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# Characterization of *Trichodesmium* spp. by Genetic Techniques

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**The genetic diversity of *Trichodesmium* spp. from natural populations (off Bermuda in the Sargasso Sea and off North Australia in the Arafura and Coral Seas) and of culture isolates from two regions (Sargasso Sea and Indian Ocean) was investigated. Three independent techniques were used, including a DNA fingerprinting method based on a highly iterated palindrome (HIP1), denaturing gradient gel electrophoresis of a *hetR* fragment, and sequencing of the internal transcribed spacer (ITS) of the 16S-23S rDNA region. Low genetic diversity was observed in natural populations of *Trichodesmium* spp. from the two hemispheres. Culture isolates of *Trichodesmium thiebautii*, *Trichodesmium hildebrandtii*, *Trichodesmium tenue*, and *Katagnymene spiralis* displayed remarkable similarity when these techniques were used, suggesting that *K. spiralis* is very closely related to the genus *Trichodesmium*. The largest genetic variation was found between *Trichodesmium erythraeum* and all other species of *Trichodesmium*, including a species of *Katagnymene*. Our data obtained with all three techniques suggest that there are two major clades of *Trichodesmium* spp. The HIP1 fingerprinting and ITS sequence analyses allowed the closely related species to be distinguished. This is the first report of the presence of HIP1 in marine cyanobacteria.**

The genus *Trichodesmium* is a group of globally significant marine diazotrophic cyanobacteria that contribute up to 80 Tg of new N input into oligotrophic waters per year (7, 21). They are cosmopolitan in tropical and subtropical oceans and can form massive surface blooms (6, 20) large enough to be seen from the space shuttle (21). *Trichodesmium thiebautii* and *Trichodesmium erythraeum* are the most frequently reported species associated with blooms in the open ocean.

It has been observed that oceanic $N_2$-fixing filamentous cyanobacteria have lower diversity than filamentous cyanobacteria found in coastal areas, freshwater, and terrestrial systems. With the notable exceptions of *Nostoc*, *Anabaena*, the endosymbiont *Richelia intracellularis* (8, 9, 37), and molecular evidence for heterocystous cyanobacteria at the Bermuda Atlantic Time-series Study (BATS) site (44), there are few heterocystous cyanobacteria in the open ocean relative to the numbers in brackish and freshwater systems. Additionally, there are few genera of nonheterocystous filamentous cyanobacteria in the open ocean, where the most notable such cyanobacteria are members of the genera *Trichodesmium* and *Katagnymene* (5).

Wille (40) described four species of *Trichodesmium* based on morphology (*T. contortum*, *T. thiebautii*, *T. tenue*, and *T. erythraeum*) and two species of *Katagnymene* (*K. spiralis* and *K. pelagica*) obtained on a cruise in the Atlantic Ocean. Later, Sournia (36) described one additional species, *Trichodesmium hildebrandtii*, and reluctantly placed *T. tenue* with *T. thiebautii* due to the large variation in filament size of the latter species (40). More recently, identification of *Trichodesmium* species based on ultrastructural and morphological characterization resulted in identification of five species of *Trichodesmium*, *T. thiebautii*, *T. erythaeum*, *T. tenue*, *T. contortum*, and *T. hildebrandtii* (19). In the latter study, species of *Trichodesmium* were found to be morphologically similar regardless of geographical origin.

Sequence analyses of *nifH*, *hetR*, and regions of the small-subunit rRNA (16S rRNA) have revealed very low genetic diversity among the species of *Trichodesmium* (4, 18, 42). The similarities of *nifH* sequences from other cyanobacterial species in a genus typically range from 92 to 95%, whereas *T. thiebautii* and *T. erythraeum nifH* sequences were found to be 98% identical (4, 42). Recently, Janson et al. (18) found *Trichodesmium* spp. to be very similar using 16S ribosomal DNA (rDNA) and *hetR* sequences and were able to resolve three clades containing (i) *T. thiebautii* and *T. hildebrandtii*, (ii) *T. contortum* and *T. tenue*, and (iii) *T. erythraeum*. In the latter study, the *hetR* sequence was more variable and gave better resolution between the species *T. erythraeum* and *T. thiebautii* than analysis of the 16S rDNA region gave.

Although the use of *hetR* in the study of Janson et al. (18) resulted in lower sequence similarity between *T. erythraeum* and *T. thiebautii*, the similarities between closely related species, such as *T. thiebautii* and *T. hildebrandtii*, were high (98%). In order to investigate the genetic diversity of the closely related species of *Trichodesmium*, genetic techniques that provide high resolution are required. In this study, we investigated the genetic diversity of *Trichodesmium* spp. by using three independent techniques that provide high resolution in order to resolve members of this closely related genus and to investigate the genetic diversity of natural populations. A PCR-based DNA fingerprinting method using base pair extended short oligonucleotide primers for HIP1 was used to distinguish *Trichodesmium* spp. and to investigate the genetic diversity within and between species. HIP1 is an octameric repetitive sequence found in many but not all cyanobacteria (33) and has

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TABLE 1. *Trichodesmium* spp. and *Katagnymene* sp. isolates used in the genetic analysis

| Species | Strain | Morphology | Cell size (μm) | | Place of origin | Year | Source |
|---|---|---|---|---|---|---|---|
| | | | Width | Length | | | |
| *T. erythraeum* | IMS 101 | Straight filaments | 6–7 | 4–7 | North Carolina coast | 1992 | L. Prufert-Bebout[a] |
| *T. erythraeum* | BE | Raft-like colonies | 6–7 | 4–7 | BATS, Sargasso Sea | 1999 | K. Orcutt |
| *T. erythraeum* | Z-5 | Straight filaments | 8–9 | 6–10 | Zanzibar, West Indian Ocean | 1998 | J. B. Waterbury |
| *T. erythraeum* | Z-9 | Straight filaments | 8–9 | 4–7 | Zanzibar, West Indian Ocean | 1998 | J. B. Waterbury |
| *T. thiebautii* | II-3 | Spherical colonies | 7–9 | 6–12 | BATS, Sargasso Sea | 1994 | J. B. Waterbury |
| *T. tenue* | Z-1 | Bow tie colonies | 5–7 | 10–19 | Zanzibar, West Indian Ocean | 1998 | J. B. Waterbury |
| *T. hildebrandtii* | #11 | Spherical or bow tie colonies | 14–17 | 6–12 | Central Sargasso Sea | 1994 | J. B. Waterbury |
| *T. hildebrandtii* | II-4 | Spherical or bow tie colonies | 16 | 5–10 | BATS, Sargasso Sea | 1998 | J. B. Waterbury |
| *K. spiralis* | KAT | Spiral filaments | 15–16 | 4–7 | Zanzibar, West Indian Ocean | 1998 | J. B. Waterbury |

[a] See reference 31.

been used in DNA fingerprinting to resolve cyanobacterial species and strains (35). The diversity of trichomes within single *Trichodesmium* colonies from natural populations and a comparison of culture isolates were examined by using denaturing gradient gel electrophoresis (DGGE) analysis of a fragment of the *hetR* gene. The sequence of the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes was determined to study inter- and intraspecific variability. In most bacteria the 5S, 16S, and 23S rRNA genes are separated by ITS regions. The spacer region between the 16S and 23S rRNA genes can encode zero, one, or two tRNA genes and can exhibit considerable length and sequence variation. Due to this variation analysis of the ITS region has been widely applied to many bacterial groups (2, 12, 17, 22) and cyanobacteria (24) in order to resolve closely related species.

