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A UNIQUE PSEUDANABAENALEAN (CYANOBACTERIA) GENUS *NODOSILINEA* GEN. NOV. BASED ON MORPHOLOGICAL AND MOLECULAR DATA[1]

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The cyanobacteria are a diverse, ancient lineage of oxygenic, phototrophic bacteria. Ubiquitous in nearly all ecosystems, the alpha-level diversity of these organisms lags behind other algal lineages due to a perceived dearth of phylogenetically useful characters. Recent phylogenetic studies of species within the genus *Leptolyngbya* have demonstrated that this is a polyphyletic assemblage. One group of strains that fits within the current circumscription of *Leptolyngbya* is genetically and phylogenetically distinct from *Leptolyngbya* sensu stricto. Members of this clade possess both a morphological synapomorphy and shared 16S-23S internal transcribed spacer (ITS) secondary structure, allowing the diagnosis of the new cyanobacterial genus *Nodosilinea*. Members of this genus are united by the unique ability to form nodules along the length of the filament. This trait has been previously observed only in the species *Leptolyngbya nodulosa* Z. Li et J. Brand, and we have chosen this species as the generitype of *Nodosilinea*. We currently recognize four species in the genus, *N. nodulosa* (Z. Li et J. Brand) comb. nov., *N. bijugata* (Kong.) comb. nov., *N. conica* sp. nov., and *N. epilithica* sp. nov.

Key index words: **16S rRNA; 16S-23S ITS; cyanobacteria; *Halomicronema*; *Leptolyngbya*; *Nodosilinea*; Pseudanabaenales; rRNA secondary structure; systematics; taxonomy**

Abbreviations: **ITS, internal transcribed spacer; ML, maximum likelihood; MP, maximum parsimony**

Cyanobacteria are a widely distributed group of oxygenic photosynthetic prokaryotes that possess chl *a* and phycobiliproteins and whose 16S and 5S rRNA sequences are similar to other members of the bacteria (Castenholz and Waterbury 1989). Despite their widespread occurrence and ecological importance, the α-level taxonomy of the cyanobacteria is currently in a state of chaos. The old botanical nomenclature for the group (Geitler 1932) has been challenged, particularly with reference to sheath characteristics, which vary widely in culture (Rippka et al. 1979, Rippka 1988). Drouet's revisions (Drouet and Daily 1956, Drouet 1968, 1973, 1978, 1981) are now considered overly drastic and incorrect.

The most recent reclassification scheme was proposed by Komárek and Anagnostidis (e.g., Anagnostidis and Komárek 1985, 1988, 1990, Komárek and Anagnostidis 1986, 1989, 1999). Their approach is similar to the botanical approach in that they use morphology and life history characters. They limit the use of sheath criteria for the definition of genera and emphasize details of cell division, hormogonia formation, tapering, polarity, and method of false branch formation, as well as other features to identify a different set of genera than that proposed by Geitler (1932). They recognize many more

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genera than Drouet and a majority of bacteriologists, although some of their generic concepts predate Geitler. The volume on the Oscillatoriales (Komárek and Anagnostidis 2005) provides a modern set of taxonomic hypotheses that can now be tested and refined with molecular data sets.

In an attempt to resolve the problems in cyanobacterial taxonomy stemming from the paucity of morphological characters and the plasticity of sheath characteristics, many researchers have turned to the use of gene sequence data. In particular, the 16S rRNA gene has been used in many studies to examine phylogenetic relationships within the cyanobacteria (Wilmotte et al. 1992, Nelissen et al. 1996, Turner 1997, Casamatta et al. 2005, Řeháková et al. 2007). While useful for separating well-diverged species, the use of this gene is best suited for generic placement (Fox et al. 1992, Johansen and Casamatta 2005). As a result, other genetic markers are being investigated such as the ITS region between the 16S and 23S gene, the phycocyanin operon, and variable region sequence polymorphisms within the 16S rRNA gene (Rudi et al. 1997, Iteman et al. 2000, Boyer et al. 2002, Teneva et al. 2005).

Within cyanobacteria, one of the least characterized, yet most diverse and species-rich groups of taxa are those species and genera in the Oscillatoriales. The most comprehensive taxonomic treatment of this order was done by Komárek and Anagnostidis (2005) who used a variety of features, mainly based on morphology and ecology, to reassign species to different genera and higher level taxa. One of the key contributions of this work was the splitting of Oscillatoriales sensu classicus into two separate subclasses, Pseudanabaenales (Synechococcophycidae) and Oscillatoriales (Oscillatoriophycidae). This revision has been anticipated for years, following the recognition that type of cell division, thylakoid arrangement, presence and type of inclusion bodies, and 16S rRNA gene sequence data are congruent in demonstrating that the Oscillatoriales sensu classicus is not a monophyletic group (Casamatta et al. 2005). In the recently defined Pseudanabaenales, the genus *Leptolyngbya* is the largest and most clearly polyphyletic, with recent studies indicating that the genetic diversity in the clade exceeds the morphological diversity (Casamatta et al. 2005, Komárek and Anagnostidis 2005, Johansen et al. 2008). *Leptolyngbya* has been known to be polyphyletic for some time (Albertano and Kovácik 1994, Turner 1997, Wilmotte et al. 1997, Castenholz 2001, Wilmotte and Herdman 2001, Taton et al. 2003, Casamatta et al. 2005, Komárek and Anagnostidis 2005), but new genera have not been described. Many clades (genera) exist within the botanical circumscription of *Leptolyngbya*, but morphological separation of these genera is difficult because they are typically character poor (Albertano and Kovácik 1994, Casamatta et al. 2005, Bruno et al. 2009). The problem is further complicated by the fact that some species in Oscillatorialean genera such as *Phormidium* have long been known to be associated more closely with the Pseudanabaenales (Komárek and Anagnostidis 2005), such as *Phormidium priestleyi* and *P. molle*, which have recently been transferred to *Phormidesmis* Turicchia et al. (2009), a new genus within the Pseudanabaenales. However, we have identified from our samples a clade that is clearly monophyletic when employing 16S rRNA gene sequence data, ITS folding patterns, and morphology. Additionally, these taxa may be capable of performing nitrogen fixation. We have erected the new genus *Nodosilinea* to encompass these organisms.

MATERIALS AND METHODS

Cultures and morphology. Strains used in this study were provided primarily by Lubomir Kovácik, with a few strains from the culture collection maintained by Jeff Johansen (Table 1). Cultures of the strains used in this study were grown on Z-8 medium (Carmichael 1986) under fluorescent lights (65 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) with a 12:12 light:dark (L:D) photoperiod. Light-limitation experiments were conducted at <4 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} for 4 weeks. Morphological examinations were performed using an Olympus BX-51 light microscope (Olympus America Inc., Center Valley, PA, USA) at x1,000 with Nomarski DIC optics. Photomicrographs were taken of each strain with a SPOT Insight 2MP digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Line drawings of strains are of representative filaments examined directly in the microscope to allow optimization of focus of granules and thylakoids.

DNA extraction and sequencing. Whole genomic DNA was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA bands were examined on a 1% agarose gel run at 100 volts for 40 min. For amplification of the 16-23S rRNA gene product, previously published primer pairs were used (Table 2). Samples were PCR amplified in an Eppendorf Mastercycler (Brinkmann Instruments Inc., Westbury, NY, USA) as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 4 min, and a final 7 min extension at 72°C.

PCR products were cleaned using a QIAquick PCR Cleanup kit (Qiagen Sciences, Valencia, CA, USA) and examined on a 1% agarose gel. Cleaned PCR products were cloned using the Promega Easy-Vector Cloning kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Nine colonies were chosen at random and PCR amplified using primers M13F and M13R. Samples were then cycle sequenced with the following profile: 40 cycles of 96° for 20 s, 55°C for 25 s, and 60°C for 4 min. Only a single operon from each strain was recovered based on fragment size. Cycle sequenced products were run on a CEQ 8000 Genetic Analysis System DNA sequencer (Beckman-Coulter, Fullerton, CA, USA). Each sequence was run a minimum of four times (two forward and two reverse reads) to increase the accuracy of the results.

Construction of secondary structures. The 16S-23S ITS region (~550 bp) was analyzed by determining secondary structure of the following conserved domains: D1-D1′ helix, Box-B antiterminator helix, V2 helix (present only in operons containing both tRNA genes), and the V3 helix. Secondary structures of specific ITS motifs were predicted using comparative analysis combined with confirmation in Mfold (Zuker 2003).

Sequence analysis and phylogenetic tree construction. ChromasPro (Technelysium Pty Ltd., Tewantin, QLD, Australia) was

TABLE 1. Strain information for taxa used in this study. The reference strain for the type species, *Nodosilinea nodulosa*, is listed first. Three strains for which sequences were available (OBB24S04, OBB19S12, OBB30S02) were not available for morphological characterization, but all are from the Bubano Basin in Italy.

