APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2005, p. 190–196 Vol. 71, No. 1
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.1.190–196.2005

Unveiling of Novel Radiations within *Trichodesmium* Cluster by *hetR* Gene Sequence Analysis

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Received 22 October 2003/Accepted 10 August 2004

The filamentous nonheterocystous cyanobacterial genus *Katagnymene* is a common diazotrophic component of tropical and subtropical oceans. To assess the phylogenetic affiliation of this taxon, two partial 16S rRNA gene sequences and 25 partial *hetR* gene sequences originating from the genera *Katagnymene* and *Trichodesmium* collected from open, surface waters of the Atlantic, Indian, and Pacific oceans were compared. Single trichomes or colonies were identified morphologically by using light microscopy and then used directly as templates in *hetR* PCR analyses. In addition, three cultured strains, identified as *Katagnymene pelagica*, *Katagnymene spiralis*, and *Trichodesmium* sp., were examined. The data show that the genus *Katagnymene* is in the *Trichodesmium* cluster and that *K. pelagica* Lemmermann and *K. spiralis* Lemmermann are most likely one species, despite their different morphologies. Phylogenetic analyses also unveiled four distinct clusters in the *Trichodesmium* cluster, including one novel cluster. Our findings emphasize the conclusion that known morphological traits used to differentiate marine nonheterocystous cyanobacteria at the genus and species levels correlate poorly with genetic data, and a revision is therefore suggested.

The world's tropical and subtropical oceans are highly oligotrophic and are therefore a suitable habitat for diazotrophic cyanobacteria. In the open ocean, the nonheterocystous cyanobacterial genus *Trichodesmium* is very common. *Trichodesmium* was first discovered in 1830 (9), and in 1961 it was found to be diazotrophic; its ecology and physiology have since been characterized in numerous studies (6, 17). Recent estimates suggest that *Trichodesmium* alone may account for 40 to 50% of the global nitrogen sequestered through biological nitrogen fixation (17). One key feature of *Trichodesmium* is its ability to form colonies, but the cells may also exist as free trichomes. The marine nonheterocystous genus *Katagnymene* occurs only as free trichomes and was recently found to be diazotrophic (22). Like *Trichodesmium*, its nitrogen fixation activity is restricted to the photophase, and nitrogenase is confined to subsets of cells termed diazocytes (10, 12), which in *Katagnymene* account for about 7% of the total cells. Thus, *Katagnymene* and *Trichodesmium* share a unique diazotrophic behavior (5, 15, 22).

Katagnymene was first described by Lemmermann (19) and was divided into two species, *Katagnymene pelagica* and *Katagnymene spiralis*, based on the degree of trichome coiling. *Katagnymene* occurs in all major tropical and subtropical oceans (18, 19, 22, 24, 30). The trichomes are characterized by cells that are shorter than they are wide and by being surrounded by a distinct mucilaginous sheath. *K. spiralis* is more or less spirally coiled, and both *Katagnymene* species may form long trichomes that sometimes are up to 15 mm long. In previous taxonomic studies, *Katagnymene* was suggested to belong to the oscillatoriacean family, together with *Trichodesmium* spp.; the former genus was placed in the subfamily *Hormoscilloidae*, and the latter genus was placed in the *Phormidiaceae* (1). Previously, Drouet (8) suggested that *K. spiralis* should be classified as *Microcoleus lyngbyaceus* based on the thickened cell walls of the trichome end cells and that *K. pelagica* var. *major* Wille should be classified as *Oscillatoria* (*Trichodesmium*) *erythraea*. Besides the two marine species, the genus *Katagnymene* also includes five fresh or brackish water species (1, 2). It has recently been suggested that *K. spiralis* is closely related to *Trichodesmium* spp. based on certain gene sequence similarities (22, 26).

Likewise, the taxonomy of *Trichodesmium* has often been revised, and only one extensive genetic study has been performed (14). This study showed that the genus harbors five marine species in the following three main clusters: (i) a cluster which includes *Trichodesmium hildebrandtii* and fusiform and spherical colonies of *Trichodesmium thiebautii*; (ii) a cluster which includes *Trichodesmium erythraeum* (including the laboratory strain *Trichodesmium* sp. strain IMS 101); and (iii) a cluster which includes *Trichodesmium tenue* and *Trichodesmium contortum*.