## MATERIALS AND METHODS

**Collection of *Trichodesmium* spp.** Natural populations of *Trichodesmium* colonies were collected in 1999 from the BATS site (31°50′N, 64°10′W) off Bermuda and during a research cruise off North Australia between Darwin and Townsville. Colonies were picked randomly and separated into *T. thiebautii* tufts (colonies with trichomes arranged in parallel), *T. thiebautii* puffs (colonies with trichomes arranged radially), and the easily recognized *T. erythraeum* colonies (colonies that are darker reddish brown and have trichomes arranged in parallel like a raft). The colonies were rinsed in filtered seawater, placed on 0.5-μm-pore-size Fluorophore FHLP filters (Millipore, Bedford, Mass.), and frozen immediately in sterile Eppendorf tubes. The samples were transported on dry ice and stored at −20°C until they were used in the PCR. Culture isolates of *Trichodesmium* spp. were collected from the Sargasso Sea off Bermuda (BATS) and from the West Indian Ocean off Zanzibar (East Africa). An isolate of *K. spiralis* (KAT) was also collected off Zanzibar. All isolates of *Trichodesmium* spp. and *Katagnymene* were identified by using the morphological descriptions of Janson et al. (19) and Geitler (15) and are listed in Table 1. A culture strain of *T. erythraeum* (BE) was isolated from the BATS site by placing several colonies into a 50:50 mixture of Whatman GF/F-filtered Sargasso seawater and YBC11 medium (11). Cultured *Trichodesmium* spp. used in the HIP1 fingerprinting and DGGE analyses were maintained at the Department of Botany, Stockholm University, Stockholm, Sweden, on YBC11 medium (11) in a climate chamber under the following conditions: daily cycle consisting of 12 h of light (52 microeinsteins $m^{-2}$ $s^{-1}$) and 12 h of darkness, 28°C, and 65% humidity. All other *Trichodesmium* spp. and *K. spiralis* strain KAT were isolated from single trichomes. These cultured isolates were maintained at the Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Mass., in a medium made of 0.75× Sargasso seawater filtered successively through 1.0- and 0.2-μm-pore-size Millipore membrane filters before it was Tyndalized by heating in a microwave oven to boiling in Teflon containers. The Sargasso seawater was diluted with steam-sterilized Millipore Q water. All chemicals added to the medium were tissue culture tested and purchased from Sigma. Concentrated stocks (1,000×) were sterilized by either filtration or steam and added to the 0.75× seawater base. The chemicals added included 1.5 μM EDTA, 8 μM phosphoric acid, 0.05 μM Fe (ferric citrate), 0.1 μM $MnSO_4$, 0.01 μM $ZnCl_2$, 0.01 μM $NaMoO_4$, 0.0001 μM $CoCl_2$, 0.0001 μM $NiCl_2$, 0.0001 μM $NaSeO_3$, and 1.5 μg of vitamin $B_{12}$ per liter. All *Trichodesmium* cultures were grown in Nalgene polycarbonate flasks. The growth conditions were typically a cycle consisting of 14 h of light (30 to 300 microeinsteins $m^{-2}$ $s^{-1}$) and 10 h of darkness and temperatures ranging from 24 to 28°C. The small-volume cultures were gently shaken daily.

**HIP1 fingerprinting.** The sequences of the oligonucleotide primers used for the PCR fingerprinting were based on the HIP1 consensus sequence 5′-GCGA TCGC-3′ with a 2-bp extension at the 3′ end as described by Smith et al. (35). Extended HIP1 primers (HIP-AT, HIP-CA, HIP-GC, and HIP-TG) were used as either single primers or in combinations of two primers. The primers were synthesized by DNA Technology A/S (Aarhus, Denmark). The PCR samples were run in 25-μl mixtures containing a colony of field-collected *Trichodesmium* that was placed on a triangular piece of a 0.5-μm-pore-size Fluorophore FHLP filter (Millipore) (3). For the cultured *Trichodesmium* and *Katagnymene* strains, a 1-μl template was made from either one colony dispersed in sterile double-distilled freshwater or 2 to 5 ml of a dense culture filtered onto a 13-mm-diameter polycarbonate filter and dispersed in 20 to 50 μl of sterile double-distilled freshwater. Each reaction mixture contained a deoxynucleoside triphosphate mixture (Finnzymes OY, Espoo, Finland) (40 nM each), buffer (1/10 of the appropriate 10× buffer supplied by the manufacturer), 1.0 U of DyNAzyme DNA polymerase (Finnzymes OY), and primers (50 pmol each). All PCR samples were overlaid with sterile mineral oil. The PCR conditions were similar to those described by Smith et al. (35); reaction mixtures were cycled with a Perkin-Elmer Cetus DNA thermal cycler 480 using a temperature profile of 95°C for 5 min, 30 cycles of 95°C for 30 s, 30°C for 30 s, and 72°C for 60 s, and finally one cycle of 72°C for 5 min. Aliquots (12 μl) of each amplified PCR product were separated on a 1.5% agarose gel, visualized with ethidium bromide, and photographed under UV light using a Kodak DC 120 digital camera and Kodak Digital Science 1D Analysis software. A pGEM DNA marker (Promega, Madison, Wis.) was included on the agarose gel. At least 20 individual colonies of each species (*T. thiebautii* and *T. erythraeum*) and morphology (*T. thiebautii* puffs and tufts) were analyzed from both cruises (off Bermuda and off North Australia), and the cultured isolates were each examined with the PCR at least five times.

The specificity of the HIP1 primers was tested by using DNA templates from strains known to not contain HIP1 repeats (*Prochlorococcus marinus* strain MED4, *Synechococcus* sp. strain 8103, and *Synechococcus* sp. strain 7803) as a negative control. No PCR products were generated from these strains.