Species name and strain designation	Cell width (μm)	Cell length (μm)	Location of isolation	Constricted	End cell	Special features
Nodosilinea nodulosa UTEX 2910	1.1–1.5	1.2–2.4	South China Sea (marine), plankton tow, 10 m depth	Distinct	Rounded	Capable of anaerobic N fixation; inflated sheaths; cells mostly isodiametric, flattened
Nodosilinea nodulosa PCC7104	1.0–1.5	1.0–1.5	Rock at shoreline (marine), Montauk Point, New York, USA	Distinct	Bluntly rounded	Capable of anaerobic N fixation; cells mostly isodiametric
Nodosilinea species 0BB32S02	1.0–1.5	1.0–1.6	Planktonic (freshwater), Bubano Basin, Imola, Italy	Distinct	Bluntly rounded	
Nodosilinea epilithica Kovácik 1998/7	1.5–2.5	1.2–8.0	Vieste, Peninsula Gargano, Italy, green biofilm on wall of a house	Distinct	Rounded	Cells typically barrel shaped after cell division, cylindrical in nondividing trichomes
Nodosilinea epilithica Kovácik 1990/52	1.5–1.8	1.0–3.2	Kesariani Monastery, Imitos Mountains, Greece, wet stone wall of the drinking fountain	Distinct	Rounded	Cells typically barrel shaped after cell division, cylindrical in nondividing trichomes
Nodosilinea bijugata Kovácik 1986/5a	1.5–1.7	1.5–6.2	Eutrophic Lake Piaseczno, Poland. Psammic in the littoral zone.	Distinct	Rounded	Cells typically barrel shaped to spherical; inflated sheaths
Nodosilinea species Řeháková 1960/20	2.0–2.5	2.0–4.5	Czech Republic, Celakovice, pool Cerna	Distinct	Rounded	Abundant nodule formation
Nodosilinea conica SEV4-5c1	2.5–2.7	0.9–2.4	Sevilleta LTER site, New Mexico, Chihuahuan Desert, soil	Slight	Bluntly conical	The bluntly conical end cells are unique in this clade
Nodosilinea species FI2-2HA2	1.5–2.0	1.5–4.5–(12)	Fort Irwin, California, Mojave Desert, soil	Slight	Rounded	Swollen end cells in old culture, asymmetric cell division
Nodosilinea species NB1a-A5	1.6–2.0	1.6–4.0	Natural Bridges National Monument, Utah, Colorado Plateau, desert soil	Slight	Rounded	Trichomes becoming doubled within sheath

TABLE 2. Primers used in PCR reactions and cycle sequencing of newly sequenced taxa.

Designation[a]	Sequence 5′-3′	16S position[b]
8F[c]	AGTTGATCCTGGCTCAG	8–24
VRF2[d]	GGGGAATTTTCCGCAATGGG	359–378
CYA781F[d]	AATGGGATTAGATACCCCA GTAGTC	781–805
WAW1486R[e]	AAGGAGGTGATCCAGCCACA	1,486–1,467
1407F	TGTACACACCGCCCGTC	1,407–1,423
B23SR	CTTCGCCTCTGTGTGCCTAGG	23S
M13F	GTTTTCCCAGTCACGAC	
M13R	CAGGAAACAGCTATGAC	

[a]F (forward) and R (reverse) correspond to the direction of the primer.
[b]Relative to *Escherichia coli*.
[c]After Lane (1991).
[d]After Nubel et al. (2000).
[e]After Wilmotte et al. (1993).

used to combine and edit all sequence data. Our 16S sequences were combined with sequences from GenBank designated as *Leptolyngbya* with a partial sequence length of at least 1,059 nucleotides. We additionally included any taxa that showed ≥97.5% sequence similarity via BLAST searches and had the required sequence length. Several other pseudanabaenalean strains were included that we have sequenced previously but not reported in the literature. The combined taxon sampling using these criteria provided a data set containing 117 operational taxonomic units (OTUs). The sequences were aligned together using the ClustalX web interface (Thompson et al. 1997) and manually checked and edited using Maclade v.4.06 (Maddison and Maddison 2000).

The GTR+I+gamma model was selected under the uncorrected Akaike information criterion using PAUP* 4.0b10 (Swofford 2002) and Modeltest 3.7 (Posada and Crandall 1998) and was used for all model-based analyses (16S rRNA and 16S-23S ITS). The software MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) was used for Bayesian inference. A Dirichlet (1,2,1,1,2,1) prior was used for the substitution rate parameters, a Dirichlet (2) prior was used for base frequencies, and a uniform (0,1 prior) was applied to the pinvar parameter. An exponential (1.0) prior was set on branch lengths and gamma shape parameters. Two runs were run for 10^7 generations, using one cold chain and three heated chains and sampling every 100 trees. The first 40,000 samples of each run were discarded as burnin phase. The stability of model parameters and the convergence of the two runs were confirmed using Tracer v1.4.1 (Rambaut and Drummond 2003) and AWTY (Wilgenbusch et al. 2004). A maximum-likelihood (ML) analysis was performed using GARLI (Zwickl 2006). A GTR+I+gamma model was applied, and bootstrap support values were obtained from 100 pseudoreplicate data sets. Additionally, an unweighted maximum-parsimony (MP) analysis was carried out using PAUP* 4.0b10 (Swofford 2002), and bootstrap supports were obtained from 1,000 pseudoreplicate data sets.

The 16S-23S ITS regions align very poorly in distantly related cyanobacterial taxa. However, within genera it is possible to achieve a reasonable alignment (Johansen and Casamatta 2005, Lukešová et al. 2009). We aligned all 16S-23S ITS regions for *Nodosilinea* strains for which we had sequence (six strains). MP was chosen as the optimality criterion for this analysis as it was possible to treat gaps as a new character state. A heuristic search, with the TBR swapping algorithm and 1,000 nreps was performed with 1,000 bootstraps to gauge support employing the GTR+I+G model for the data set. This same data set was also analyzed with ML and distance methods (gaps = missing), and bootstrap values (1,000 replicates) were mapped on to the MP phylogeny.

TEM images. To compare the ultrastructure of cells, all strains were studied using TEM. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and later postfixed with 2% osmium tetroxide. The fixed material was dehydrated in an acetone series (30, 50, 70, 80, 90, 95, and 100%) and embedded in Spurr's resin (Spurr 1969). Ultrathin cross-sections were contrasted with uranylacetate and Pb-citrate and examined in a JEOL 1010 (JEOL Ltd., Peabody, MA, USA) electron microscope.

RESULTS

Phylogenetic analysis. The Bayesian analysis of 116 pseudanabaenalean OTUs plus *Gloeobacter* as an outgroup taxon yielded a tree that clearly separated *Nodosilinea* from other genera in the order, such as *Leptolyngbya, Tapinothrix, Pseudophormidium,* and *Phormidesmis.* A total of 14 strains belong to a clade that contains the reference strain of the type species of *Nodosilinea, N. nodulosa* UTEX 2910 (Fig. 1A). The sister taxon to *Nodosilinea* among strains sequenced thus far is *Halomicronema* or one of two strains, MBIC 10087 and MBIC 10012, but all three of these putative sister taxa share <94% similarity with all *Nodosilinea* strains (*Halomicronema* 91.2%–93.6%, MBIC 10087 90.5%–92.5%, and MBIC 10012 90.1%–92.7%). There are several genera associated in the larger clade containing *Nodosilinea,* including *Halomicronema, Prochlorothrix, Limnothrix,* and *Synechococcus.* It is evident that some OTUs are misidenti-

