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Nodosilinea chupicuarensis sp. nov. (Leptolyngbyaceae, Synechococcales) a subaerial cyanobacterium isolated from a stone monument in central Mexico

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Abstract

Microorganisms of subaerial biofilms, which develop on the surface of stone monuments, have been widely studied due to their impact on Cultural Heritage deterioration. Nevertheless, these extremophile microorganisms are poorly characterised. In this study, a cyanobacterial strain isolated from an archaeological zone in north-central Mexico was characterised through a polyphasic study based on ecological, morphological, and molecular data. Phylogenetic analyses were done using sequences of the 16S rRNA gene and the 16S-23S ITS region. Based on the polyphasic data, a new species corresponding to the genus *Nodosilinea* is described. This alkaliphilic species has optimum growth at pH of 9. Its fatty acid profile indicates that C18:1, 9*E* and C16:0 are its most abundant fatty acids. Extracellular polymeric substance production was verified on this strain. Only a few species of the genus *Nodosilinea* have been documented and described, and this is the first report of their presence in central Mexico. Hence the name *Nodosilinea chupicuarensis sp. nov.* is proposed.

Keywords: Leptolyngbyaceae, cyanobacterial exopolysaccharides, Internal Transcribed Spacer (ITS), 16S rRNA gene, subaerial biofilms

Introduction

Subaerial biofilms are complex microbiological ecosystems composed of heterotrophic and phototrophic microorganisms (Gorbushina & Broughton 2009). These microbiological communities develop on mineral surfaces, commonly rock, and are continuously exposed to the atmosphere (Gorbushina 2007). Among the subaerial biofilm microorganisms, the phototrophs play a very important role, since the input of nitrogen and carbon to the biofilm depends on their metabolism. Furthermore, phototrophs synthesize most of the biofilm exopolysaccharide matrix (Cary *et al*. 2010, Vázquez-Martínez *et al*. 2014, Rossi & De Philippis 2015, Vázquez-Nion *et al*. 2016).

Most cultural heritage sites consist of anthropogenic structures built from rock. Stone monuments and some engineering works provide unique niches on which the subaerial biofilms can commonly develop, and these biofilms then contribute to the degradation of the stone structure through their enhancement of weathering processes (Warscheid & Braams 2000, Scheerer *et al*. 2009, Vasanthakumar *et al*. 2013). Mexico has more World Heritage sites than any other country in the Americas and is 6th in the world (UNESCO 2016). Some of these monuments have been exposed to the atmosphere for centuries, developing centennial biofilms, while others, although they were built centuries ago, have been more recently re-exposed and have developed younger biofilms. There are just a few studies about the microflora that develops on these stone structures (Ramirez *et al*. 2010, Ortega-Morales *et al.* 2006, Novelo *et al*. 2011), so this niche is a source of novel and poorly characterised microorganisms.

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While a fair amount of study has been conducted on community composition of subaerial communities on soil, the subaerial floras of man-made stone structures are less well known. These structures provide an especially harsh habitat that repeatedly is wetted and dried in rapid succession and often bakes at high temperature under sunlight. A number of novel eukaryotic microalgal species, such as *Mesotaenium testaceovaginatum* Fučíková, Hall, Johansen *et* Lowe (2008: 31), *Hylodesmus singaporensis* Eliáš, Němcová, Škaloud *et* Neustupa *in* Eliáš *et al*. (2010: 1126), *Leptochlorella corticola* Neustupa, Veselá, Němcová *et* Škaloud *in* Neustupa *et al*. (2013: 379), *Kalinella apyrenoidosa* Neustupa, Veselá, Němcová *et* Škaloud *in* Neustupa *et al*. (2013: 382), and *Polulichloris henanensis* Song, Zhang, Liu *et* Hu (2015b: 140), and have been isolated and described from subaerial surfaces in the past few years. New cyanobacterial genera have been less commonly found, although the genus *Oculatella* Zammit, Billi *et* Albertano (2012: 352) stands as an exception, having been described from subterranean catacombs and having two additional subaerial taxa described since (Osorio-Santos *et al*. 2014).