**PCR amplification of *hetR*.** The forward primer used in the PCR was *hetR*1 (5′-AARTGYGCNATHTAYATGAC-3′) described by Janson et al. (18) with an additional 39-bp GC clamp at the 5′ end. The reverse primer (5′-GCATCA GGCATARTTGAAGGA-3′) was designed to generate a 272-bp fragment by alignment of *hetR* genes from *Trichodesmium*, using the GenBank accession numbers reported by Janson et al. (18). Each reaction mixture contained a 2-μl template treated as described above for the HIP1 PCR, a deoxynucleoside triphosphate mixture (Finnzymes OY) (20 nM each), buffer (1/10 of the appro-

priate 10× buffer supplied by the manufacturer), 1.0 U of DyNAzyme DNA polymerase (Finnzymes OY), and primers (100 pmol each) and was overlaid with sterile mineral oil. The PCR conditions were those described by Janson et al. (18); reactions were cycled with a Perkin-Elmer Cetus DNA thermal cycler 480 using a temperature profile of 95°C for 6 min, 30 cycles of 93°C for 60 s, 52°C for 60 s, and 70°C for 60 s, and finally one cycle of 70°C for 10 min.

**Analysis of PCR products by DGGE.** DGGE was performed by using the Dcode universal mutation detection system as described in the manufacturer's manual (Bio-Rad, Hercules, Calif.). Aliquots (15 μl) of each PCR product were combined with 15 μl of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol) and applied directly onto 6% (wt/vol) polyacrylamide gels in 0.5× TAE buffer (0.02 M Tris base, 0.01 M acetic acid, 0.5 mM EDTA; pH 8.0) with a linear 20 to 45% denaturant gradient (100% denaturant was defined as 7 M urea and 40% [vol/vol] formamide). Gel electrophoresis of cultured strains (*T. tenue* Z-1, *T. hildebrandtii* #11 and II-4, and *K. spiralis* KAT) and natural samples (*T. thiebautii* puffs and tufts) was performed at 60°C (constant temperature) for 6.5 h at 150 V; the *T. erythraeum* strains (IMS 101, BE, and Z-9) and natural samples of *T. erythraeum* were electrophoresed under the same conditions for 24 h at 150 V. For quality control in the DGGE analysis, *Nostoc* sp. strains PCC 9229 and PCC 9231 were included to ensure that denaturation had occurred (32). After electrophoresis the gel was stained for 10 min with ethidium bromide and photographed as described above.

**Sequencing of the ITS region.** A high-fidelity PCR protocol with the *Pfu* polymerase (Promega) was used to amplify the ITS region from the following culture isolates: *T. erythraeum* strains IMS 101 and Z-9, *T. tenue* strain Z-1, *T. hildebrandtii* strains II-4 and #11, *T. thiebautii* strain II-3, and *K. spiralis* strain KAT (Table 1). The DNA sequence of the ITS was obtained by using some modifications of the method described by Rocap et al. (34). The cultured cells ($3 \times 10^6$ cells $ml^{-1}$) were filtered onto 5-μm-pore-size polycarbonate membranes to dryness, resuspended in 100 μl of sterile double-distilled freshwater, heated at 95°C for 10 min, and centrifuged in an Eppendorf microfuge at 14,000 rpm for 5 min. The supernatant fraction was used as the template for high-fidelity PCR with *Pfu* polymerase and primers tri16S-1247F (5′-CGTACTACAATGGTTGG G-3′) and 23S-241R (5′-TTCGCTCGCCRCTACT-3′). Primer tri16S-1247F was designed to be specific for filamentous nonheterocystous cyanobacteria (*Trichodesmium* and *Oscillatoria*) by using the Ribosomal Database Project check probe functionality (26). The reverse primer 23S-241 sequence was taken from the study of Rocap et al. (34), and only one band was obtained for each strain. Amplified products from two independent reaction mixtures were purified from the agarose gel by using a Qiaquick gel extraction kit (Qiagen, Valencia, Calif.) and were sequenced entirely on both strands at the University of Maine DNA Sequencing Center. Two additional primers, AlaF (5′-TWTAGCTCAGTTGG-TAGAG-3′) and AlaR (5′-CTCTACCAACTGAGCTAWA-3′), were used in order to get complete coverage of the ITS region (34).

**Analysis of ITS sequences.** The DNA sequences were aligned by using Macvector (Oxford Molecular, Oxford, United Kingdom) and ClustalX (39) software. The evolutionary relationships of the cultures were determined with PAUP (38) by using optimality settings of distance, maximum likelihood, and maximum parsimony. In these analyses the insertions and deletions were treated as missing data. The HKY85+gamma model was used for the likelihood analyses, while LogDet was used for neighbor joining. The gamma parameter and nucleotide frequency were determined empirically. Bootstrap values were generated for all three methods by using 1,000 replicates. Similar relationships were inferred for the remaining *Trichodesmium* species when the three *T. erythraeum* strains were omitted.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the sequences determined in this study are as follows: *Trichodesmium* strain Z-9, AF399646; *Trichodesmium* strain IMS 101, AF399647; *Trichodesmium* strain II-3, AF399648; *Trichodesmium* strain #11, AF399649; *Trichodesmium* strain II-4, AF399650; *Trichodesmium* strain Z-1, AF399651; and *Katagnymene* strain KAT, AF399652.

## RESULTS

**HIP1 fingerprinting.** Each of the four HIP1 extended primers was tested for its ability to distinguish between cyanobacterial cultures of the different species. The DNA of *Trichodesmium* spp. contain large numbers of HIP1 sequences that produced smearing banding patterns when nonextended HIP1 primers were used (data not shown). Therefore, both single extended primers and two extended primers in combination were tested (Fig. 1). The single HIP-TG extended primer gave distinct fingerprints for *T. erythraeum* strain BE and *T. thiebautii* strain II-3 but with few products that were similar in size in both species (Fig. 1). In order to optimize the fingerprint resolution by producing more complex banding patterns, double-primer combinations were used (Fig. 1). The combination HIP-GC and HIP-CA gave the highest resolution and was able to distinguish between species in culture (Fig. 2). Cultured isolates of *T. thiebautii* strain II-3, *T. hildebrandtii* strains II-4 and #11 , *T. tenue* strain Z-1, and *K. spiralis* strain KAT displayed fingerprints that were similar but had banding patterns that were distinguishable from each other. *T. erythraeum* strains IMS 101 and Z-9 displayed a pattern that was distinctly different from the other patterns (Fig. 2). However, within the *T. erythraeum* species group it was possible to distinguish the HIP1 patterns of strains IMS 101 and Z-9 (Fig. 2). In contrast, the two strains of *T. hildebrandtii* (#11 and II-4) collected 4 years apart from the Sargasso Sea had virtually identical fingerprint patterns (Fig. 2).