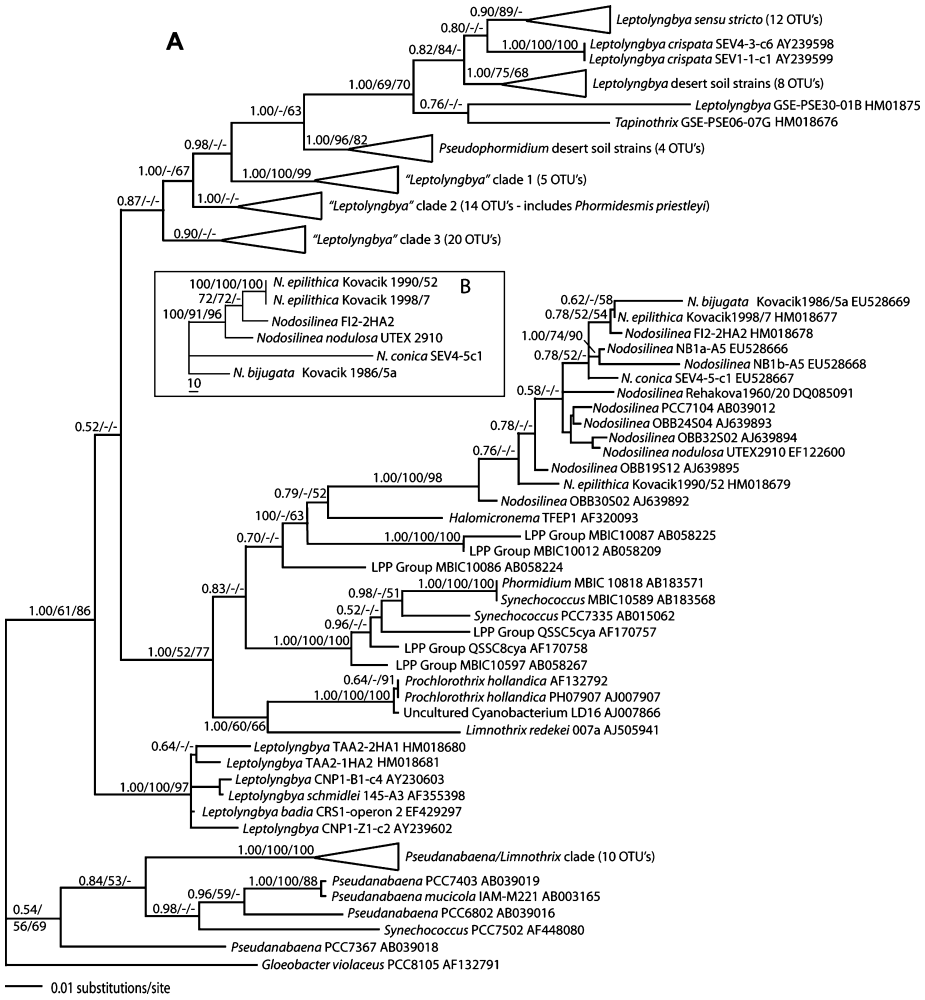

FIG. 1. Phylogenetic analyses. (A) Bayesian analysis of 116 OTUs of Pseudanabaenales plus *Gloeobacter violaceus* from the Gloeobacteriales based on a 1,059 nt fragment of the 16S rRNA gene. Node support is indicated as Bayesian posterior probabilities/bootstrap support from parsimony analysis/bootstrap support from likelihood analysis; ''-'' means support <0.50 or 50% parsimony/likelihood bootstrap support, respectively. (B) Phylogenetic analysis of six *Nodosilinea* strains based on sequence of the 16S-23S ITS. Node support is indicated as bootstrap support using parsimony, maximum likelihood, and distance as optimality criteria.

fied strains (e.g., *Phormidium* MBIC 10818 from another subclass) or at least represent polyphyletic genera if they are identified correctly (e.g., *Leptolyngbya, Synechococcus,* and *Limnothrix*). All strains in the new genus shared from 95.2% to 99.6% sequence similarity (Table 3).

One GenBank sequence, *Oscillatoria neglecta* IAM-M82, fell into our *Nodosilinea* clade (tree not shown). *Oscillatoria neglecta* Lemmerm. has been moved to *Jaaginema neglectum* (Lemmerm.) Anagn. et Komárek, and if this strain is correctly identified, it would mean that this species belongs in *Nodosilinea.* However, we do not have morphological information on this strain, nor is it a designated reference strain for the species, so we do not make any taxonomic changes at this time. *Jaaginema* obligately lacks sheath production, and thus *Nodosilinea* (which produces sheath material) is morphologically distinct from this genus.

Leptolyngbya sensu stricto contains several taxa, including *Leptolyngbya boryana, Leptolyngbya foveolarum, Leptolyngbya tenerrima,* and *Leptolyngbya angustata* (Fig. 1A, top cluster). Sister to this clade are a number of allied clades containing putative *Leptolyngbya, Tapinothrix, Pseudophormidium,* and *Phormidesmis* (Fig. 1A). These clades likely need more intensive taxon sampling before we can determine species and genera represented. However, it is very clear at this point that to place *Nodosilinea* into *Leptolyngbya,* it would be necessary to collapse the following genera into a single genus: *Leptolyngbya, Nodosilinea, Tapinothrix, Pseudophormidium, Phormidesmis, Halomicronema, Prochlorothrix,* and possibly *Synechococcus.* This step seems to us very unadvisable and would reverse much of the modern taxonomic work done in the Pseudanabaenales. The existence of *Nodosilinea* nested in ''*Leptolyngbya*'' makes it even more imperative to revise *Leptolyngbya* to recognize more genera in this polyphyletic taxon.

Although the phylogenetic analysis based on the 16S-23S ITS region was possible for only six strains, it did provide more resolution than the 16S rRNA phylogeny (Fig. 1B). Kovácik 1998/7 and Kovácik 1990/52 had nearly identical ITS regions and together with FI2-2HA2 and UTEX 2910 formed a well-supported clade distinct from SEV4-5c1 and Kovácik 1986/5a. However, this only splits the genus into two clades and unfortunately does not contain all of the strains for which we have 16S rDNA sequence data. The only conclusion one can draw from this phylogeny is that SEV4-5c1 and Kovácik 1986/5a do not belong in *N. nodulosa.*

Analysis of secondary structure of 16S-23S ITS motifs. ITS regions were amplified, and the D1-D1′ helix was folded for use in phylogenetic analyses (Fig. 2). The D1-D1′ helices for all *Nodosilinea* taxa consisted of 62–63 nt, with a 6 nt unilateral bulge that has a highly conserved sequence (CACUCU) present in five of the six taxa sequenced (Fig. 2, A–C, E, and F). The exceptional sequence was SEV4-5c1

TABLE 3. A partial similarity matrix (P-distance) generated using 16S rDNA gene sequence.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Nodosilinea* Kovácik 1986/5a															
2. *Nodosilinea* Kovácik 1998/7	98.22														
3. *Nodosilinea* FI2-2HA2	97.75	99.61													
4. *Nodosilinea* NB1a-A5	97.46	98.91	98.73												
5. *Nodosilinea* NB1b-A5	96.08	97.12	96.78	97.85											
6. *Nodosilinea* SEV4-5-c1	96.68	98.51	98.34	98.83	96.88										
7. *Nodosilinea* Řeháková 1960/20	96.78	97.42	97.08	97.17	96.10	97.17									
8. *Nodosilinea* PCC7104	97.03	97.82	97.62	98.12	96.52	97.71	97.82								
9. *Nodosilinea* 0BB24S04	96.93	98.12	97.62	98.41	96.62	98.01	98.11	99.31							
10. *Nodosilinea* OBB32S02	97.03	97.62	97.22	97.72	96.33	97.32	97.62	98.41	98.71						
11. *Nodosilinea nodulosa* UTEX 2910	97.03	97.63	97.42	97.92	96.33	97.51	97.62	98.61	98.91	99.41					
12. *Nodosilinea* OBB19S12	97.65	97.73	97.37	97.86	96.69	97.47	97.76	98.72	98.61	99.11	98.71				
13. *Nodosilinea* Kovácik 1990/52	96.83	97.42	97.03	97.33	95.84	96.92	97.03	97.42	97.52	97.72	97.12	98.12			
14. *Nodosilinea* OBB30S02	96.00	96.44	96.30	96.59	95.22	96.20	96.49	97.62	97.32	97.52	97.12	97.95	95.89		
15. *Halomicronema* TFEP1	91.99	93.06	93.08	92.79	91.23	93.59	91.91	92.35	92.65	92.36	92.45	92.20	92.16	93.08	
16. *Prochlorothrix hollandica*	90.60	91.28	91.33	91.03	89.67	90.45	90.54	91.61	91.37	90.48	90.58	91.03	90.48	90.31	91.13

(Fig. 2D), which had a unilateral bulge with 7 nt (UCACUCU). The loop at the apex of the helix contains 9–12 nt, and is subtended by a helix which characteristically contains a GAA-UUA stem (unusual for the noncanonical G-A pairing). This latter structure is present outside of the genus in some taxa (Fig. 2, H, I, and K). The primary central helix is similar in structure in all *Nodosilinea,* although SEV4-5c1 has a unique bilateral bulge (Fig. 2D). All other taxa for which we have sequences had D1-D1′ helices different in some particulars from the common themes seen and described above in *Nodosilinea* (Fig. 2, G–O).

The Box-B helix within *Nodosilinea* strains was more variable (Fig. 3, A–F). All were similar in length (36–41 nt) and shared the same basal pairings (AGCA-UGCU). Some had a similar bilateral bulge near the base of the helix (Fig. 3, B, C, E, and F). Kovácik 1998/7 and Kovácik 1990/52 were identical to each other; all others were unique in several aspects.

Many cyanobacteria have a helix between the two tRNA genes, known as the V2 helix. Most *Nodosilinea* lacked this structure, having only 6–7 nt between the tRNA genes. However, one strain, SEV4-5c1, had 54 nt between the tRNA genes, and a V2 of 35 nt was predicted to form in Mfold. This is a singular difference and is even more significant since many of the taxa sister to *Nodosilinea* produce no V2.