In the present study, collected *Katagnymene* and *Trichodesmium* specimens first were identified by using light microscopy and then were used as templates in PCR in order to generate sequences that could be directly linked to a specific morphology. A 448-bp fragment of the *hetR* gene, proposed to be involved in diazocyte differentiation (10), was amplified and analyzed for cyanobacteria identified as *Katagnymene* spp. or *Trichodesmium* spp., which were collected in various geographic regions, and for a few cultured specimens. Our aim was to determine the relationship between *Katagnymene* spp. and *Trichodesmium* spp. using both 16S rRNA gene and *hetR* gene sequences isolated from natural populations and cultures of members of both genera.

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TABLE 1. Morphological characteristics of *K. spiralis* and *K. pelagica* given in the original descriptions[a]

Author	Sizes (μm)			
	K. pelagica		*K. spiralis*	
	Trichome	Sheath	Trichome	Sheath
Lemmermann	16	93–100	20–22	150–168
Wille	28–44	+[b]	20	>100
Karsten	21–27	100–165	10–14 (21)	17–28
Norris	ND[c]	ND	17–28	85–90

[a] See references 18, 19, 24, and 30. The value in parentheses is from exceptional specimens.
[b] +, sheath present.
[c] ND, not determined.

MATERIALS AND METHODS

Sample collection. Cyanobacteria were collected during three cruises, in 1998 during a cruise of the R/V *Roger Revelle* in the southwestern Pacific Ocean from New Zealand to Fiji, in 1999 during a cruise of the R/V *Maurice Ewing* off northern Australia, and in 2001 during a cruise of the R/V *Seward Johnson* in the midwestern Atlantic Ocean off Barbados. Samples were collected from the surface down to a depth of 20 m by using plankton tows. The morphological descriptions of Lemmermann (19), Wille (30), and Karsten (18) were used for identification (Table 1). The samples obtained in 1999 and 2000 were identified and photographed with a light microscope (Fig. 1 and Table 2). Then in each case the same trichome was recovered and transferred in a micropipette in 4 droplets of filtered seawater and 1 droplet of Milli-Q water before it was placed in a PCR tube containing 10 μl of Milli-Q water. The samples were frozen, thawed, and used directly as templates in PCR.

Cultures. Cultures PLA1 (preliminarily identified as *K. pelagica*) and PLA2 (no affiliation) were isolated (by P.L.) as single trichomes during the 2001 cruise in the western Atlantic Ocean. The culture of *K. spiralis* (JWI1) was isolated by J. Waterbury (Woods Hole Oceanographic Institution, Woods Hole, Mass.) from the Zanzibar Channel in Tanzania in 1999. The cultures are maintained in a nitrogen-free amended seawater (Sigma) medium containing trace metals, EDTA, and vitamins as described previously (7) and supplemented with 15 μM phosphoric acid, and they are grown with a daily cycle consisting of 12 h of darkness and 12 h of light (40 μmol of photons $\cdot m^{-2} \cdot s^{-1}$) at 27°C.

PCR amplification and sequencing. Partial 16S rRNA gene sequences were obtained by using the cyanobacterium-specific primers CYA106F and CYA781R (25). Each PCR was performed with an initial denaturation step consisting of 95°C for 4 min, followed by 30 cycles of 1 min at 93°C, 1 min at 50°C (57°C for the 16S rRNA gene), and 1 min at 72°C and a final extension step consisting of 72°C for 4 min. For all of the samples except those obtained in 1998, the trichomes were used directly as templates. Each PCR mixture (total volume, 25