The genus *Leptolyngbya* Anagnostidis *et* Komárek (1988: 390) was described based on the clade of thin simple filaments known as LPP group B Rippka *et al.* (1979). The genus is characterized by filaments with facultative to obligate sheath formation, thin (0.5–3.5 μm) trichomes, with or without false branching, with cells shorter than wide to longer than wide, with or without necridia, and parietal thylakoids (Rippka *et al.* 1979, Komárek & Anagnostidis 2005). Unfortunately, this circumscription is so broad that numerous genetically distinct strains and species were assigned to *Leptolyngbya* based on morphology, even though recent phylogenetic studies have demonstrated that the genus is polyphyletic and in need of taxonomic revision (Johansen *et al.* 2011, Osorio-Santos *et al.* 2014). This revisionary work has commenced, and over a dozen novel genera have been split out of *Leptolyngbya* thus far (Abed *et al.* 2002, Turicchia *et al.* 2009, Perkerson *et al.* 2011, Taton *et al.* 2011, Zammit *et al.* 2012, Dadheech *et al.* 2012, Vaz *et al.* 2015, Song *et al.* 2015a, 2015b, Miscoe *et al.*, 2016, Sciuto et Moro 2016, Sciuto *et al.* 2017, Strunecký *et al*. 2017).

Nodosilinea Perkerson *et* Casamatta *in* Perkerson *et al.* (2011: 1404) was separated from *Leptolyngbya* based primarily on phylogenetic evidence, although the genus does possess the facultative ability to form nodules in the filaments under low-light conditions (<4 μmol photons m^2 s^{-1}). *N. nodulosa* (Li *et* Brand 2007: 397) Perkerson *et* Casamatta *in* Perkerson *et al.* (2011: 1405) was isolated from marine phytoplankton in the China Sea, but other species in the genus are subaerial on rocks (*N. epilithica* Perkerson *et* Kovacik *in* Perkerson *et al*. 2011: 1405), in soils (*N. conica* Perkerson *et* Johansen *in* Perkerson *et al*. 2011: 1407), or aquatic (*N. bijugata* (Kongisser 1925: 70) Perkerson *et* Kovacik *in* Perkerson *et al.* 2011: 1406, see also Stoyanov *et al*. 2014). *Nodosilinea* species have filaments typically with a single trichome, but sometimes can be multiseriate. Trichomes are immotile and slightly to distinctly constricted at the cross-walls. They also usually present thin, soft and colourless sheaths. The cells appear more or less isodiametric or longer than wide with peripheral thylakoids. Some species are able to perform nitrogen fixation Perkerson *et al.* (2011). If only a few individual trichomes are observed in nature, the nodules are typically not evident, and the genus is morphologically indistinguishable from *Leptolyngbya*. Consequently, this will likely be a genus that is recognised only when sequence data for strains is available.

In this manuscript, we report the polyphasic characterization of a new species of *Nodosilinea*, found growing on the surface of a stone monument in central México. Herein we characterize and describe this species as *Nodosilinea chupicuarensis sp. nov*.

Materials and Methods

Origin and culture conditions:—The strain was isolated from a subaerial biofilm in the archaeological zone *Cañada de la Virgen* in the municipality of San Miguel de Allende, Guanajuato, Mexico. This zone was the centre of the political and religious power of the Lerma river central-watershed territory during the Late Preclassical Period (800 BCE to 200 CE). The Chupícuaro culture that established the stone monuments preserved in this archaeological zone was one of the most important and influential Mesoamerican cultures for over 500 years (Noé Porter 1956, Zepeda-García-Moreno 2008, 2010). Specifically, samples were taken from a stairway of Complex A or *Casa de los Trece Cielos* at 5 cm above the south drainage channel of the central-courtyard (20°51'29.6" N, 100°55'41.0" W), Fig. 1. Samples were taken using the non-destructive technique of adhesive tape (Cutler *et al.* 2012), in October 2012. In the laboratory, samples were enriched in liquid BG-11 medium (Waterbury 2006). After two weeks of enrichment, microorganisms were isolated and an axenic culture of the strain was obtained through classic microbiological techniques after several months of culture using both solid and liquid BG-11 media. Cultures are maintained under 16 h light (50 μmol photons

m^2 s^{-1}) / 8 h dark photoperiod at 25°C. Light-limitation experiments were conducted at <4 µmol photons m^2 s^{-1} for 4 weeks in order to observe any morphological change.