The length of the 16S-23S ITS was fairly similar in *Nodosilinea*, with 493–516 nt total. The most variable regions in length were the spacer between the D1-D1′ helix and the $tRNA^{Ile}$ gene and the spacer between the $tRNA^{Ala}$ gene and the Box-B helix. The first spacer was 102–104 nt in the three closely related strains Kovácik 1990/52, Kovácik 1998/7, and FI2-2HA2. The reference strain, *N. nodulosa* UTEX 2910, had 91 nt, Kovácik 1986/5a had 81 nt, and SEV4-5c1 was most distinct with only 54 nt. The second spacer had 24 nt in all strains except SEV4-5c1, which had 14 nt.

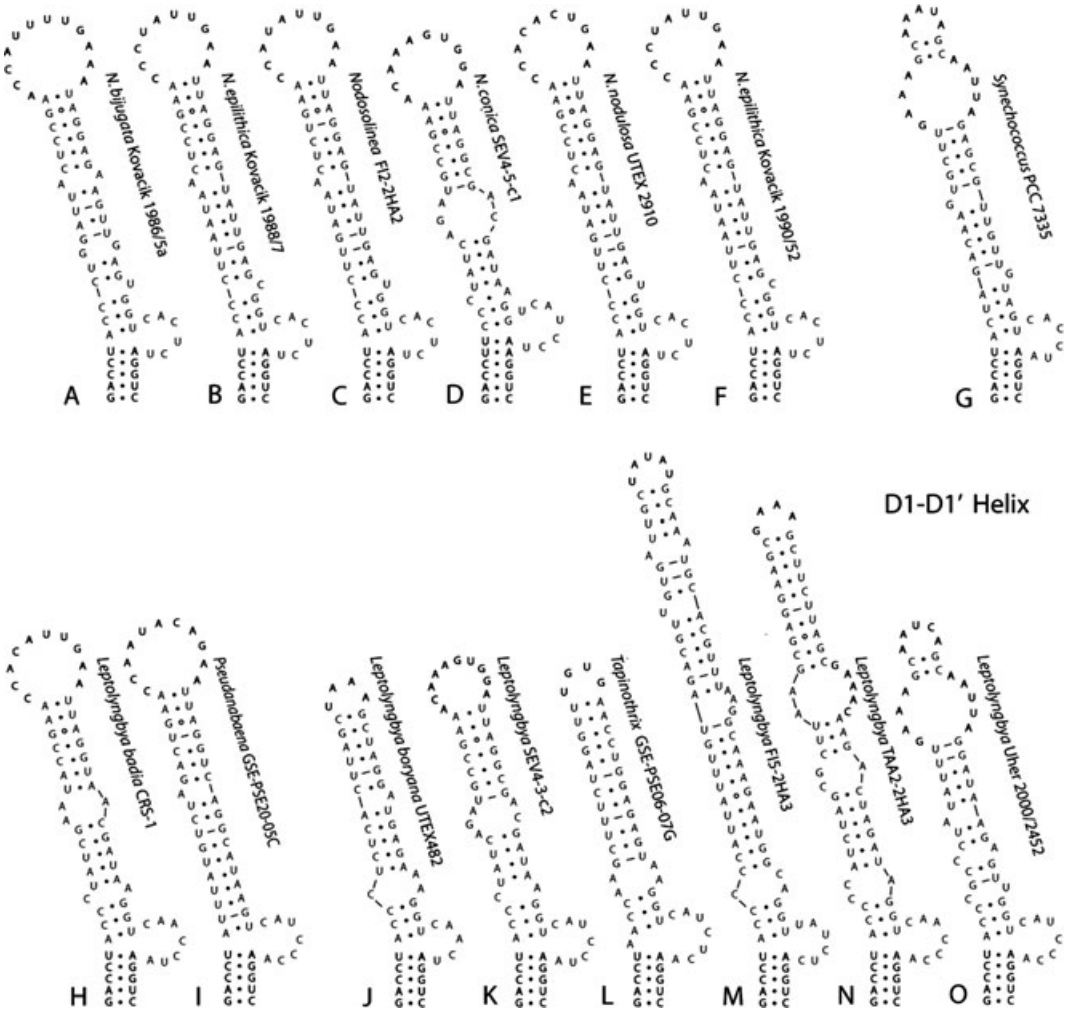

FIG. 2. D1-D1′ transcript secondary-structure helices from newly sequenced and representative Pseudanabaenalean taxa. (A–F) *Nodosilinea.* (G) *Synechococcus* PCC7335. (H and O) Representative *Leptolyngbya, Tapinothrix,* and *Pseudanabaena.*

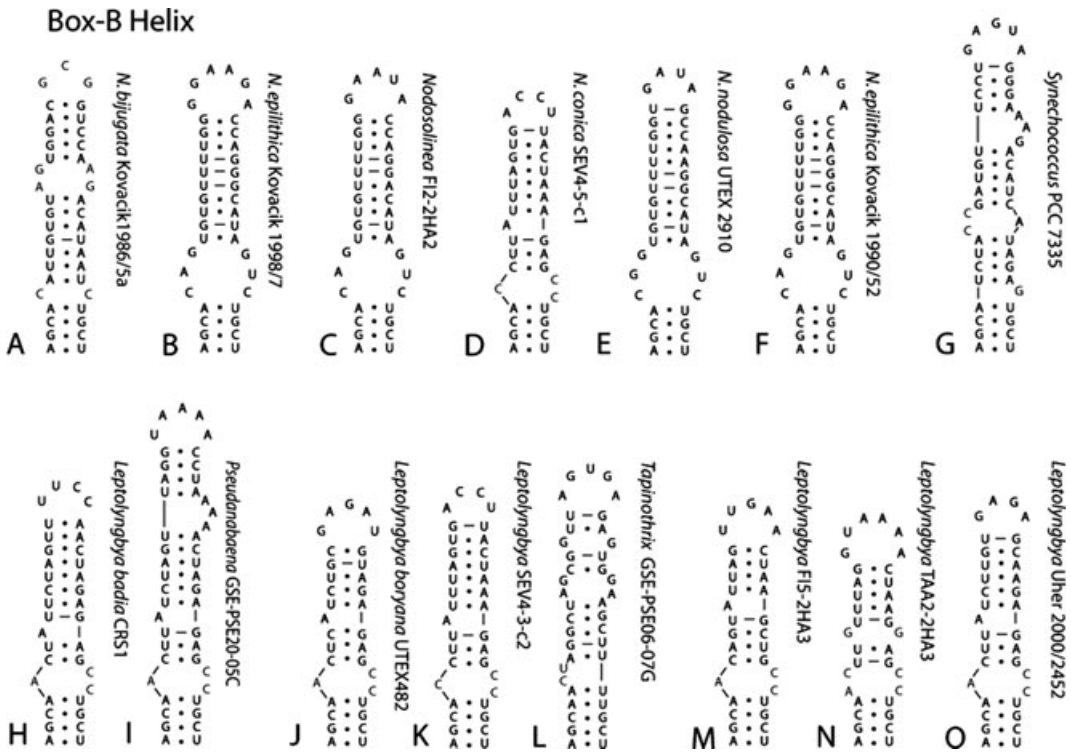

FIG. 3. Box-B transcript secondary-structure helices from newly sequenced and representative sister taxa. (A–F) *Nodosilinea.* (G) *Synechococcus* PCC7335. (H and O) Representative *Leptolyngbya, Tapinothrix,* and *Pseudanabaena.*

TEM images. All strains in the genus *Nodosilinea* showed similar ultrastructure, possessing peripheral thylakoids and pseudanabaenacean-type (type B) cell division, and therefore only two representatives are illustrated (Fig. 4).

Nodule formation. All newly described strains in this clade exhibited nodules when grown under low-light conditions (<4 μmol photons $\cdot m^{-2} \cdot s^{-1}$; Figs. 5, J and L; 7B; and 8B).

Taxonomy. We find it difficult to conclude that strains from such diverse habitats as marine phytoplankton, marine benthos, freshwater lakes, stone walls, and desert soils from Asia, Europe, and North America all belong to the same species. If one accepts either the evolutionary species concept or the ecotypic species concept for cyanobacteria (Johansen and Casamatta 2005), these cannot all be the same species. The 16S rDNA sequence data were insufficient to resolve the internal evolutionary relationships within the genus, with no bootstrap support for any internal groups except two strains isolated from the same soil sample (NB1a-A5 and NB1b-A5). However, low sequence similarity among some strains (<97.5%, see Table 3) suggests that we have multiple species represented in our sample of strains.

The reference strain for the type species, *N. nodulosa*, was isolated from marine phytoplankton but was fairly euryhaline (Li and Brand 2007). The marine strains (UTEX 2910, PCC7104) were relatively thin (trichomes 1.0–1.5 μm wide) and had high similarity in 16S rDNA sequence (98.6% similarity). Several strains from the Bubano Basin in Italy (OBB24S04, OBB32S02, OBB19S12) matched morphology (Table 1) and had even higher 16S rDNA similarity to *N. nodulosa* UTEX 2910 (98.6–99.4, Table 2). We have difficulty considering these completely freshwater strains equivalent to a marine species, but the lack of both thorough morphological comparison and the 16S-23S ITS sequence prevents us from concluding either that the Bubano Basin strains are the same species as *N. nodulosa* or that they represent a different lineage. We consequently designate these strains *Nodosilinea* species pending further study. The fourth strain from the Bubano Basin had considerably lower similarity to the marine taxa (97.1–97.6) than the other three strains, and it is likewise designated *Nodosilinea* species.