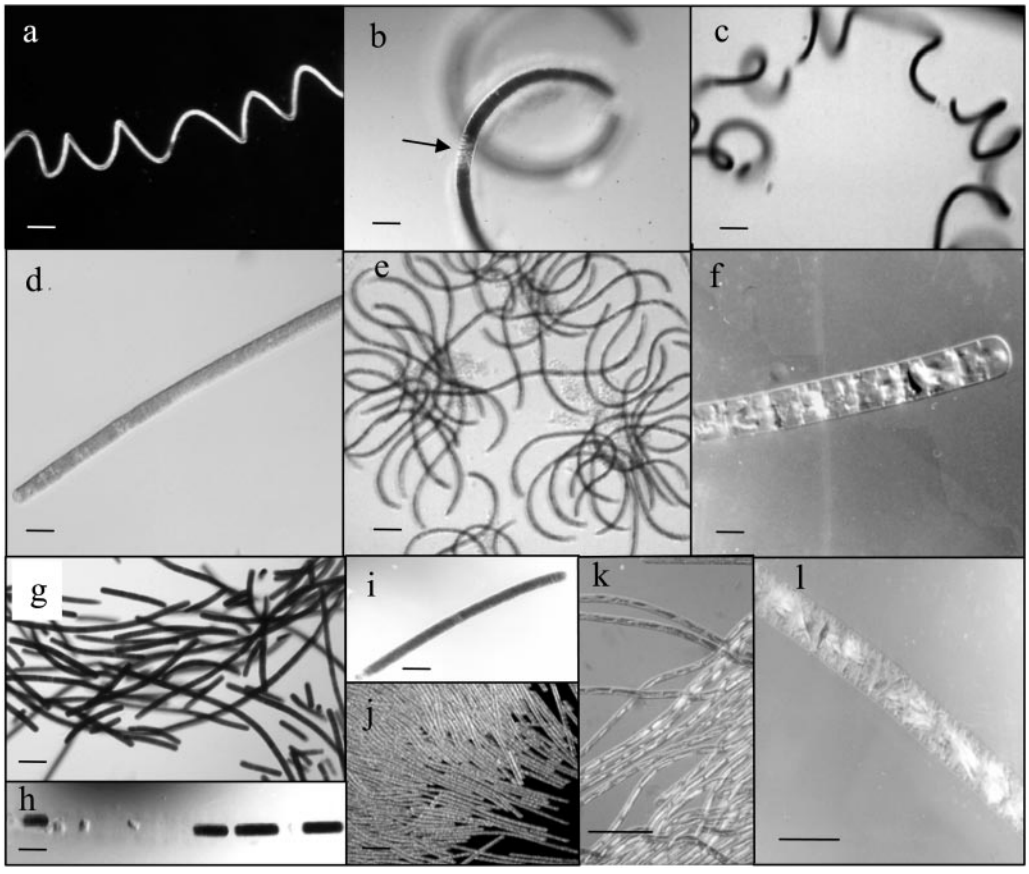

FIG. 1. Representative morphologies of some of the species of *Katagnymene* and *Trichodesmium* included in the phylogenetic analyses. Further details are given in Table 2. The morphotypes belong to cluster I (a to e), cluster II (f), cluster III (g to j), and cluster IV (k and l). (a) Cultured JWI1 (*K. spiralis*). Bar, 100 μm. (b) Specimen A27 (*K. spiralis*), with a pronounced mucilaginous sheath and with less pigmentation and segmentation (arrow). Bar, 50 μm. (c) Specimen B41-1 (*K. spiralis*). Bar, 100 μm. (d) Cultured PLA1 (*K. pelagica*). Bar, 50 μm. (e) Specimen A25, puff-shaped colony of *Trichodesmium* sp. with curved trichomes. Bar, 100 μm. (f) Specimen B49 (*Trichodesmium* sp.). Note the scattered gas vacuoles appearing as light-reflecting objects. Bar, 50 μm. (g) Cultured PLA2 (*Trichodesmium* sp.). Bar, 100 μm. (h) Part of a trichome of specimen B41-3 (*Trichodesmium* sp.) showing segmentation. Bar, 100 μm. (i) Specimen A13 (*Trichodesmium* sp.). Bar, 100 μm. (j) Part of a colony of *T. erythraeum*. Bar, 100 μm. (k) Part of a colony of *T. tenue.* Note the presence of the few light-reflecting large gas vesicles in the narrow cells. Bar, 50 μm. (l) Specimen B46 (*T. contortum*). Note the presence of several clustered light-reflecting gas vesicles. Bar, 50 μm.

TABLE 2. Morphological characteristics, collection sites, and accession numbers for the trichomes and colonies used for *hetR* and 16S rRNA gene sequencing