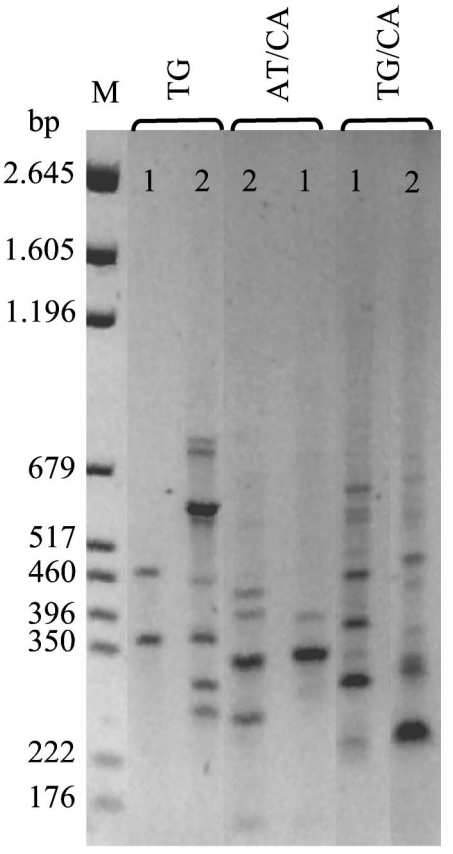


FIG. 1. HIP1 fingerprints obtained with different extended primers, including a single primer (HIP-TG [TG]) and combinations of two primers (HIP-AT and HIP-CA [AT/CA], HIP-TG and HIP-CA [TG/CA]), for culture isolates of *T. erythraeum* strain BE (lanes 1) and *T. thiebautii* strain II-3 (lanes 2). Lane M contained DNA molecular weight standards.

Natural populations of *T. thiebautii* puffs and tufts collected off Bermuda at the BATS site and off North Australia gave nearly identical fingerprint patterns in the 222- to 460-bp region (Fig. 3). Morphologically different *T. thiebautii* colonies

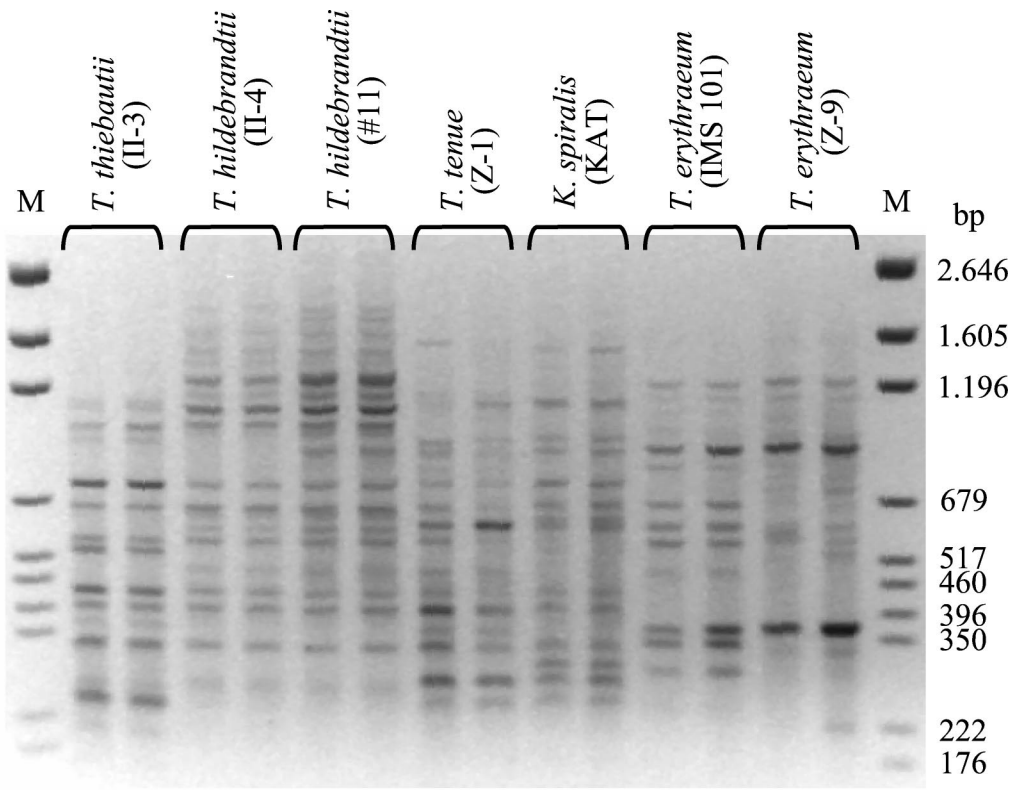


FIG. 2. Fingerprint patterns of culture isolates of *Trichodesmium* spp. and *Katagnymene* sp. obtained with a combination of primers HIP-GC and HIP-CA. The results for two replicate samples of each strain are shown, and lanes M contained DNA molecular weight standards.

had similar fingerprints, even though there were differences in trichome width within a colony (range, 4 to 10 μm) or in the arrangement of trichomes as puffs or tufts (Fig. 3). In addition, the cultured *T. thiebautii* strain II-3 puffs gave a fingerprint identical to that of the natural populations of *T. thiebautii* puffs and tufts (Fig. 3). Natural populations of *T. erythraeum* from both hemispheres had similar banding patterns in the 176- to 679-bp region (Fig. 4). However, cultured isolates of *T. erythraeum* strains IMS 101, BE, and Z-5 showed small genetic differences compared to each other (Fig. 4).

**Analysis of the *hetR* fragment by DGGE.** DGGE analysis of the PCR-amplified 272-bp *hetR* fragment was performed for both natural populations and cultured isolates. The *hetR* fragments from the heterocystous cyanobacteria *Nostoc* strains PCC 9229 and PCC 9231 were included as controls in the DGGE analysis to ensure that denaturation occurred. The amplified *hetR* fragments from the *T. thiebautii* tufts collected from natural populations off Bermuda at the BATS site and off North Australia were all identical to each other (Fig. 5A), as were the amplified *hetR* fragments from the morphologically distinctive *T. thiebautii* puffs and tufts from the BATS site (Fig. 5B). All of the DGGE analyses of colonies from natural populations showed that individual colonies contained trichomes that were similar, forming one distinct band on the gel (Fig. 5A and B). The cultures of *Trichodesmium* isolates, including *T. thiebautii* strain II-3, *T. hildebrandtii* strains #11 and II-4, *T. tenue* strain Z-1, and *K. spiralis* strain KAT, exhibited small differences in the *hetR* fragment compared to the fragment of the *T. erythraeum* strains, which showed the largest difference in mobility (Fig. 5C to E). The *hetR* fragments of cultured *T. thiebautii* strain II-3 and *T. hildebrandtii* strains #11 and II-4 had migration patterns similar to each other and could not be distinguished easily (Fig. 5C). Similarly, the *hetR* fragments of *T. tenue* strain Z-1 and *K. spiralis* strain KAT migrated close to each other on the DGGE gel (Fig. 5D). The largest difference observed between cultured species was that separating *T. erythraeum* strains IMS 101, BE, and Z-9 from the group encompassing *T. thiebautii* strain II-3, *T. hildebrandtii* strains #11 and II-4, *T. tenue* strain Z-1, and *K. spiralis* strain KAT. The *hetR* fragment of *T. erythraeum* denatured higher in the gel on a lower gradient compared to the *hetR* fragment of the *T. thiebautii* group and required a melting time as long as 24 h to resolve a single band in the DGGE analysis (Fig. 5E). The *hetR* fragments from natural populations of *T. erythraeum* obtained from the BATS site and off North Australia comigrated (data not shown), and the *hetR* fragments from *T. erythraeum* strains IMS 101, BE, and Z-9 from different geographical areas (Table 1) were found to have similar mobilities (Fig. 5E).