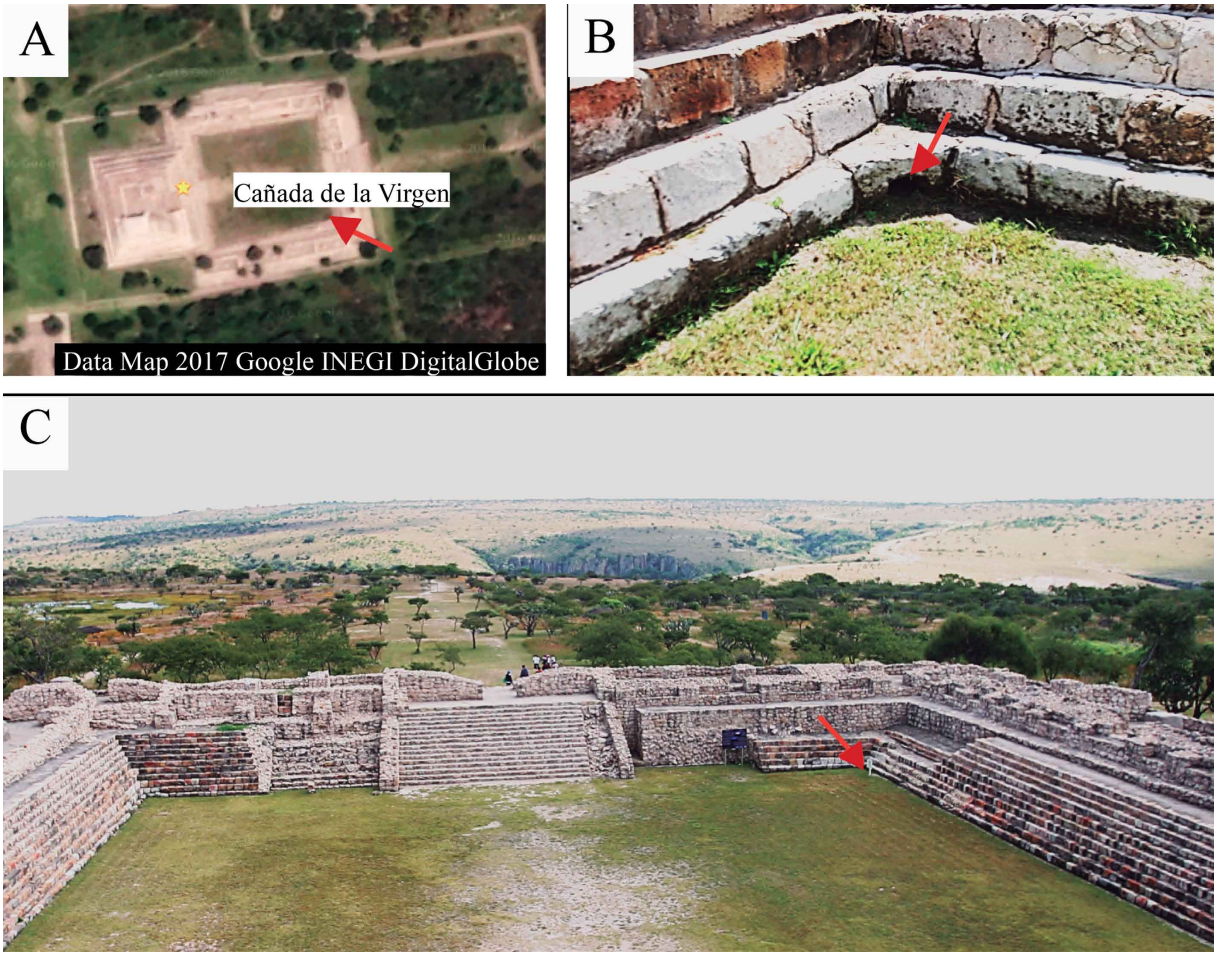

FIGURE 1. Sampling site at the Archaeological zone of *Cañada de la Virgen*. A. Aerial view of the Complex A (image taken from Google Maps, 2016). B. South drainage channel of the Complex A central-courtyard. C. Complex A central-courtyard. Red arrows indicate the specific sampling point.

Morphological characterization:—The main microscopic features were observed under a light microscope model DMB3-223 (Thomas Scientific, Inc., NJ, USA). Scanning electron microscopy (SEM) was done using a JEOL JSM microscope model 5910LV-27 (JEOL Ltd., Tokyo, Japan). For SEM, samples were fixed and dehydrated according to Li & Brand (2007), then covered with a gold coat produced by sputtering in a Denton Vacuum Desk II XLS sputter coater (Denton Vacuum, LLC, NJ, USA). In addition, fresh samples were observed without treatment under low vacuum conditions.