In the strains recognized as definitely not belonging to *N. nodulosa*, trichome diameters were greater (1.5–2.7 μm wide) and cell lengths were often much longer (up to 12 μm) (Table 1). The most morphologically distinct strain was SEV4-5c1, which had conical end cells and the largest trichome diameters (2.5–2.7 μm). Given the distinctive ecology (desert soil), morphology, and ITS sequence and structures of this strain, we recognize it as a new species separate from *N. nodulosa* and will refer to it as *N. conica* sp. nov. in the remainder of this paper (Table 1).

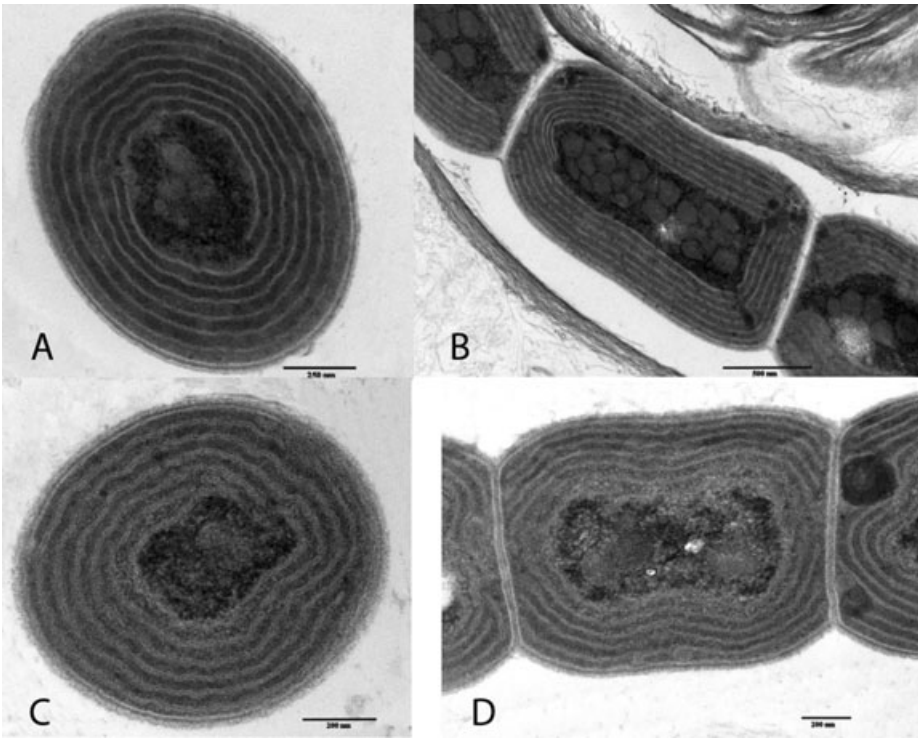

FIG. 4. TEM of selected representative strains used in this study. (A) *Nodosilinea* Řeháková 1960/20 cross-section (scale bar = 250 nm). (B) *Nodosilinea* Řeháková 1960/20 longitudinal section (scale bar = 500 nm). (C) *N. bijugata* Kovácik 1986/5a cross-section (scale bar = 200 nm). (D) *N. bijugata* Kovácik 1986/5a longitudinal section (scale bar = 200 nm).

Another strain that is distinct from *N. nodulosa* is Kovácik 1998/7. This strain is larger in diameter (trichomes 1.5–2.5 μm wide) has much longer cells (1.2–8.0 μm long) and is additionally distinctive in its ecology (subaerophytic on stone walls). We refer to this species as *N. epilithica* sp. nov. in the remainder of this paper. Kovácik 1990/52 had nearly identical ITS sequence and structure and was also isolated from a stone wall. While we did not observe the exceptionally long cells, the longest cells were notably longer than wide (3.2 μm long), and we have insufficient evidence to consider this taxon a different species from *N. epilithica* (Table 1).

Nodosilinea Kovácik 1986/5a was very distinctive in terms of its ITS sequence and structure but was a near perfect fit in terms of morphology and ecology to *Phormidium bijugatum* Kong. [=*Leptolyngbya bijugata* (Kong.) Anagn. et Komárek], a taxon that up until this point has not been illustrated in the literature and is poorly known. We are proposing a new combination for this species, *Nodosilinea bijugata* (Kong.) comb. nov.

We had several strains of *Nodosilinea* that clearly belong to the genus, but for which we have no 16S-23S ITS sequence. We are reluctant to attempt application of a species epithet to these less completely characterized strains. However, we do characterize these species morphologically in the section that follows so that the morphology associated with these sequences is documented. We believe that more strains need to be characterized in this genus before full species recognition is possible among all strains.

Nodosilinea Perkerson et Casamatta **gen. nov.**

A *Leptolyngbya* Anagn. et Komárek et speciebus omnibus Pseudanabaenacearum filis nodulosis in luce sublustri formantibus differt.

Fila typice trichomis singulis, sed interdum multiseriatescentes, nodulosis in luce sublustri formantes. Vagina plerumque praescens, tenua, mollis, incolor. Trichomae immotiles, ad septum leviter vel distincte constrictae. Cellulae plus minusve isodiametrae vel longiorae quam latiorae, vesiculae gaseosae nullae, thylakoidibus parietalibus.

Diagnosis: Differs from *Leptolyngbya* Anagn. et Komárek and all other species in the Pseudanabaenaceae in that filaments form nodules under low light conditions.

Description: Filaments typically with a single trichome, but sometimes becoming multiseriate, with nodules forming under low light conditions. Sheath usually present, thin, soft, colorless. Trichomes immotile, slightly constricted to distinctly constricted at the cross walls. Cells more or less isodiametric or longer than wide, lacking aerotopes, with peripheral thylakoids. Nitrogen fixation in at least some species of the genus.

Etymology: *Nodosilinea* = ''knotted line.''