Specimen[a]	Diam of trichome (μm)	Filament shape/segments	Colony	Geographic origin[b]	Current affiliation	Cluster	Accession no.
hetR sequences							
JWI1	14–17	Coiled/+	−	Zanzibar, IO	*K. spiralis*	I	AF490673
PLA1	15–20	Straight/+	−	AO	*K. pelagica*	I	AF490676
PLA2	25–35	Straight/+	+	AO	ND[c]	III	AF490686
A13	60	Straight/−	−	PO/IO	ND	III	AF490675
A25	13	Curled/−	+	PO/IO	*Trichodesmium*	I	AF490684
A27	20	Coiled/−	−	PO/IO	*K. spiralis*	I	AF490681
A28	15	Straight/−	−	PO/IO	ND	III	AF490678
A31	20	Straight/−	−	PO/IO	ND	III	AF490688
A32-1	15	Straight/+	−	PO/IO	*K. pelagica*	I	AF490690
A32-2	18	Straight/+	−	PO/IO	ND	III	AF490689
B41-1	15	Coiled/−	−	10°01′N, 44°57′W	*K. spiralis*	I	AF490677
B41-2	12	Coiled/−	−	10°01′N, 44°57′W	*K. spiralis*	I	AF490687
B41-3	35	Straight/−	−	10°01′N, 44°57′W	ND	III	AF490674
B46	30	Straight/−	−	09°00′N, 52°01′W	*T. contortum*	IV	AF490685
B49	20	Straight/−	−	10°60′N, 56°17′W	ND	II	AF490680
B51-1	20	Straight/−	−	11°30′N, 55°00′W	ND	I	AF490679
B51-2	20	Straight/−	−	11°30′N, 55°00′W	*K. pelagica*	I	AF490683
B51-3	25	Straight/−	−	11°30′N, 55°00′W	ND	II	AF490682
F8-1	20	Straight/−	−	25°57′S, 167°39′E	*K. pelagica*	I	AF490694
F8-3	35	Straight/−	−	25°57′S, 167°39′E	*K. pelagica*	I	AF490695
F8-5	17	Coiled/−	−	25°57′S, 167°39′E	*K. spiralis*	I	AF490697
F8-6	17	Coiled/−	−	25°57′S, 167°39′E	*K. spiralis*	I	AF490691
F8-8	25	Straight/−	−	25°57′S, 167°39′E	*K. pelagica*	I	AF490696
F47-6	12–23	Coiled/−	−	19°17′S, 175°35′E	*K. spiralis*	I	AF490693
F45-1	17	Coiled/−	−	18°57′S, 175°40′E	*K. spiralis*	I	AF490692
16S rRNA gene sequences							
F34-8	30	Straight/−	−	PO	ND	IV	AF518770
JWI1	14–17	Coiled/−	−	Zanzibar, IO	*K. spiralis*	I	AF518769

[a] The origins of the samples are indicated as follows: F, 1998 southwest Pacific Ocean cruise; A, 1999 cruise off northern Australia (Indian Ocean or Pacific Ocean); and B, 2001 cruise in the midwestern Atlantic Ocean.
[b] Abbreviations: AO, Atlantic Ocean; IO, Indian Ocean; PO, Pacific Ocean.
[c] ND, not determined.

μl) contained each deoxynucleoside triphosphate at a concentration of 200 nM, 0.5 mM $MgCl_2$, 0.5 U of Dynazyme polymerase, Dynazyme reaction buffer, 0.5 μM primer PH1, and 0.5 μM primer PH2. Primers PH1 (5′-TGY GCK ATT TAY ATG ACC TA-3′) and PH2 (5′-ATG AAN GGT ATK CCC CAA GGA-3′) were constructed based on previously published *Trichodesmium hetR* sequences for amplification of a 448-bp fragment. The PCR fragments were separated on a 1.5% agarose gel. When a band was weak, the band was purified and used as a template in a second PCR. The gel fragments were purified and subjected to one of the following two treatments. The PCR products were either sequenced directly or cloned into the pCR2.1 vector (Invitrogen, San Diego, Calif.) by using a Rapid DNA ligation kit (Boehringer, Mannheim, Germany) and transformed by using One Shot competent cells (Invitrogen). Plates were screened for white colonies. These colonies were checked to make sure that they contained the correct insert by colony PCR, and positive colonies were grown in Luria-Bertani medium overnight. Then DNA preparations were checked for the correct insert by enzymatic restriction. For sequencing of cloned inserts primers T7 and M13 reverse were used. When the purified PCR products were sequenced directly, primers PH1 and PH2 were used. cDNA strands were sequenced by using BigDye terminators (PE Applied Biosystems) with a Perkin-Elmer model ABI 377 automated sequencer.

Sequence analysis. When necessary, the alignments of sequences were edited manually with Seaview (13). *hetR* trees were constructed by using PHYLIP (version 3.6a; Department of Genetics, University of Washington, Seattle) (http://evolution.genetics.washington.edu/phylip.html). The sequences of *Aphanizomenon* sp. strain KAC 15 and *Leptolyngbya* sp. strain PCC 73110 were used as outgroups. When distance matrices were used to generate the trees, the correction of Jukes and Cantor (16) was employed. For the nucleotide maximum-likelihood tree, the DNAML program was used (11), with the transition/transversion ratio set to 1.69 and the base frequencies set to 0.291 (A), 0.189 (C), 0.214 (G), and 0.305 (T). The transition/transversion ratio and base frequencies were determined by using PhyloWin. For maximum-likelihood analyses of the translated amino acid sequences of *hetR*, TREE-PUZZLE 5.0 was used (27). The analysis with TREE-PUZZLE was performed with the default settings,