Molecular Methods:—Genomic DNA was extracted using the phenol-chloroform protocol, from approximately 100 mg of fresh cell material, previously frozen and pulverised in liquid nitrogen. The extracted DNA was washed with 70% ethanol, and dried and re-suspended in TAE buffer. DNA integrity was verified in a 1% ultrapure agarose gel.

Amplification of most of the 16S gene (approximately 1600 bp) was done using the 27F 5'-AGA GGT TTG ATC CTG GTC AG-3' and R1494 5'-TAC GGR TAC CTT GTT ACG AC-3' oligonucleotide primer pair (Lane 1991). The reaction mix was composed of 1 ng of DNA (µL PCR)$^{-1}$, reaction buffer at final concentration of 1 X, dNTPs at 0.2 mM (Invitrogen, CA, USA), $MgCl_2$ at 2.5 mM, DMSO at 0.5% (v/v), each oligonucleotide primer at 0.2 µM, and 1 unit of DNA Taq polymerase (QIAGEN, NH, USA). PCR was performed with a T100 thermal cycler (Bio-Rad Laboratories, Inc.) as follows: an initial denaturing step at 95°C for 2 min, followed by 25 thermic cycles (i.e. 40 s at 95°C, 30 s at 55°C and 1.5 min at 72°C), and then a final extension step at 72°C for 7 min. Once the reaction was completed, the integrity of the PCR product was verified in a 1% agarose gel.

A cloning step was performed using the pJET1.2/blunt vector and the CloneJET PCR Cloning Kit (Thermo Scientific, MA, USA). The processes for ligation, transformation, recombinant clone analysis, 16S recombinant clones PCR, and plasmid DNA extraction were done following manufacturer instructions (http://101.200.202.226/files/prod/manuals/201210/18/449454001.pdf). The quality and integrity of plasmid DNA were verified in a 1% agarose gel.

Transformation was achieved using *Escherichia coli* DH5α chemo-competent cells. The presence of a 16S rRNA insert in each clone was checked by plasmid digestion and subsequent analysis of the product on a 1% agarose gel.

Three positive clones were chosen to obtain the 16S gene sequence. The sequences were obtained by *Sanger* capillary sequencing using two external sequencing primers and a single internal primer as follows: pJET1.2Fw 5'-CGA CTC ACT ATA GGG AGA GCG GC-3', 16S-515Fw 5'-GTG CCA GCM GCC GCG GTAA -3', and pJET1.2Rv 5'-AAG AAC ATC GAT TTT CCA TGG CAG-3' (Thermo Scientific Fermentas™ pJET™ 1.2, Lane 1991) . Sequence quality was verified using the software Sequence Scanner v 1.0. (Applied Biosystems, Thermo Fisher Scientific Inc., USA). For each clone, sequences were assembled using the software MEGA 6 (Tamura *et al.* 2013). The consensus sequence of the three assembled sequences was produced using the online platform EGassembler—GenomeNet (Masoudi-Nejad *et al.* 2006).

The same PCR, cloning and sequencing process was followed to obtain the complete sequence of the 16S-23S intergenic spacer (ITS), with the follow observations: the oligonucleotide pair used for PCR was VRF5Fw 5'-TGT ACA CAC CGG CCC GTC-3' and VRF1Rv 5'-CTC TGT GTG CCT AGG TAT CC-3' (Johansen *et al.* 2011), and the primers for sequencing was pJET1.2Fw and pJET1.2Rv (Thermo Scientific Fermentas™ pJET™ 1.2).

Phylogenetic analyses:—A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed in order to identify the closest matches for the consensus sequence. A total of 47 *Nodosilinea* sequences at least 1100 bp in length were included as the ingroup. An extensive list of 222 thin filamentous taxa was added as outgroup taxa. The full list with accession numbers of each sequence can be found in the 16S rRNA phylogeny given in supplemental materials (Figure S1). The query sequence and the NCBI-GenBank downloaded sequences were aligned using the MUSCLE tool of MEGA 6. In addition, we examined secondary structures of conserved regions to make sure that they were aligned correctly (Řeháková *et al.* 2014). The alignment was submitted to MrBayes on XSEDE (3.2.6) available on CIPRES Science Gateway v.3.1 with the following parameters: NST=6, Rates=invgamma, MCMC Ngen=30,000,000. All other parameters were left as defaults. The analysis was run until the average standard deviation of split frequencies was < 0.01. Minimum ESS was confirmed to be >500 for all parameters, and PSRF = 1.00 for all parameters. Maximum Likelihood analysis was run on RaxML, also through the CIPRES Portal, using the model GTR+G+I, with 1000 bootstrap replicates. Maximum Parsimony analysis was run on PAUP, also through the CIPRES Portal, with gapmode=newstate, steepest=no, multrees=yes, swap=TBR, nreps=1000, with 1000 bootstrap replicates. Bootstrap values from both the maximum likelihood and parsimony analyses were mapped on to the Bayesian Inference phylogeny.