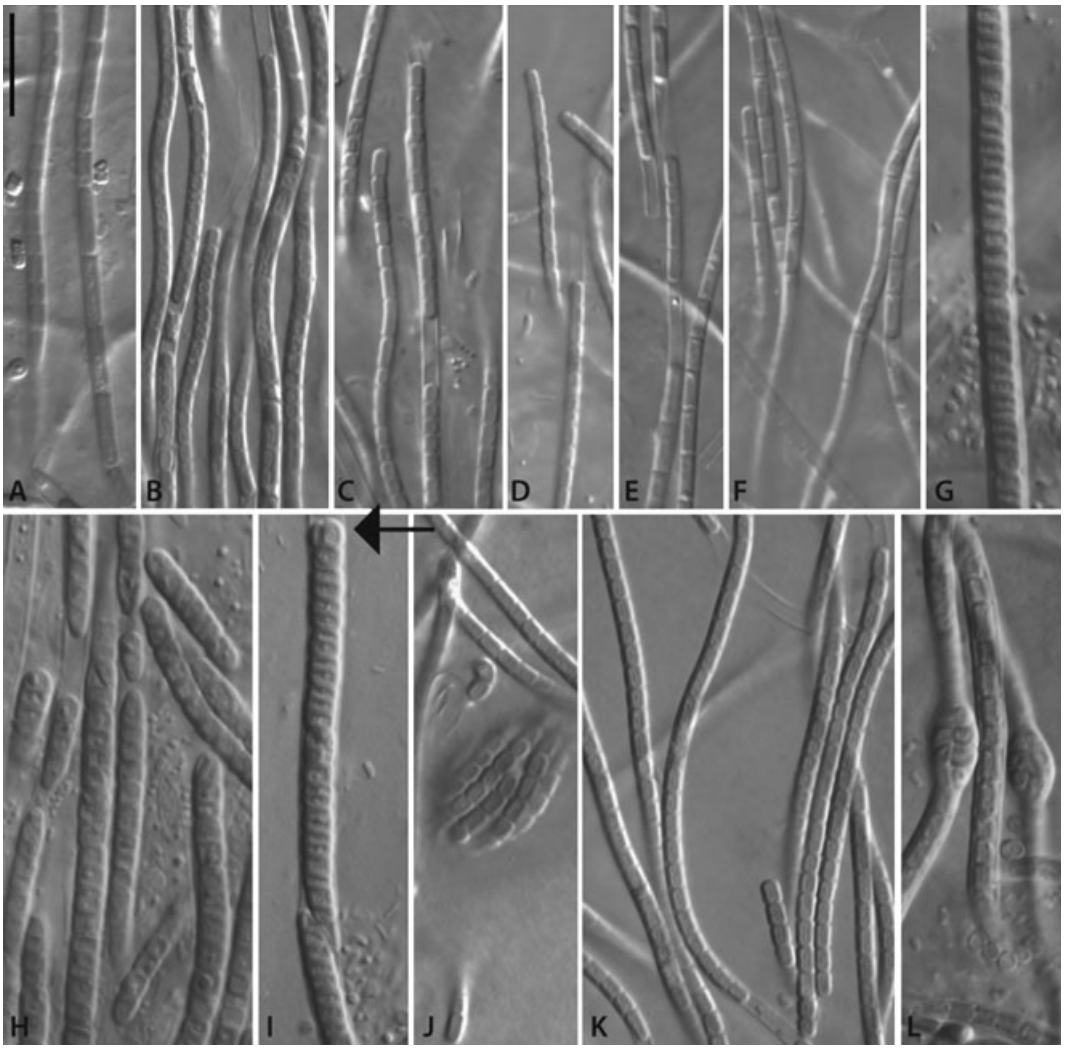

FIG. 5. *Nodosilinea* in LM. (Aand B) *N. epilithica* Kovácik 1988/7. (C) *N. epilithica* Kovácik 1990/52. (D–F) *N. bijugata.* (G–I) *N. conica* SEV4-5-c1; note cell division apparently in two planes (arrow). (J and K) *Nodosilinea* Řeháková 1960/20. (L) *Nodosilinea* FI2-2HA2. Scale bars = 10 μm.

Type species of the genus: Nodosilinea nodulosa (Z. Li and J. Brand) Perkerson and Casamatta comb. nov. Basionym: *Leptolyngbya nodulosa* Z. Li and J. Brand (2007, p. 397, figs. 1–12).

Nodosilinea epilithica Perkerson et Casamatta **sp. nov.** (Figs. 5, A–C; 6, A and B).

N. nodulosa affinis sed trichormis latioribus notabilis et habitat in saxo aquis dulcis madefacto.

Fila typice non ramosa, raro pseudoramosa, noduli in luce sublustri, vaginis praeter culturas juniores plerumque praesentibus. Vagina incolor, typice tenuis interdum latescens et diffluescens. Trichomae leviter vel distincte constrictae ad septum, necridiis nullis, partibus meristematibus nullis, cellulis in trichomis penitus dividentibus, cellulis partium nodulosarum ut videtur divisione cellulosa in plano duobus, 1.5–2.5 μm wide. Hormogonia plerumque formata. Cellulae typice doliiformes, thylakoidibus parietalibus, saepe granulis singulis vel binis in centroplasmate evidenti, cellulis diametro longiora vel paulo breviora, 1.0–8.0 μm longae. Cellulae apicales rotundatae, cellulis intercalaribus similibus.

Diagnosis: Akin to *N. nodulosa,* but notable for its wider trichomes and its occurrence on stone wetted by freshwater.

Description: Filaments typically unbranched, rarely pseudobranched, forming nodules in low light, with sheaths present in all, but the most rapidly dividing cultures. Sheath typically thin, colorless, occasionally becoming wide and diffluent. Trichomes slightly constricted to strongly constricted at the cross walls, lacking necridia, lacking meristematic zones, with cells dividing throughout the trichome, with cells of nodular regions seemingly with cell division in more than one plane, 1.5–2.5 μm wide. Hormogonia formation common. Cells typically barrel shaped, with peripheral thylakoids, often with one to two granules evident in the centroplasm, shorter to longer than wide, 1.0–8.0 μm long. End cells rounded, similar to intercalary cells.

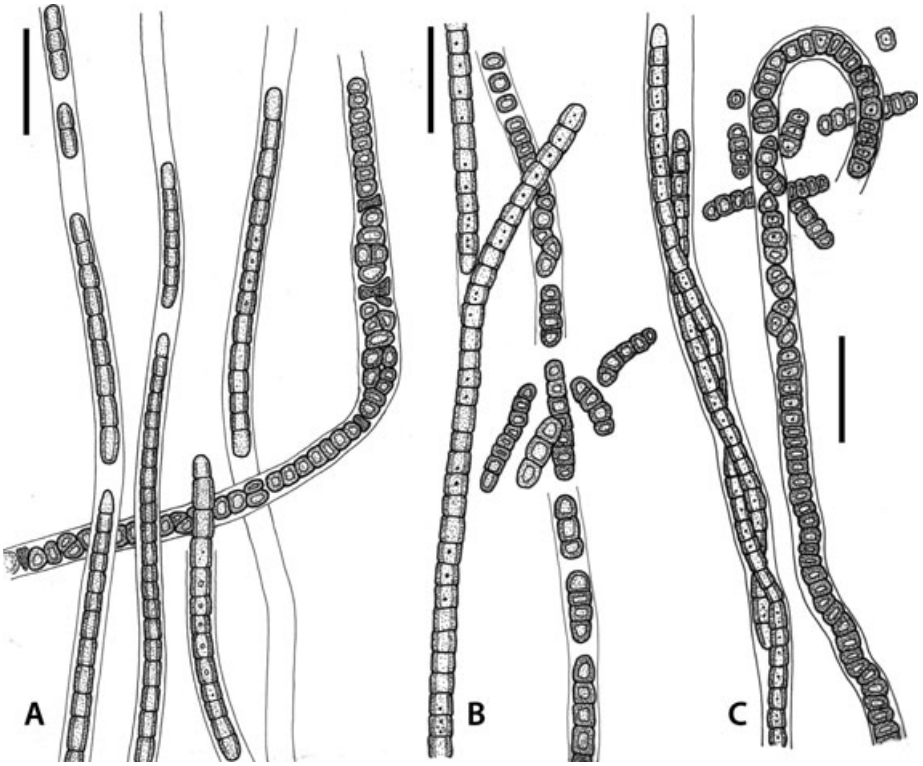

FIG. 6. *Nodosilinea epilithica*. (A) *N. epilithica* Kovácik 1988/7; note cell division apparently in two planes. (B and C) *N. epilithica* Kovácik 1990/52; note abundant hormogonia production, filament with two trichomes in a common sheath, and false branching. Scale bars = 10 μm.

Type locality: Italy, Peninsula Gargano, town of Vieste (Foggia), green biofilm on the wall of the house, coll. Kapusta on June 1998.

Holotype here designated: BRY 37707.

Reference strain: Nodosilinea epilithica Kovácik 1998/7.

Additional strain: *Nodosilinea epilithica* Kovácik 1990/52.

Etymology: *Nodosilinea epilithica* = ''the epilithic *Nodosilinea*.''

The 16S-23S ITS sequences of the two strains of *N. epilithica* isolated thus far are identical, and consequently, the secondary structures are identical as well. The ITS secondary structure of this species is nearly identical to that found in the type species *N. nodulosa* in the D1-D1′ helix (Fig. 2, B, E, and F), Box-B helix (Fig. 3, B, E, and F), and V3 (not shown), but the sequence differs. The spacer region between the D3 and $tRNA^{Ile}$ is 11 nt shorter in *N. nodulosa* than in *N. epilithica*. The secondary structure of the D5 helix is not possible to determine without having the 23S-5S ITS region as well (see Lukešová et al. 2009). However, the D5 region of these two species is identical in number of nucleotides (57) and has high sequence similarity at the start of the region.

Nodosilinea bijugata (Kong.) Perkerson et Kovácik **comb. nov.** (Figs. 5, D–F; and 7A).

Description: Filaments unbranched, rarely forming nodules, with sheaths often absent. Sheaths thin, colorless. Trichomes slightly constricted at the cross walls, lacking necridia, lacking meristematic zones, with cells dividing throughout the trichome length, 1.5–1.7 μm wide. Hormogonia absent. Cells cylindrical, with peripheral thylakoids scarcely evident, often with one to two granules at the cross walls, isodiametric to longer than wide, 1.5–6.2 μm long. End cells rounded, typically elongated.

Basionym: *Phormidium bijugatum* Kong. (1925, p. 70).

Reference strain: Nodosilinea bijugata Kovácik 1986/5a.

The description of this taxon in Komárek and Anagnostidis (2005) gives a trichome width of 1.0–2.8 μm and a cell length of 1.4–4.7. We suspect that more than one species may produce the granules at the cross walls, and that the range of widths given in Komárek and Anagnostidis (2005) may encompass more than one species.