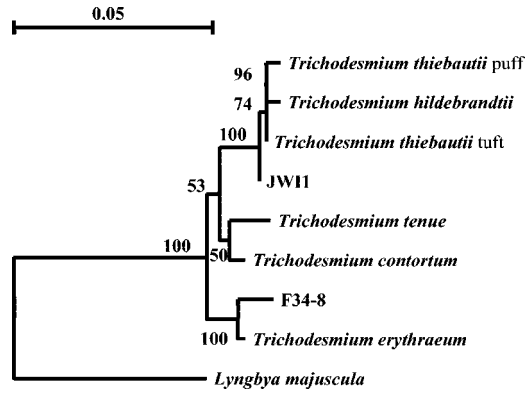

FIG. 2. Phylogenetic comparison of F34-5 (*Trichodesmium* sp.), JWI1 (*K. spiralis*), and the extant *Trichodesmium* cluster (14) based on partial 16S rRNA gene sequences. The F34-5 (*Trichodesmium* sp.) and JWI1 (*K. spiralis*) sequences both fall in the *Trichodesmium* cluster with 100% bootstrap support. The sequences were analyzed by calculating the distances between pairs of sequences by using the distance correction of Tajima and Nei (28), followed by construction of a phylogenetic tree by the neighbor-joining method and bootstrap resampling with 500 replicates. The analysis was carried out with the TREECON software package (29). *Lyngbya majuscula* was used as an outgroup. Scale bar = 0.05 substitution per sequence position.