The phylogeny based on the ITS region was constructed by comparing the query sequence with other *Nodosilinea* ITS sequences deposited in NCBI-Nucleotide. All sequences chosen were verified as belonging to the genus *Nodosilinea* by phylogenetic analysis of 16S rRNA gene sequence data, even though some were misidentified in the data base. The query sequence and the NCBI-GenBank downloaded sequences were aligned using MUSCLE and the alignment was manually examined. In order to include indels in the analysis of the ITS region, parsimony was chosen as the criterion, with missing bases coded as a fifth base. The tree was run in PAUP v. 4b10 with the following parameters: heuristic search, multrees = yes, steepest descent = no, swap=TBR, nreps = 1000. A total of 10,000 bootstrap replicates were performed on the single best tree. The same alignment was then modified to add a scoring of the indels in the ITS (1 = base present, 0 = base absent), and the mixed data alignment was then analysed with MrBayes. The trees had nearly identical topologies; the Bayesian Inference phylogeny is reported with bootstrap values from the parsimony analysis mapped on to the nodes. Sequence dissimilarity among ITS regions in *Nodosilinea* was calculated as uncorrected p-distance using PAUP v. 4b10.

For the secondary structure folding analysis, the ITS sequences were folded using the online platform Mfold Web Server (Zuker 2003), selecting secondary structure predictions with minimum free energy under default settings at 37°C. The sequence of the tRNA genes was determined using the software tRNAscan-SE version 1.21 (Lowe & Eddy 1997). ITS secondary structures of the D1-D1', Box-B, and V3 helices were analysed in order to determine the intragenic variability between the query and representative *Nodosilinea* species (some misidentified in NCBI as *Leptolyngbya*, *Oscillatoria* Vaucher *ex* Gomont (1892: 198), and *Phormidium* Kützing *ex* Gomont (1892: 179), but all confirmed as belonging to the genus *Nodosilinea* by phylogenetic analysis of the 16S rRNA gene). The secondary structures were redrawn in Adobe Illustrator CS6 version 16.0.0 (Adobe Systems Inc.)

Fatty acid profile:—In recent studies, the fatty acid profile has been used as a taxonomic biomarker for microalgae (Lang *et al.* 2011). With the aim of obtaining further taxonomic information for future reference, the fatty acid profile, as methyl ester derivatives, was determined as follows: 50 mg of fresh cell material was added with 1 mL of a methanolic solution of 0.5 M NaOH and incubated at 90°C for 1 h. Afterwards, the sample was tempered to room temperature and 1 mL of a methanolic solution of 14% BF_3 was added (Sigma-Aldrich Chemie, Steinheim, DEU), with a second incubation at 90°C for 30 min. Once the reaction was completed, the sample was tempered to

room temperature, then 2 mL of water and 4 mL of hexane were added and the mix was emulsified in a vortex. The emulsion was centrifuged 5 min at 3000 rpm, and the hexane phase was collected. The hexane phase was dried under nitrogen flux and the residue was dissolved in 200 µL of isooctane. Samples were immediately analysed by GC-EIMS, in an Agilent Technologies 7890 Gas Chromatograph coupled to a 5975 inert XL MDS Triple-Axis Detector using a J&W DB-1UI (60 m x 250 µm x 0.25 µm) capillary column. GC oven temperature program was as follows: initial temperature of 150°C held for 3 min, then a ramp of 4°C per min to 280°C held for 25 min. Heptadecanoic acid methyl ester at final concentration of 150 µg mL^{-1} was added as internal standard. The identification of each fatty acid was achieved using the software products Mass Hunter Workstation version B.06.00 (Agilent Technologies, Inc.), the NIST spectral database and library MS Search version 2.0 (National Institute of Standards and Technology, 2011), and the respective standard.

