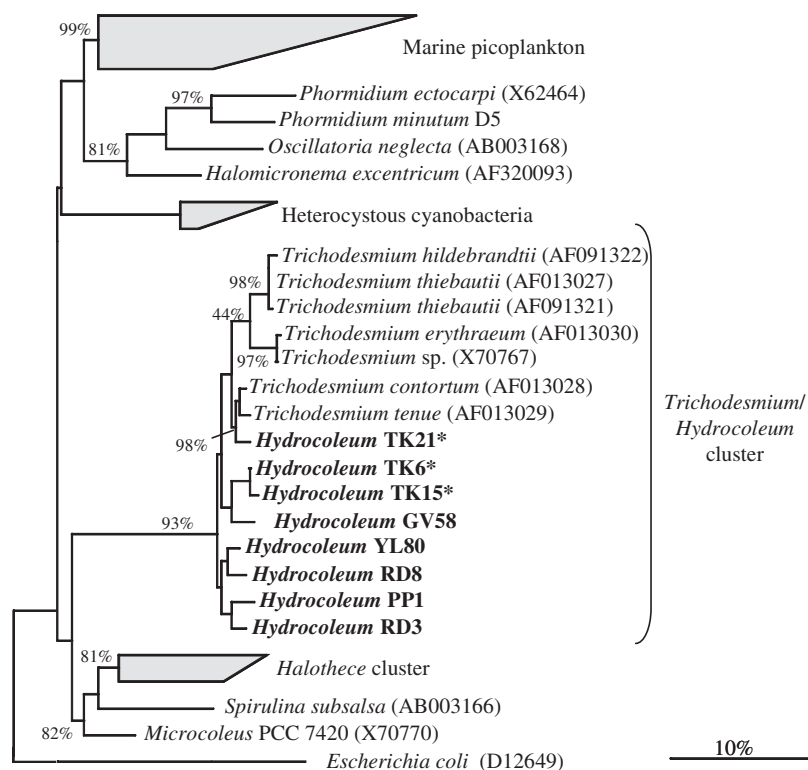
Fig. 5. Phylogenetic tree of *nifH* gene showing the position of sequences from three representative *Hydrocoleum* populations (RD3, RD8 and GV58). Bootstrap values from 1000 trees are included and indicated as percentage at relevant nodes. The bar indicates 10% sequence divergence.

16S rRNA gene sequences. *Hydrocoleum nifH* gene sequences clustered together and were most closely aligned with *Trichodesmium erythraeum* IMS101, *Katagnymene spiralis* and *Trichodesmium thiebautii*. *Katagnymene* is a sheathed solitary nitrogen-fixing oscillatoriacean, phylogenetically closely related to *Trichodesmium* (Lundgren *et al*., 2001). The phylogenetic divergence within this cluster was less than 6.2%. However, most of the divergence stems from the distance imposed by the deep-branching strain *Trichodesmium thiebautii*, while the others clustered tightly within less than 3.5% sequence divergence.

Distances between the *Hydrocoleum* and *Trichodesmium* sequences are much shorter than those separating some other cyanobacterial genera (> 7% sequence divergence) such as, for example, *Anabaena* and *Nostoc* (Henson *et al*., 2002). Detection of *nifH* gene in all *Hydrocoleum* populations studied implies their potential to fix atmospheric nitrogen. Indeed, nitrogen fixation measured in the mats of the Tikehau lagoon, French Polynesia by acetylene reduction (Charpy-Roubaud *et al*., 2001) showed a consistent increase in daytime values indicating a significant cyanobacterial contribution. Similar results were shown for sea-grass epiphytes on Great Barrier Reef, Australia, Papua New Guinea and Fiji Islands where the mats were also dominated by the same cyanobacterium (Iizumi, 1994; Iizumi & Yamamuro, 2000).

Additional analyses were carried out regarding sequencing and DNA polymorphisms of the phycocyanin and phycoerythrin intergenic spacers: *cpcBA*-IGS and *cpeB-cpeA* gene clusters (Neilan *et al*., 1995). The 695 bp PCR amplification products were detected for both genes in all populations tested. The sequence analysis showed that *Hydrocoleum* populations were 98% similar to each other and displayed 94% similarity to the sequence of

Trichodesmium erythraeum IMS101. Amplification of *cpeB-cpeA* fragment gave a single product of 517 bp. *Hydrocoleum* population PP1 was 88% similar to *Trichodesmium erythraeum* IMS101 (this study) and only 78% similar to the sequences of the freshwater planktonic gas-vesicle containing *Planktothrix rubescens* (Accession number AJ132245; Beard *et al.*, 1999).

Our results present clear evidence of strong similarities in genetic makeup and structural properties of the two genera that constitute the most abundant filamentous cyanobacteria in the plankton and benthos of tropical oceans. This finding is intriguing and inevitably lead to the question of their common origins, prevalence and diversification. Like the cluster of coccoid cyanobacteria in marine picoplankton (Castenholz *et al.*, 2001), the *Trichodesmium/Hydrocoleum* cluster occupies a distinct position in the phylogenetic trees suggesting that this lineage may have branched from other cyanobacteria relatively early. The timing of this common origin, however, remains unknown. The current criteria defining prokaryotic species are inadequate and incapable of keeping pace with the levels of microbial diversity that are being uncovered in nature. The species concept in Bacteria and accordingly in Cyanobacteria is still very problematic. Species are defined as genetic clusters, which are separated by genetic gaps. However, stable eco- and morphotypes do exist inside clusters and often across gaps. Future prospects of species and speciation concepts should certainly incorporate ecological data, which will allow for better taxonomic assignments. Furthermore, the ability of these benthic populations to fix dinitrogen points out to a significant contribution to nitrogen budget of the euphotic benthic habitats of the world's oceans, which may have been underestimated in earlier assessments.

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