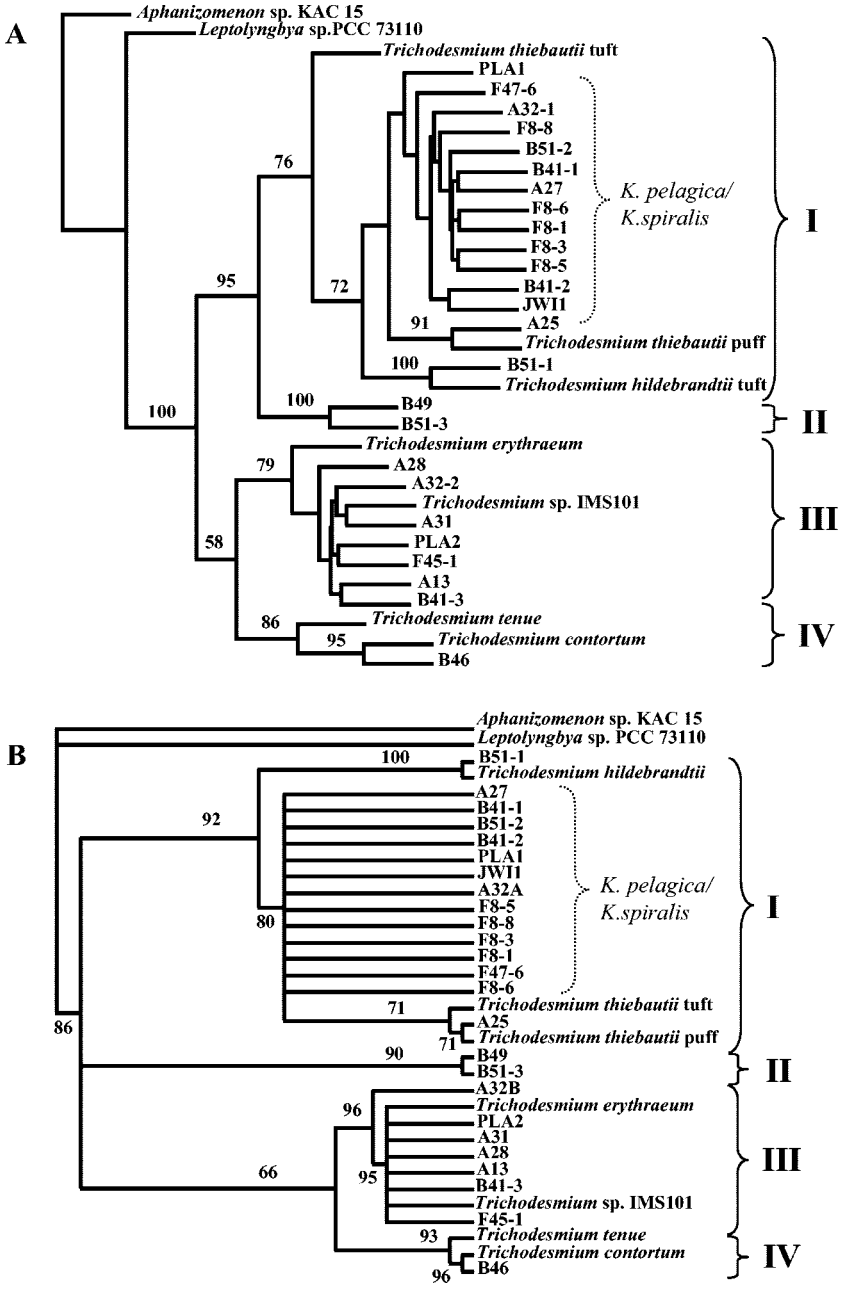

FIG. 3. Phylogenetic trees inferred from partial *hetR* sequences obtained in the present study from natural and cultured populations of *Katagnymene* and *Trichodesmium* species and from the extant *Trichodesmium* cluster (14). *Aphanizomenon* sp. strain KAC15 and *Leptolyngbya* sp. strain PCC 73110 were used as outgroups. The brackets with roman numbers indicate the four main clusters in the *Trichodesmium* cluster. The brackets labeled *K. pelagica*/*K. spiralis* indicate the 13 sequences derived from trichomes originally identified as *K. pelagica* or *K. spiralis*. (A) Maximum-likelihood analysis of partial *hetR* nucleotide sequences. (B) Maximum-likelihood analysis of partial *hetR* amino acid sequences. For the analyses of the amino acid sequences encoded by *hetR*, TREE-PUZZLE 5.0 (27) was performed with the default settings, except that the model of rate heterogeneity was chosen as a gamma distributed rate. Abbreviations: IMS, culture collection of the Institute of Marine Science, University of North Carolina, Morehead City; KAC, Kalmar Algae Collection; PCC, Pasteur Culture Collection.

except that the model of rate heterogeneity was chosen as a gamma distributed rate.

The 16S rRNA gene sequences were analyzed by calculating distances between pairs of sequences by using the distance correction of Tajima and Nei (28), followed by construction of a phylogenetic tree by the neighbor-joining method and bootstrap resampling with 500 replicates (Fig. 2). This analysis was carried out by using the TREECON software package (29). *Leptolyngbya* sp. strain PCC 73110 was used as the outgroup in the 16S rRNA gene-based tree.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences determined in the present study are given in Table 2.

RESULTS

Microscopic observations. Our microscopic observations and the origins of the collected specimens are summarized in

Table 2. For identification of *Katagnymene* we used the following criteria described by Lemmermann (19) for the two marine species: (i) trichomes in which the cell diameter varies from 12 to 35 μm, with cells that are shorter than they are wide; (ii) trichomes surrounded by a mucilaginous sheath; (iii) trichomes coiled to straight; (iv) trichomes often segmented, yet retained in the same sheath; and (v) trichomes solitary (trichomes do not aggregate into colonies). Seven specimens, including the cultured JWI1 specimen, were identified as *K. spiralis*. The trichomes were coiled, and the cell diameters ranged from 12 to 30 μm (Fig. 1a to d). The degree of coiling varied, and the trichomes were surrounded by a thick mucilaginous sheath. Regions with less pigmentation were often observed; this may have been an initial step in the formation of necrotic cells, leading to segmentation of trichomes (Fig. 1b and c). Six specimens, including the cultured PLA1specimen, were identified as *K. pelagica*. The trichomes were straight, had widths that ranged from 15 to 35 μm, and were surrounded by a mucilaginous sheath (Table 2 and Fig. 1e and f). Regions where there was less pigmentation and segmentation of the trichome were also observed in *K. pelagica* (Fig. 1e).

The remainder of the collected but unidentified single cyanobacterial trichomes and one colony were separated into three different groups on the basis of morphological characteristics (i.e., morphotypes). None of the morphotypes observed clearly fit descriptions given previously. These included A25, collected off northern Australia in 1999, which formed loosely aggregated colonies composed of curled trichomes. The trichomes ranged in diameter from 10 to 14 μm (Fig. 1e). Colonies with similar morphologies have also been observed in the Zanzibar Channel in Tanzania (unpublished observations). Another morphotype was observed in the midwestern Atlantic Ocean in 2001. The trichomes were 20 to 25 μm wide (B49 and B51-3) and sheathless and had randomly scattered gas vacuoles. The trichomes were golden and did not form colonies (Fig. 1f). Moreover, on all three cruises yet another morphotype was observed; in this morphotype the trichome diameters varied from 18 to 60 μm, and the cells were shorter than they were wide (they were one-fifth to one-eighth times as long as they were wide). The trichomes were often highly segmented, and colonies were not observed. However, the cultured PLA2 organism is a representative of this morphotype, and it can form loose colonies at least under laboratory conditions (Fig. 1g to i). This morphotype is identical to one described in previous work (15) and does not correspond to the description of either *Katagnymene* or *Trichodesmium*. Trichome morphology identical to that of *T. contortum* (14) was detected in the midwestern Atlantic Ocean in 2001. The trichomes were 30 to 35 μm wide and pale brown (B46) (Fig. 1l). The trichome morphologies of *T. erythraeum* and *T. tenue* are included for comparative purposes in Fig. 1 (Fig. 1j and k), while no sequences are reported here for either of these species.