**Analysis of the ITS region.** Six ITS sequences were determined from cultured isolates of *Trichodesmium* spp., and one ITS sequence was determined from *Katagnymene* sp. strain KAT (Fig. 6). These sequences did not contain the duplications seen in cultured strain NIBB 1067 analyzed by Wilmotte et al. (41). All of the cultured *Trichodesmium* strains in this study and *Katagnymene* sp. strain KAT showed high sequence similarity (Fig. 6). Strains of *T. erythraeum*, including isolates from the Atlantic (IMS 101), Pacific (NIBB 1067), and Indian (Z-9) oceans, were readily separated from *T. thiebautii* strain

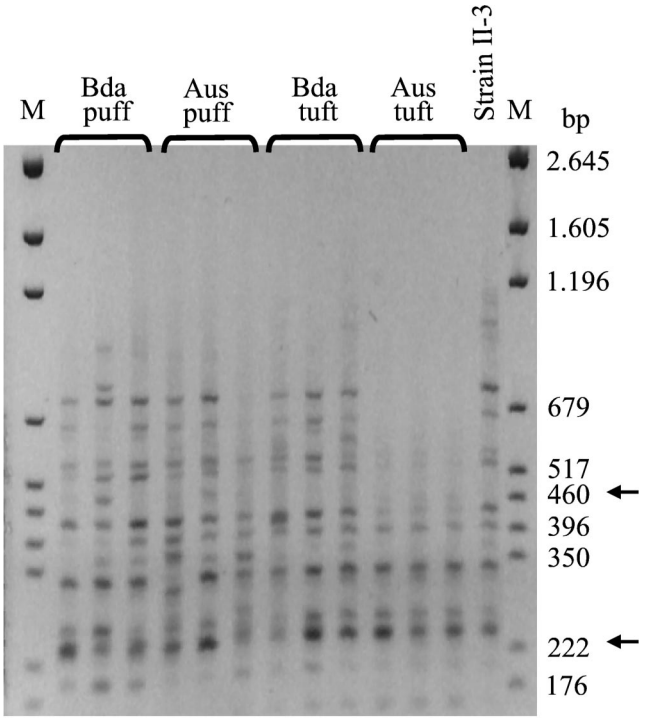


FIG. 3. Fingerprint patterns obtained with a combination of primers HIP-GC and HIP-CA for *T. thiebautii* puffs and tufts collected at the BATS site near Bermuda (Bda) and in the Arafura Sea off North Australia (Aus). For each group the results for 3 of a minimum of 20 samples analyzed are shown. A culture of *T. thiebauttii* strain II-3 was used for comparison. The arrows show the range of the primary bands used for pattern identification, and lanes M contained DNA molecular weight standards.

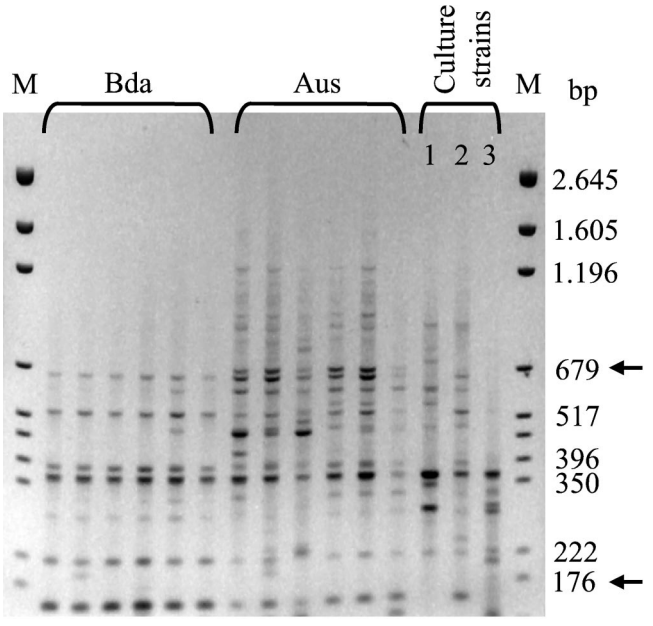


FIG. 4. Fingerprint patterns obtained with a combination of primers HIP-GC and HIP-CA for natural populations of *T. erythraeum* collected at the BATS site (Bda) and off North Australia (Aus). Culture isolates of *T. erythraeum* strain IMS 101 (1) and *T. erythraeum* strain BE (2), both from the North Atlantic, as well as *T. erythraeum* strain Z-5 (3) collected off Zanzibar, were included for comparison. For each station the results for 6 of a minimum of 20 samples analyzed are shown. The arrows show the range of the primary bands used for pattern identification, and lanes M contained DNA molecular weight standards.

II-3, *T. hildebrandtii* strains #11 and II-4, *T. tenue* strain Z-1, and *K. spiralis* strain KAT (Fig. 7). The members of the group containing *T. thiebautii* strain II-3, *T. hildebrandtii* strains #11 and II-4, *T. tenue* strain Z-1, and *K. spiralis* strain KAT were all very similar. *K. spiralis* strain KAT was most similar to *T. tenue* strain Z-1. However, all isolates sequenced in this study could be distinguished from each other (Fig. 6 and 7).

## DISCUSSION

Our results strongly support the results of other studies that found *Trichodesmium* spp. to be closely related (4, 18, 42). Genetic characterization of *Trichodesmium* spp. based on HIP1 fingerprinting, DGGE analysis of the *hetR* fragment, and ITS sequencing showed that *Trichodesmium* spp. cluster into two major groups. This suggests that two distinct clades are present in the oceans, one including the closely related species *T. thiebautii*, *T. tenue*, *T. hildebrandtii*, and *K. spiralis* and the other containing only *T. erythraeum* (Fig. 7).