This strain bears a strong resemblance to *Leptolyngbya appalachiana* Johansen et Olsen (Johansen et al. 2008), both in regard to dimensions and the appearance of granules at the cross walls. However, the phylogenetic placement of *L. appalachiana* is very distant from *Nodosilinea* (Fig. 1A, ''*Leptolyngbya*'' clade 2), and the secondary structure of the 16S-23S ITS of *L. appalachiana* is notably different (Johansen et al. 2008). The D1-D1′ helix of *N. bijugata* is very similar to that of both *N. nodulosa* and *N. epilithica*, differing only in the appearance of the central part of the helix, which has two mismatched pairs that

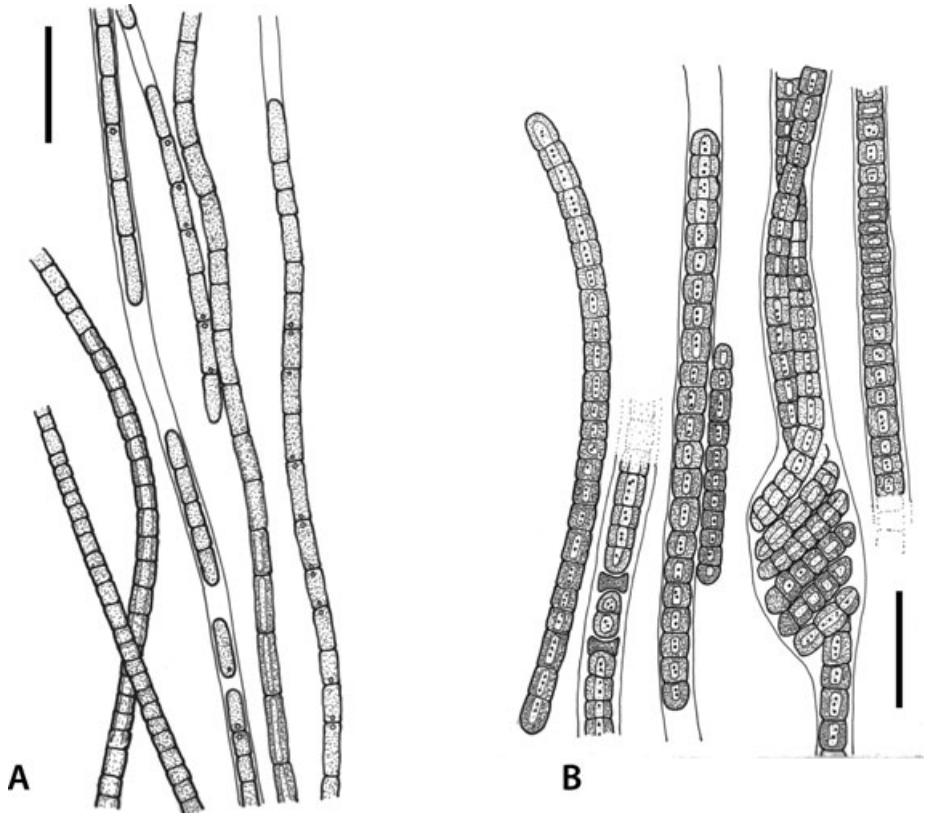

FIG. 7. *Nodosilinea* species. (A) *N. bijugata* Kovácik 1986/5a. (B) *Nodosilinea* Řeháková 1960/20. Scale bars = 10 μm.

cause small bilateral bulges (Fig. 2A). The Box-B, however, was unique among all *Nodosilinea* (Fig. 3A).

Nodosilinea conica Perkerson et J. R. Johans. **sp. nov.** (Figs. 5, G–I; and 8A).

A speciebus omnibus *Nodosilineae* trichomis latioribus et cellulis apicalibus conicis distinguenda.

Fila non ramosa, raro nodulis. Vagina mollis tenuis incolor. Trichomae leviter constrictae ad septum, necridiis nullis, cellulis in trichomis penitus vel in partibus meristematibus dividentibus, ad apicem abrupte contractae, 2.5–2.7 μm wide. Hormogonia plerumque formata. Cellulae thylakoidibus parietalibus evidentibus, non granulares, breviorae quam latiorae vel fere isodiametrae, 0.9–2.4 μm longae. Cellulae apicales conicae in trichomis maturis.

Diagnosis: Distinguished from all other *Nodosilinea* species by its wider trichomes and conical end cells.

Description: Filaments unbranched, rarely forming nodules. Sheaths soft, thin, colorless. Trichomes slightly constricted at the cross walls, lacking necridia, with cells dividing throughout the trichome length or in meristematic regions, tapering abruptly at the ends, 2.5–2.7 μm wide. Hormogonia often present. Cells with evident peripheral thylakoids, shorter than wide to almost isodiametric, 0.9–2.4 μm long. End cells conical in mature trichomes.

Type locality: Sevilleta Long Term Ecological Research site, Socorro County, New Mexico, USA. Chihuahuan Desert, soil, 34°12′43.5′ N latitude, 106°45′28.5′ W longitude.

Holotype here designated: BRY 37708.

Reference strain: Nodosilinea conica SEV4-5-c1.

Etymology: *Nodosilinea conica* = ''the conical *Nodosilinea*.''

This is the most easily differentiated species of the four species in *Nodosilinea*. It had the largest trichome widths, distinctly conical end cells, tapering in some trichomes, and a distinct biotope (desert soil). Furthermore, the 16S-23S ITS of this strain was very distinctive. It was the only ITS region recovered that had a V2 helix and had unique secondary structure in both the D1-D1′ helix and Box-B helix (Figs. 2D and 3D). The spacers between the D1-D1′ and $tRNA^{Ile}$ and between the $tRNA^{Ala}$ and Box-B were both shortest in this strain as well.

Nodosilinea K. Řeháková 1960/20 (Figs. 5, J and K; and 7B).

Description: Filaments unbranched, abundantly forming nodules, with sheaths evident. Sheaths thin, colorless. Trichomes slightly constricted at the cross walls, lacking necridia, lacking meristematic zones, with cells dividing throughout the trichome length, 2.0–2.5 μm wide. Hormogonia absent. Cells cylindrical, with peripheral thylakoids, often with one to two granules at the cross walls, occasionally with one or two granules in the centroplasm, isodiametric to longer than wide, 2.0–4.5 μm long. End cells rounded.

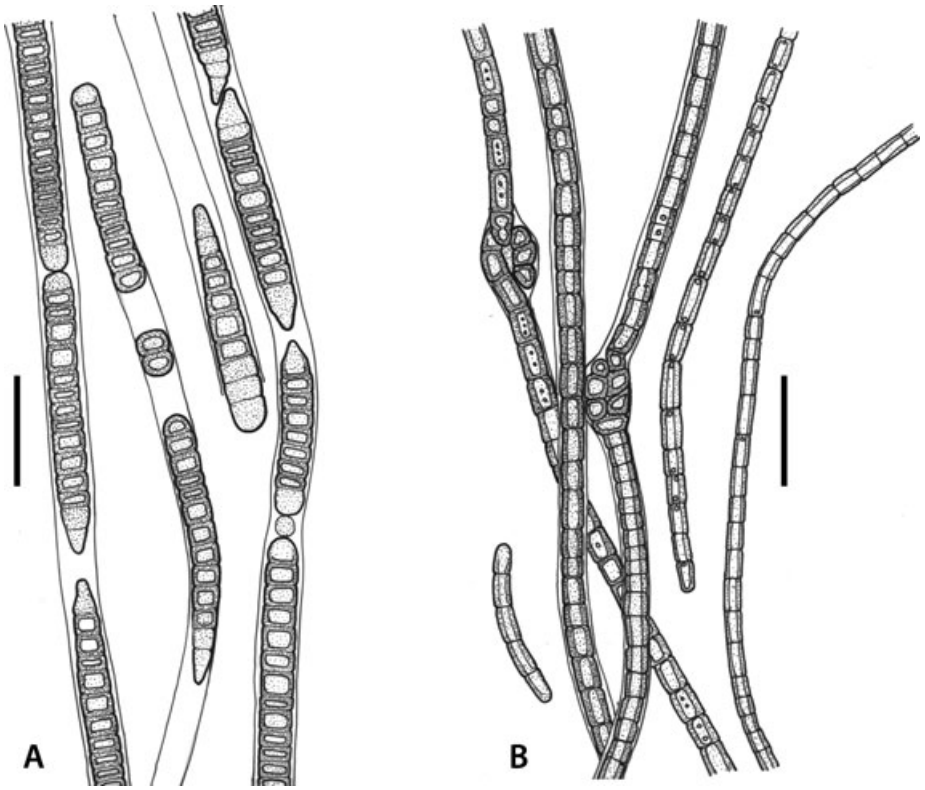

FIG. 8. *Nodosilinea* species. (A) *N. conica* SEV4-5-c1. (B) *Nodosilinea* FI2-2HA2. Scale bars = 10 μm.

Reference strain: Nodosilinea K. Řeháková 1960/20.

Nodosilinea FI2-2HA2 (Figs. 5L and 8B).

Description: Filaments unbranched, abundantly forming nodules, with sheaths evident. Sheaths thin, colorless. Trichomes slightly constricted at the cross walls, lacking necridia, lacking meristematic zones, with cells dividing throughout the trichome length, 1.5–2.0 μm wide. Hormogonia present. Cells cylindrical, with peripheral thylakoids, often with one to two granules at the cross walls, occasionally with one or two granules in the centroplasm, isodiametric to longer than wide, 1.5–4.5–(12) μm long. End cells rounded.