Optimum pH for growth:—The ability of the strain to survive and grow under distinct pH conditions was tested. The strain was cultured in BG-11 liquid medium and pH was adjusted with 1 M HCl or 2M NaOH. Experiments were done in 125 mL flasks containing 50 mL of medium. Different pH values were tested: 4, 6, 7, 8, 10 and 12. Flasks were incubated for 21 days under the conditions described above; pH value was measured at the end of incubation. Growth was determined on a dry weight basis. Experiments were performed in triplicate.

This strain is maintained in axenic culture at the Laboratorio de Fitobioquímica ceparium in CINVESTAV-IPN Unidad Irapuato, Mexico. The same strain is deposited in the CIBNOR culture collection (B.C.S., Mexico) with deposition number CIB87 and is the source of the herbarium materials (MEXU 4813!, 4814!, 4815!) deposited at the Herbario Nacional at the Universidad Nacional Autónoma de México.

Results

Taxonomic description

The investigated strain shows the diagnostic traits of the genus *Nodosilinea*. However, it possesses characteristics that do not correspond to any previously described species, and it is phylogenetically distinct with respect to both the 16S rRNA gene and the associated 16S-23S ITS region; thus we here name it as a new species.

Class **Cyanophyceae**

Subclass **Synechococcophycideae**

Order **Synechococcales**

Family **Leptolyngbyaceae**

***Nodosilinea chupicuarensis* Vázquez-Martínez, Gutierrez-Villagomez and Molina-Torres *sp. nov.* (Fig. 2 and 3)**

Diagnosis:—Differing from all other described *Nodosilinea* species through possession of regularly spiralled filaments in the population.

Description:—Thallus creeping on the rock surface forming a blue-green patina, embedded in mucilage. Filaments long (up to more than 350 cells long), under high light straight or gently curved, uniseriate, without false branching, 1.1–1.3 µm wide, under low light intensity (<4 µmol photons m^2 s^{-1}) appearing curved and forming spirals, in some instances filaments multiseriate, occasionally forming tight, compact nodules. Sheaths clear, thin, occasionally extending beyond trichome ends. Trichomes slightly motile, particularly at the 10–20 cells at the tips, constricted at the cross-walls, 0.9–1.2 µm wide. Cells lacking aerotopes, barrel-shaped (0.9 µm wide x 1.2 µm long), occasionally disc-shaped (1.2 µm wide x 0.6 µm long) or isodiametric (0.9–1.2 µm in diameter), with clear centroplasm and chromoplasm often visible in LM (=peripheral thylakoids). Apical cells dome-shaped, non-capitate, sometimes elongated, without calyptra. Reproducing by motile hormogonia.

Etymology:—*Nodosilinea chupicuarensis*, *Nodosilinea* = "Knotted line" Perkerson *et al.* (2011); *chupicuarensis* (chu.pi.cua.re´n.sis) N.L. fem. adj. chupicuarensis = origin from Chupícuaro, the ancient name of the region where the type strain was isolated (from *Purépecha* "*chupicua*" the name of a plant of the *Impomea* genus used to obtain a blue stain combined with "*ro*" meaning "place"; consequently *chupicuaro* = "blue place").

Habitat:—Stone surface, epilithic.

Type Locality:—MEXICO. Guanajuato: San Miguel de Allende, archaeological zone of *Cañada de la Virgen*, Complex A, 20°51'29.6" N, 100°55'41.0" W, *J. Vázquez-Martínez*, *J.M. Gutierrez-Villagomez*, *J. Molina-Torres*, *12-10-2012.*