In particular, our ITS data indicate that the genus *Trichodesmium* is composed of a closely related group of species (Fig. 6 and 7). The conservation of length and sequence variation within the ITS of the strains examined so far is quite remarkable, but nonetheless differences appear to be sufficient to be able to delineate species clusters. In contrast to the *hetR* sequence analysis described by Janson et al. (18), it appears that the ITS sequences are able to distinguish *T. thiebautii* strain II-3 from *T. hildebrandtii* strains #11 and II-4.

We have demonstrated that DNA fingerprinting using the repetitive sequence of HIP1 combined with ITS sequence analysis provides sufficient resolution to identify closely related species in the genus *Trichodesmium*. However, DGGE analysis of the *hetR* fragments could not resolve the small differences between closely related species, such as *T. thiebautii* (strain II-3) and *T. hildebrandtii* (strains #11 and II-4). Therefore, the DGGE technique could only determine that trichomes within an individual colony were not a mixture of members of the two clades of *Trichodesmium* spp. in natural populations.

The use of HIP1 fingerprinting provided sufficient resolution in an analysis of the genetic diversity of natural populations of *Trichodesmium* from two distant geographical locations (Fig. 3 and 4). Although whole colonies from the natural environment can be used directly as templates in the PCR, variation in template size (variable colony size) can affect the amplification efficiency of some PCR products. For example, we experienced incomplete amplification of large PCR products when small colonies were used (Fig. 3 and 4). Smaller *T. erythraeum* colonies were found off Bermuda at the BATS site than in the waters off North Australia, and this may have resulted in a lower efficiency of amplification of large PCR products from the BATS samples (Fig. 4). The presence of polysaccharides that are common in some cyanobacteria may also cause inhibition in the PCR (35). The Fluorophore filters were useful for collecting and transporting *Trichodesmium* colonies from the

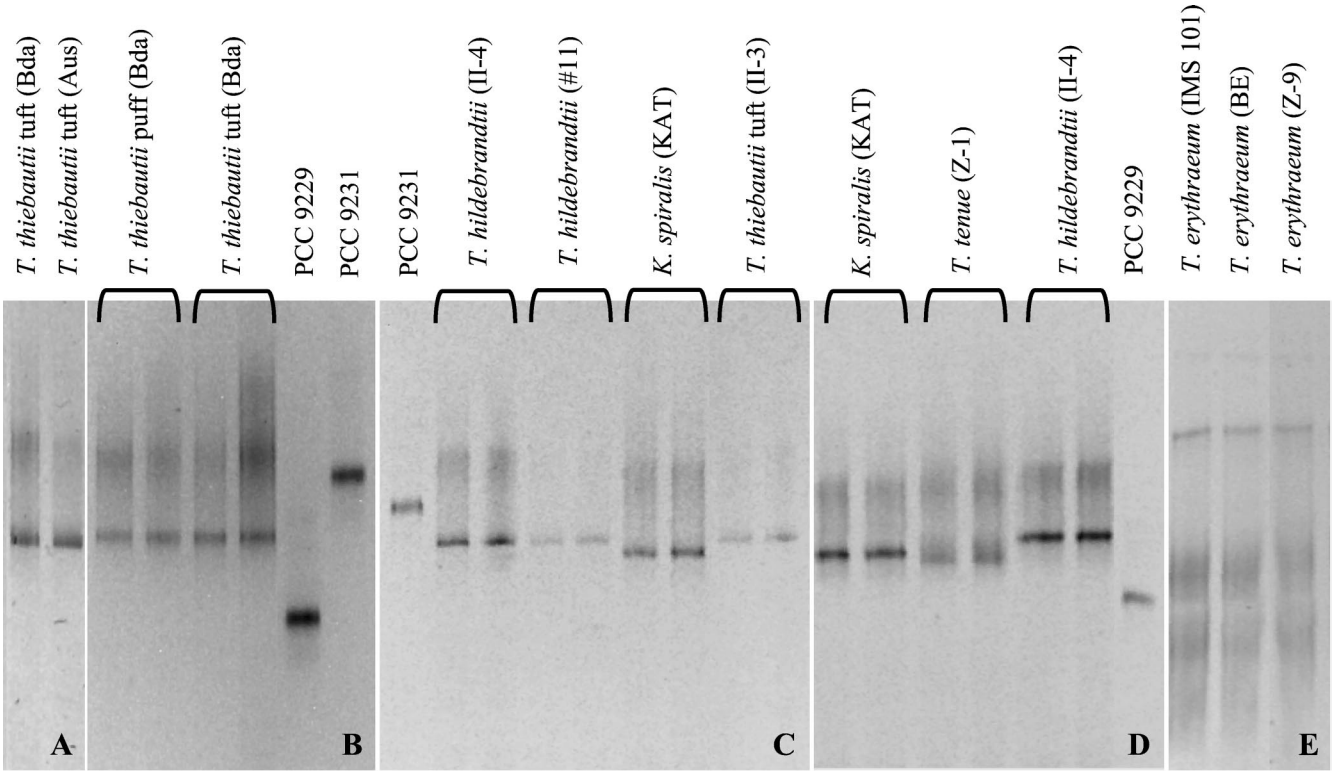


FIG. 5. DGGE analysis of the *hetR* fragment from natural populations of *T. thiebautii* tufts collected at the BATS site (Bda) and off North Australia (Aus) (A); natural populations of *T. thiebautii* puffs and tufts collected at the BATS site and *Nostoc* strains PCC 9229 and PCC 9231 (B); culture isolates of *T. hildebrandtii* strains II-4 and #11, *K. spiralis* strain KAT, and *T. thiebautii* tuft strain II-3 (C); cultured isolates of *K. spiralis* strain KAT, *T. tenue* strain Z-1, and *T. hildebandtii* strain II-4 (D); and cultured isolates of *T. erythraeum* strains IMS 101, BE, and Z-9 (E).

field to the laboratory and can be used directly in the PCR (3). Finally, due to the large number of products obtained with HIP1 primers, any folding of the DNA template prevents primers from annealing and results in faint bands or a loss of bands in the DNA fingerprints. Therefore, multiple samples are recommended for any investigation of natural populations.