Genetic analyses. Part (584 bp) of the highly conserved gene sequence of the small-subunit rRNA gene was sequenced for some of the cyanobacteria collected. In the resulting phylogenetic tree the cultured JWI1 specimen (Table 2 and Fig. 1a), assigned to the species *K. spiralis*, and the morphotype F34-5 (Table 2 and Fig. 1i to k) both fell in the *Trichodesmium* cluster with 100% bootstrap support (Fig. 2). Furthermore, the phylogenetic analyses revealed that the 16S rRNA gene sequence of JWI1 was closely related to those of *T. thiebautii* and *T. hildebrandtii*, while F34-5 (*Trichodesmium* sp.) was closely related to *T. erythraeum*.

As *Trichodesmium* spp. display low genetic diversity, the more variable gene *hetR* was used previously to analyze the intrageneric phylogeny (14). In view of this, we used partial *hetR* gene sequences (448 bp) to further resolve the relationship between *Katagnymene* spp. and *Trichodesmium* spp. The 25 *hetR* sequences amplified in the present study were all from organisms located in the *Trichodesmium* cluster with 100% bootstrap support (Fig. 3a). Both the resulting *hetR* nucleotide and amino acid trees, containing novel and previously described *Trichodesmium* spp. (the latter sequences were included for comparative purposes), contained four well-defined clusters of related sequences (Fig. 3). Cluster I comprised *T. thiebautii*, *T. hildebrandtii*, two of the novel sequences, and all 13 sequences from members of *K. spiralis* and *K. pelagica*. Cluster II exclusively encompassed two identical sequences from a novel morphotype. Cluster III comprised *T. erythraeum* along with seven sequences from the large dark-pigmented trichomes. Cluster IV contained *T. tenue* and *T. contortum*, as well as one sequence from *T. contortum* from this study. As Fig. 3B shows, the 13 *hetR* sequences derived from trichomes morphologically identified as *K. spiralis* or *K. pelagica* formed a distinct radiation within *Trichodesmium* cluster I. These sequences included sequences from *K. pelagica* collected from six different sampling sites on the three cruises, and seven sequences were generated from *K. spiralis* collected on the three cruises and one cultured isolate from the Indian Ocean (Table 2 and Fig. 3). Morphologically, *K. spiralis* from the Pacific Ocean had a larger cell diameter than the corresponding trichomes from the Atlantic Ocean. Smaller morphological differences were observed for *K. pelagica* from the different geographic areas sampled (Table 2). The overall topologies of the *hetR* nucleotide and deduced amino acid trees were similar (Fig. 3). However, there was one exception in cluster I: in the nucleotide-based tree (Fig. 3A) the sequence of the *T. thiebautii* tuft appeared to be ancestral to all other sequences in cluster I with a weak supporting bootstrap value (72), but in the amino acid tree (Fig. 3B) *T. thiebautii* was ancestral to the A25 and *T. thiebautii* puff sequences only. This makes classification of the different *T. thiebautii* phylotypes and morphotypes difficult, and further studies are needed in order to resolve this issue. The different tree topologies observed for cluster I could be explained by a high degree of homoplasy within the group of sequences, to which the maximum-likelihood method is less sensitive. The distance and parsimony analyses of the *hetR* nucleotide sequences resulted in similar topologies, but the sequence of the cultured PLA1 specimen was grouped outside the cluster containing the other sequences from *K. pelagica* and *K. spiralis* (data not shown).

DISCUSSION

Our data clearly demonstrate that the classification of the marine cyanobacteria hitherto classified as belonging to the genus *Katagnymene* is not supported by the 16S rRNA gene and *hetR* phylogenies presented here. Rather, all the marine nonheterocystous filamentous cyanobacteria collected and analyzed fall in the same major cluster, the *Trichodesmium* clus-

ter. Our data also for the first time demonstrate that *Trichodesmium* is divided into four main clusters, thereby extending previous studies (14, 26) that identified three clusters by analysis of the same *hetR* sequence and identified two clusters with a smaller number of strains by 16S-23S rRNA intergenic transcribed sequence (ITS) analyses.