Reference strain: *Nodosilinea* FI2-2HA2.

Nodosilinea NB1a-A5 (Fig. 9B).

Description: Filaments unbranched, rarely forming nodules, with sheaths evident. Sheaths thin, colorless, becoming diffluent. Trichomes slightly constricted at the cross walls, lacking necridia, lacking meristematic zones, with cells of nodular regions having the appearance of division in more than one plane, with cells dividing throughout the trichome length, 1.6–2.0 μm wide. Hormogonia present. Cells cylindrical, with peripheral thylakoids, typically with two or three granules in the centroplasm, shorter to longer than wide, 1.6–4.0 μm long. End cells rounded.

Reference strain: *Nodosilinea* NB1a-A5 was lost subsequent to characterization and sequencing.

DISCUSSION

Our studies of the form-genus *Leptolyngbya* clearly indicate that the genetic diversity in the clade exceeds the morphological diversity (Casamatta et al. 2005, Johansen et al. 2011). The genus is defined by the following characters: thin (0.8–3 μm wide) trichomes facultatively producing sheaths, with one trichome per sheath, with peripheral thylakoids, and cell division in which new cross walls formation does not occur before the previous cross walls are completely formed. Species are differentiated at present based on differences in color, trichome width, cell length, end cell morphology, and biotope. Many of the strains we have examined closely have dissimilar 16S rRNA genes, and it is evident from our phylogenetic analyses of *Leptolyngbya* and related members of the Pseudanabaenaceae that there are not only many new and undescribed species in *Leptolyngbya* (Casamatta et al. 2005, Komárek 2007, Johansen et al. 2008, 2011), but additionally there are a number of undescribed genera as well (Fig. 1). *Leptolyngbya* has been known to be polyphyletic for some time (Albertano and Kovácik 1994, Turner 1997, Wilmotte et al. 1997, Castenholz 2001, Wilmotte and Herdman 2001, Taton et al. 2003, Casamatta et al. 2005, Komárek and Anagnostidis 2005), but new genera have been slow to be recognized. *Phormidesmis* is the only new

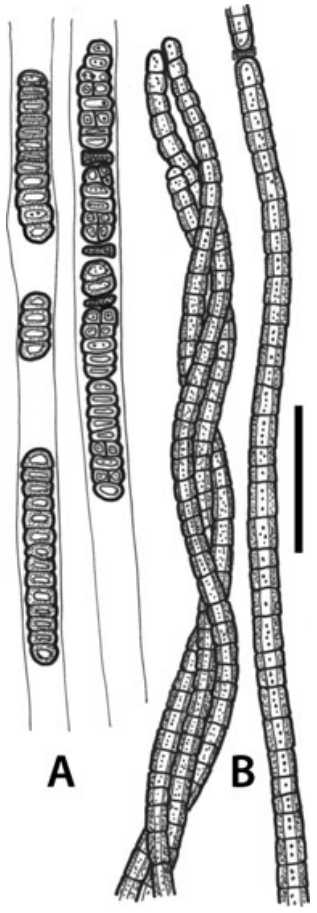

FIG. 9. *Nodosilinea* NB1a-A5. (A) Mature trichomes showing cell division in two planes. (B) Trichomes from young cultures showing the granular nature of the centroplasm observed in this and other species of *Nodosilinea*. Scale bar = 10 μm.

genus to have been split out of *Leptolyngbya* thus far (Komárek et al. 2009, Turicchia et al. 2009). As Komárek and Anagnostidis (2005, p. 175) observed ''the whole genus needs urgently the most widely conceived revision.''

We are confident that *Nodosilinea* is a monophyletic taxon diagnosable from other members of the Pseudanabaenaceae including *Leptolyngbya*. Other researchers have also recovered the same clade in phylogenetic analyses, but thus far no one has proposed a formal name for these organisms. Cuzman et al. (2010) recently recovered the same topology in their investigations of phototrophic taxa from fountains in Italy and Spain. They assigned a species epithet to one strain that fell within the cluster we are calling *Nodosilinea*, *Leptolyngbya margaretheana,* based on morphology and the presence of granules at the cross walls, and thus this may represent a new species of *Nodosilinea* in the future. Some authors have pointed out that lack of clearly articulated species concepts and failure of the 16S rRNA gene to resolve species level taxonomic decisions makes elucidating cyanobacterial species difficult (Flechtner et al. 2002, Johansen and Casamatta 2005, Řeháková et al. 2007). For example, some cyanobacterial populations or strains that are morphologically quite similar can be genetically very distinct (Ward et al. 1992, Casamatta et al. 2003, Perkerson et al. 2010). In our current case, however, we have three lines of evidence supporting the monophyly and clear separation of our new genus under a ''total evidence'' approach.

First, our new genus is genetically quite unique from other cyanobacteria based on 16S rDNA sequence data. In all phylogenetic analyses we conducted, with varied taxon sampling, optimality criteria, and tree search options, the *Nodosilinea* clade always formed and was always supported in the associated bootstrap analyses. In addition, sequence similarity to the putative sister taxon *Halomicronema* and morphologically similar *Leptolyngbya* was low. We recognize that sequence similarity by itself is neither sufficient nor ideal for delineating new species as has been pointed out in the literature (e.g., Johansen and Casamatta 2005, Řeháková et al. 2007), but it has been considered as part of the evidence for erecting some new cyanobacterial genera, such as *Halospirulina*, *Halomicronema*, *Rexia*, *Brasilonema*, *Mojavia,* and *Coleofasciculus* (Nubel et al. 2000, Abed et al. 2002, Casamatta et al. 2006, Fiore et al. 2007, Řeháková et al. 2007, Siegesmund et al. 2008).

Second, all of our strains have a highly conserved 16S-23S ITS secondary structure that is unique from other clades of cyanobacteria. Secondary structure of the 16S-23S ITS region has been suggested as a character useful for taxonomy (Wilmotte et al. 1993, Gugger et al. 2005, Johansen and Casamatta 2005, Casamatta et al. 2006) and has recently been incorporated into the diagnoses of both new species and new genera (Řeháková et al. 2007, Johansen et al. 2008, 2011, Siegesmund et al. 2008, Lukešová et al. 2009). The ITS region varies greatly from genus to genus, and thus aligning sequences between genera is not always possible (Johansen and Casamatta 2005, Johansen et al. 2011). To avoid the potential problem of interoperon variability, we employed cloned PCR products and utilized only the ITS regions with both tRNAs (Johansen et al. 2008, 2011).

Third, while some authors have pointed out that cyanobacterial morphology can vary greatly based upon environmental conditions (Stanier et al. 1971, Palinska et al. 1996, Casamatta and Vis 2004), our new strains all share a clearly visible morphological character (the presence of nodules) under appropriate low-light conditions. The formation of nodules under low-light conditions has never been reported from any other taxa in the Oscillatoriales (Li and Brand 2007). Li and Brand (2007) first reported on this unique character from *Leptolyngbya nodulosa* (=*N. nodulosa*) but were unable to determine the role(s) of these nodules. Putative roles included nitrogen fixation, buoyancy, and antiherbivory, but preliminary data indicated that none of these were definitive (Li

2007, Li and Brand 2007). We analyzed several of our newly described strains to determine if they were capable of nitrogen fixation, but the results were inconclusive for all strains except the two in *N. nodulosa*. The ontogeny of nodule formation is unclear, but it appears in at least some instances that cell division can occur in two planes in these strains (Figs. 5I; 6A; and 9A). If division in two planes is indeed occurring, it is a unique feature among the Pseudanabaenales. This morphological criterion alone would be sufficient evidence for erecting a new genus, and given the additional molecular evidence (monophyly, ITS structure), description of this new genus is justified. It should be noted, however, that nodule formation has not been assessed in *Halomicronema*, the sister genus, but thus far no report of nodules has been made.

This study showed that while the 16S rRNA gene sequence is very useful for phylogenetic analysis and recognition of monophyletic clusters of species, it was not informative at the species level within the genus *Nodosilinea*. This finding is in agreement with other studies in which species were not resolved well within clades representing genera. In particular, the *Leptolyngbya* sensu stricto clade contains morphologically distinct species that differ in ITS structure but are not resolved into clear species groups (Johansen et al. 2008, 2011). Additional taxon sampling may improve resolution, but given the high sequence similarity in both *Leptolyngbya* and *Nodosilinea*, we suspect that we will need multiple gene sequences to understand the phylogenetic relationships within these genera.

The recognition of *Nodosilinea* makes the polyphyletic nature of *Leptolyngbya* even more striking. To include all strains assigned to *Leptolyngbya* into a monophyletic group, one would have to collapse many genera, including *Halomicronema, Prochlorothrix, Nodosilinea, Phormidesmis, Tapinothrix,* and *Pseudophormidium*. The more desirable option to us is to further study and characterize the clades in *Leptolyngbya* to be able to correctly diagnose separate genera. We expect this work will be forthcoming in the near future.

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