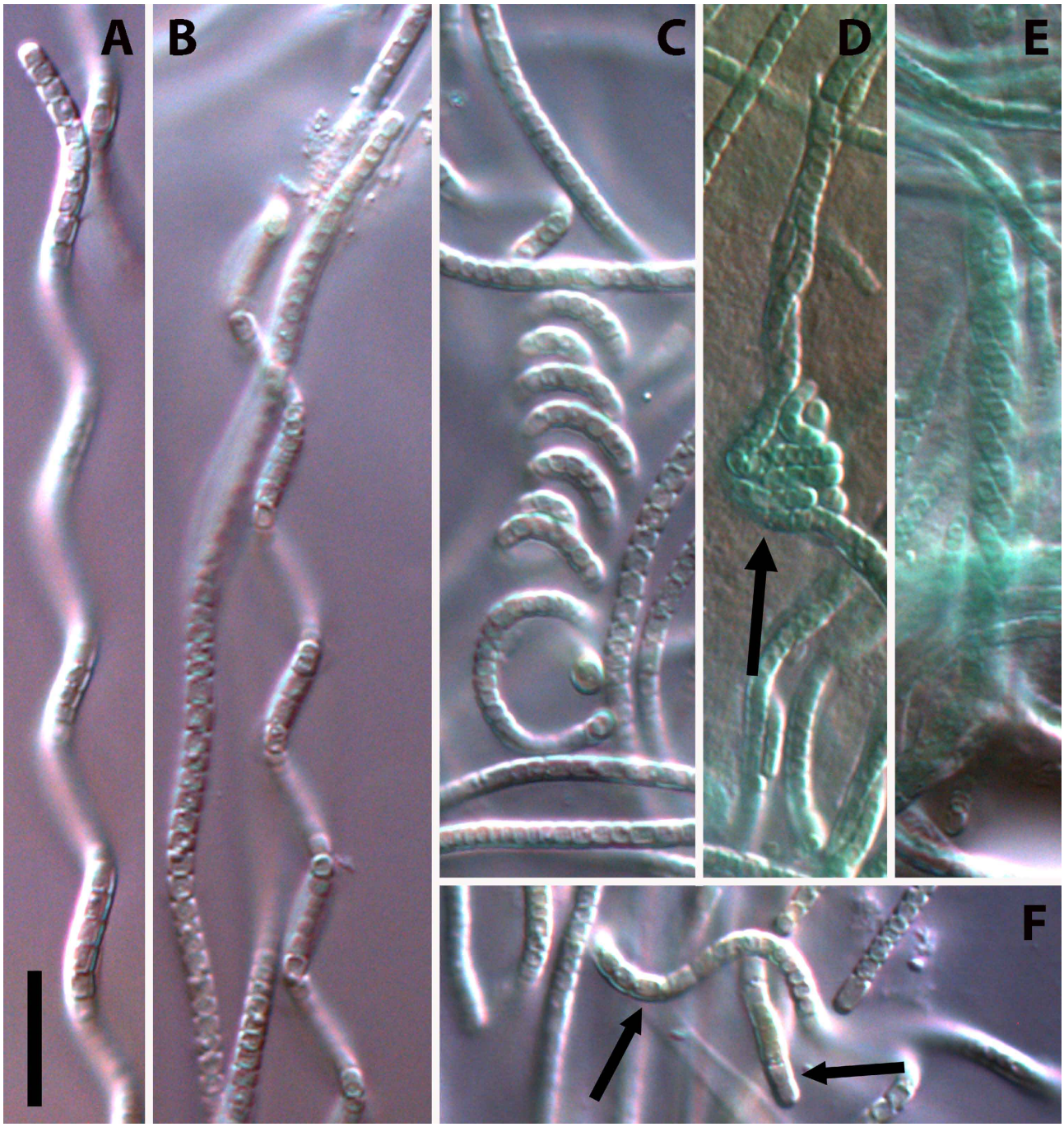

FIGURE 2. Main characteristics of *Nodosilinea chupicuarensis*. A-C. Mature filaments forming loose to tight spirals. D. A characteristic nodule (arrow). E. Multiseriate filament. F. Filaments with mature, elongated end cells (arrows). All figures to same scale, scale = 10 μm.

Holotype here designated:—MEXU 4813! in the Herbario Nacional at the Universidad Nacional Autónoma de México at Mexico City, Mexico.

Isotypes here designated:—MEXU 4814!, MEXU4815! in the Herbario Nacional at the Universidad Nacional Autónoma de México at Mexico City, Mexico.

Observations:—Under low light intensity conditions (3–4 weeks of culture), filaments are curved to form spirals that can be very compact or very relaxed. In some instances, trichomes become multiseriate due to cells inside the filament dividing in multiple planes. In some cases, these multiseriate regions resemble nodules. Within these multiseriate regions, nodules occasionally occur with the characteristic morphology of nodules found in other

Nodosilinea species. This strain presents a distinctive spirally coiled morphology under the same low light intensity conditions that produce nodules in other species, with only rare nodule formation.