The large cluster I, besides known *Trichodesmium* species, contained all the morphologically identified *Katagnymene* spp. sequences. The partial *hetR* sequences from the 13 specimens of *K. pelagica* and *K. spiralis* appeared to be randomly intermixed in this cluster. Hence, our data suggest that these two species should be merged into one species in spite of the morphological diversity represented by trichomes whose widths range from 12 to 35 μm and the great differences in the degree of coiling. Furthermore, it is clear that phenotypes such as appearing in a noncolonial state, as solitary trichomes, and as coiled trichomes are inconsistent with the genetic data presented here. The original descriptions of *Katagnymene* and *Trichodesmium* (9, 19) relied on such phenotypic traits, and revision is therefore needed. Consistent with this, analyses of Baltic Sea *Nodularia* demonstrated that the distinction between coiled and noncoiled trichomes was not supported by phycocyanin-intergenic sequence, *gvpA*-IGS, or rDNA-ITS genotypic grouping (3). Furthermore, strains of the coiled cyanobacterium *Arthrospira* are not closely related to other coiled cyanobacteria based on 16S rRNA gene sequences, and coiling of trichomes can be lost during culturing (20, 23). The capacity to form colonies and the colony shapes were not correlated to the genetic data, since different colony shapes of the colony-forming organisms (*Trichodesmium*) and the non-colony-forming organisms (*Katagnymene*) were intermixed in cluster I. Moreover, in cluster III the tuft-shaped *T. thiebautii* colonies were also shown to be genetically identical to the puff-shaped colonies of the same species when other genetic markers were used (4, 26). However, the only partial *hetR* sequence from a puff-shaped colony examined here clustered with the sequence reported previously. The close relationship between *Katagnymene* spp. and *Trichodesmium* found here was substantiated by previous data obtained by using partial *nifH* sequencing (22), as well as sequencing of the 16S-23S rRNA ITS combined with *hetR* denaturing gradient gel electrophoresis analyses and HIP1 fingerprinting (26).

Cluster II, represented by gold-pigmented colonies (Table 2 and Fig. 3), is a novel cluster compared to the three clusters identified by Janson et al. (14). The cluster II specimens most likely represent a new species. However, due to a lack of reference material other than the *hetR* sequences, a formal description cannot be presented at this time.

Our data and those in previous analyses (14, 26) collectively demonstrate that *T. erythraeum* is the most distinctly separate cluster (cluster III) in the *Trichodesmium* radiation. Also included in this cluster are sequences from large (>30-μm) dark-pigmented trichomes with a high degree of segmentation, previously referred to as *T. contortum* due to the morphological overlap (15). This was unexpected as *T. erythraeum* is characterized by a cell width of 6 to 12 μm (30) and forms raft-shaped colonies (Fig. 1l), while the dark-pigmented morphotype examined here was typically more than 30 μm wide and colonies were not observed. Like cluster I, cluster III apparently encompasses sequences from a wide array of morphotypes, which, however, showed more than 98% *hetR* nucleotide sequence similarity.

Furthermore, a *hetR* sequence was retrieved from *T. contortum* (sample B-46) (Fig. 1m and 3). The specimen was morphologically and genotypically (100% sequence identity) homologous to the specimen described previously (14), and both matched the original morphological description (30). Our data therefore extended a recent study (26) by demonstrating a morphological and genotypic difference between *T. contortum* and *K. spiralis*.

In conclusion, the genus *Trichodesmium* was resolved into four main clusters, which are composed of morphologically diverse strains. One of these clusters (cluster I) also contains a radiation comprising the two previously described marine *Katagnymene* species, and cluster II is a novel *Trichodesmium* lineage. One apparent link between the members of the genus *Katagnymene* and the members of the genus *Trichodesmium* is their nitrogen fixation behavior, which is unique among cyanobacteria (5, 15, 21, 22). We therefore propose that the genus description of *Trichodesmium* must be extended to include species and strains which predominantly live as individual trichomes (organisms classified as *Katagnymene*) and that *K. pelagica* and *K. spiralis* should be united into one species.

ACKNOWLEDGMENTS

We are grateful to E. J. Carpenter (San Francisco State University, San Francisco, Calif.) and D. Capone (The Wrigley Institute for Environmental Studies, University of California at Los Angeles, Los Angeles) for inviting us to participate in research cruises. J. Waterbury (Woods Hole Oceanographic Institution, Woods Hole, Mass.) is acknowledged for the cultured JWI1 specimen (*K. spiralis*).

We also acknowledge financial assistance from STINT (The Swedish Foundation for International Cooperation in Research and Higher Education) and Sida/SAREC (Swedish International Development Cooperation Agency) to B.B. and the Swedish Research Council for financial support provided to B.B. and S.J.

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