The fact that HIP1 is present in the *Trichodesmium* genome is an interesting result in itself. HIP1 sequences are involved in adaptive response and were first identified in cells of Cd-tolerant *Synechococcus* strain PCC 6301, where they traversed an excised region of the metallothionein locus (16). HIP1 sequences are unique among repetitive sequences in that they are abundant in protein-encoding regions of the genome (33). No HIP1 sequences have been identified in the genomes of marine *Synechococcus* sp. strain WH8102 and *Prochlorococcus* sp. strain MED4 (27), and it was suggested that marine cyanobacteria might not contain HIP1 sequences due to the homeostatic environment of the open ocean (10). To our knowledge, this is the first report of the presence of HIP1 sequences in a marine cyanobacterium. Therefore, the possible role of HIP1 sequences in the adaptive response and genome plasticity of *Trichodesmium* spp. will be of great interest in future studies.

Genetic comparisons of bacterioplankton from the Atlantic and Pacific oceans have shown that certain microbial species are widely distributed in subtropical oceans (29). For example, high- and low-light-adapted *Prochlorococcus* isolates from two different regions of the North Atlantic were found to have high sequence similarity in their 16S rRNA genes (28). Molecular evidence also suggests that transequatorial mixing of Arctic and Antarctic populations of foraminiferans occurs (13). Our data based on HIP1 fingerprinting indicate that members of the genus *Trichodesmium* have low genetic diversity in natural populations and that individual species may have a global distribution.

The cultured isolates of *T. erythraeum* strain BE from the BATS site, *T. erythraeum* strains Z-5 and Z-9 from the West Indian Ocean, and *T. erythraeum* strain IMS 101 from Gulf Stream waters off the Carolinas exhibited similar primary banding patterns but also slight differences (Fig. 2 and 4). These results could indicate that there are several subspecies within *T. erythraeum*. However, the HIP1 fingerprints of natural populations of *T. erythraeum* from the two hemispheres showed little genetic diversity within this species (Fig. 4). The changes in banding patterns observed in culture isolates compared to natural populations may be indicative of gene rearrangements that can occur as a result of laboratory maintenance in culture (33). This phenomenon appears to be unique to *T. erythraeum* as isolates of *T. hildebrandtii* strains #11 and II-4 had virtually identical fingerprints even though they were isolated 4 years apart (Fig. 2). Similarly, *T. thiebautii* strain II-3 isolated from the BATS site in 1994 had the same fingerprint as the natural samples collected in 1999 (Fig. 3).

Colonies of *T. thiebautii* puffs and tufts were found to be identical as determined by HIP1 fingerprinting and DGGE analysis of the *hetR* fragment (Fig. 5B). These results are

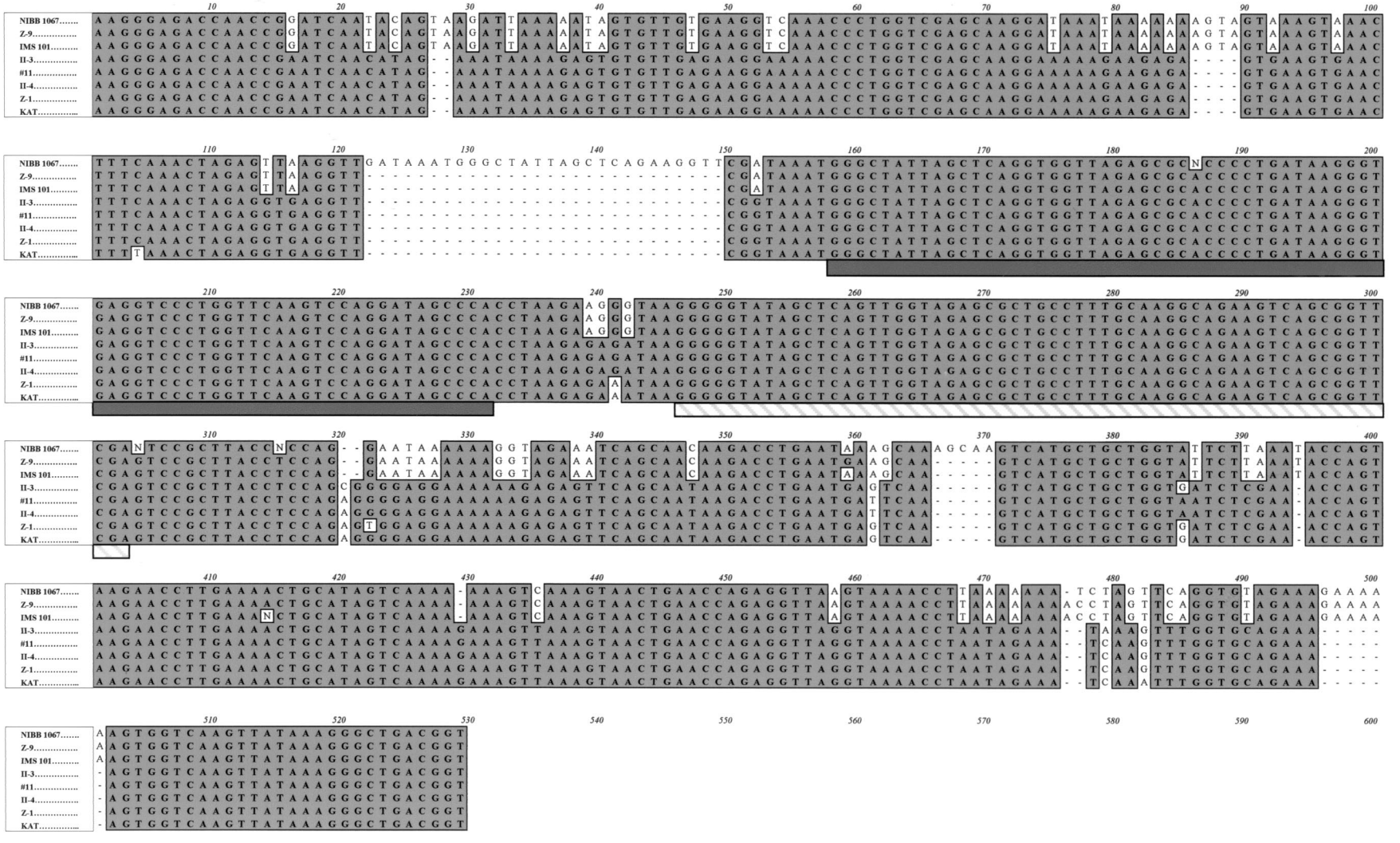


FIG. 6. Alignment of ITS sequences (16S-23S rDNA). The sequences were aligned by using ClustalW in the MacVector program (Oxford Molecular). Conserved bases are shaded, and $tRNA^{Ile}$ and $tRNA^{Ala}$ are depicted as boxes below the sequences (shaded and cross-hatched, respectively). NIBB 1067 (41), Z-9, and IMS 101 are all strains of *T. erythraeum*, while #11 and II-4 are strains of *T. hildebrandtii.* Strains Z-1, KAT, and II-3 are strains of *T. tenue*, *K. spiralis*, and *T. thiebautii*, respectively.

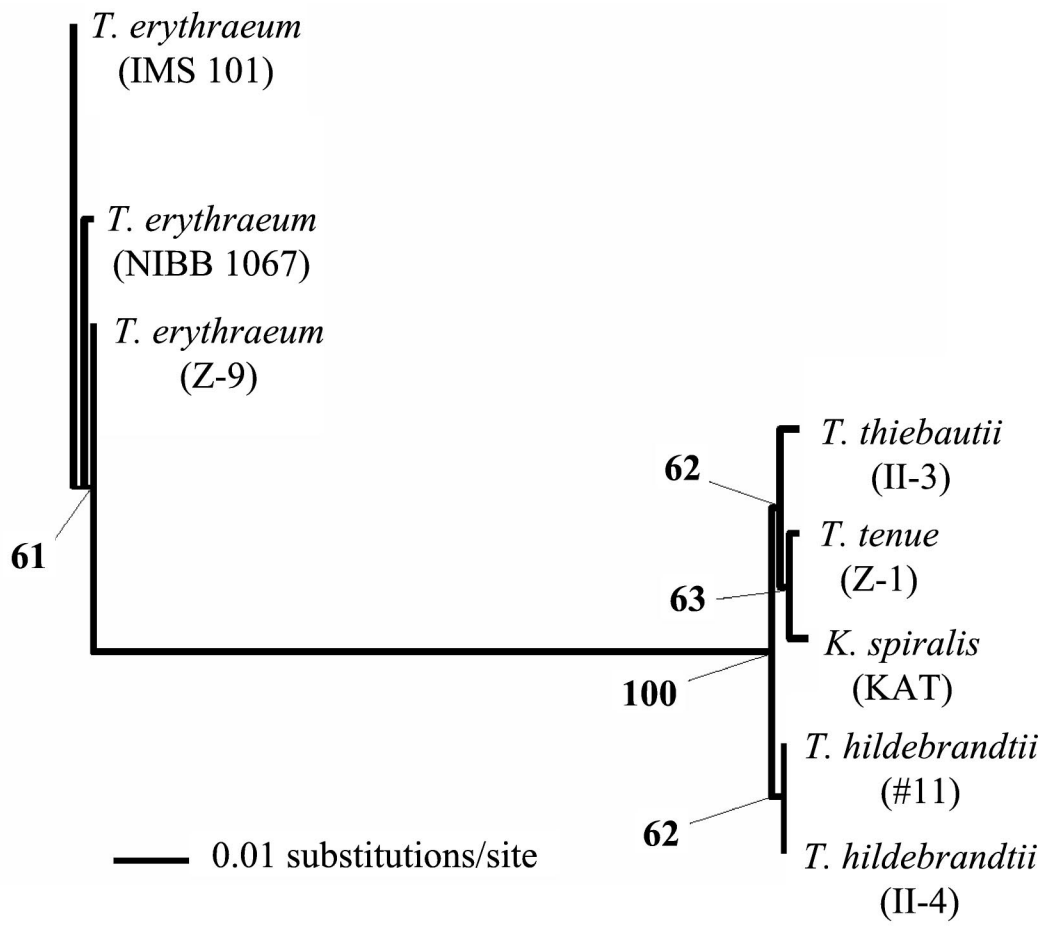


FIG. 7. Evolutionary relationships inferred from whole *Trichodesmium* ITS sequences. The consensus tree shown was constructed by using maximum-likelihood analysis and neighbor joining. Similar relationships were obtained from distance and maximum-parsimony analyses. Bootstrap values were generated from 1,000 replicates, and only values greater than 50 are shown at the nodes.

similar to those of Ben-Porath et al. (4), who reported that a *nifH* DNA sequence of a *T. thiebautii* puff colony was identical to that of a *T. thiebautii* tuft (42, 43). The DGGE analysis produced only one band for *T. thiebautii* puffs and tufts as well as for *T. erythraeum*, indicating that the colonies were comprised of trichomes with similar genetic compositions and not a mixture of the two species. This may imply that there is a sensing mechanism among trichomes for self-identification if the colonies assemble from free trichomes. On the other hand, the colonies could also be clonal, resulting from growth and division originating from a single trichome. Although in this study *T. thiebautii* puffs and tufts were found to be genetically similar, an earlier study (14) showed that protein extracts obtained from the two morphologies produced slightly different banding patterns when they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These observations suggest that the differences in morphology of *T. thiebautii* puffs and tufts are reflected at the protein level. The different morphologies of *T. thiebautii* puffs and tufts may be adaptations to the physical environment (turbulence) of the open ocean. For example, *T. thiebautii* puff colonies are the dominant form during winter conditions at the BATS site (30).

The trichome widths within *T. thiebautii* puff and tuft colonies displayed variation that overlapped the trichome widths of previously described species (19). Barker et al. (1) also found no correlation among trichome width, degree of coiling or gas vesicle strength, and genotype of Baltic Sea *Nodularia* sp. and emphasized the importance of genetic studies for species identification. In the past, morphological characteristics have been used for species identification of *Trichodesmium* isolates. However, due to overlapping characteristics (e.g., trichome width), a polyphasic approach using genetic tools coupled with morphology is probably more appropriate. For example, in one report of *T. contortum* a trichome width of 30 to 40 μm was described (19), while in another (36) the spiraled and flexed trichomes were described as >16 μm wide and as wide as 52 μm. This overlap has perhaps caused some confusion in the distinction between the genera *Katagnymene* and *Trichodesmium*. Our results based on ITS sequence, DGGE, and HIP1 fingerprinting analyses suggest that *K. spiralis* should be incorporated in the genus *Trichodesmium*. Until recently (25), *Katagnymene* was poorly characterized, as is the species *T. contortum*. *K. spiralis* is perhaps not a new species of *Trichodesmium* but is similar to the spiral trichomes described as *T. contortum* by Sournia (36) and the screw-like trichomes identified as *T. contortum* in the North Pacific Ocean by Letelier and Karl

(23). Our finding that *T. tenue* and *K. spiralis* (or *T. contortum*) were closely related was also reflected in the study of Janson et al. (18), who found a close relationship between *T. tenue* and *T. contortum*.

The use of HIP1 fingerprinting will be useful for further diversity studies of the population dynamics of this important group in oceanic new production and biogeochemistry. HIP1 analysis can be used as a screening method to resolve differences in a population prior to sequencing, which will save time and expense. Although DGGE analysis of the *hetR* fragment could not distinguish between closely related strains, the HIP1 PCR and ITS sequencing were able to resolve the small differences. The functional role of HIP1 as an adaptive response in these marine diazotrophs may also in future studies reveal important information regarding the unique occupation of a very specific niche in the global ocean.

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