The thallus is formed by a cluster of filaments that becomes a firm mat. In some instances, the filaments are embedded in an extracellular polymeric matrix, and these polymers can be visualised by alcian blue staining (data not

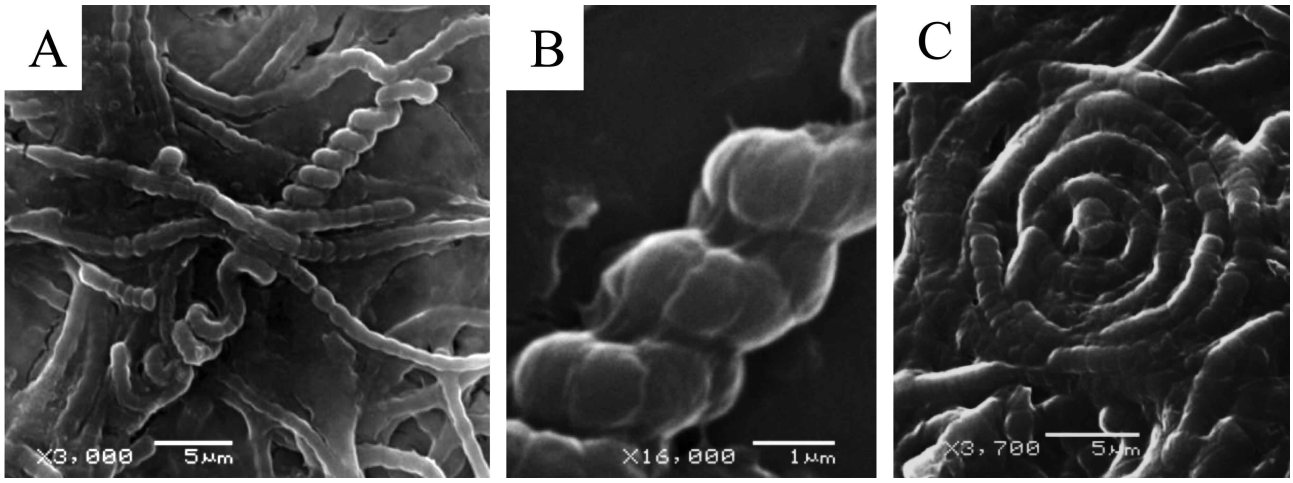

FIGURE 3. SEM micrographs of *Nodosilinea chupicuarensis.* A. Mature uniseriate filament forming a tight spiral. B. Amplification of a spiral. C. Coiled filaments.

shown). The thallus grows as a cluster of tangled filaments that are inlaid in the rock porosities forming a green-blue patina. Something similar occurs *in vitro* when the strain grows in solid BG-11 medium. Trichomes grow as spherical compact aggregates (1–4 mm diameter) when the strain is cultivated in liquid BG-11 medium under constant agitation. Hormogonia are formed by direct trichome fragmentation without formation of necridia.

TABLE 1. Comparison of 16S-23S ITS region p-distance among *N. chupicuarensis* and most closely related taxa. Strains with p-distance values less than 0.030 are likely in the same species, while values above 0.070 can be considered strong evidence for recognising separate species (Erwin & Thacker 2008; Osorio-Santos *et al.* 2014; Pietrasiak *et al.* 2014).

	Nodosilinea chupicuarensis	*Nodosilinea* CXA0007.4	*Nodosilinea epilithica* Kovacik 1990/52	*Nodosilinea* WJT8-NPBG4	*Phormidium* AA	*Nodosilinea nodulosa* UTEX 2910A	*Nodosilinea* ATA11-6B-CV9	*Nodosilinea bijugata* Kovacik 1986/5a	*Nodosilinea* FI2-2HA2
Nodosilinea chupicuarensis									
Nodosilinea CXA0007.4	0.067								
Nodosilinea epilithica Kovacik 1990/52	0.078	0.051							
Nodosilinea WJT8-NPBG4	0.078	0.045	0.021						
Phormidium AA	0.074	0.068	0.084	0.078					
Nodosilinea nodulosa UTEX 2910A	0.082	0.098	0.115	0.106	0.086				
Nodosilinea ATA11-6B-CV9	0.101	0.118	0.124	0.122	0.095	0.107			
Nodosilinea bijugata Kovacik 1986/5a	0.113	0.080	0.103	0.088	0.110	0.115	0.098		
Nodosilinea FI2-2HA2	0.111	0.060	0.094	0.082	0.066	0.103	0.118	0.081	
Leptolyngbya antarctica ANT.LACV6.1	0.128	0.159	0.161	0.155	0.125	0.123	0.102	0.122	0.141

Phylogenetic analysis:—According to the phylogenetic analysis using 16S rRNA gene sequences, *Nodosilinea chupicuarensis* is most related to *Nodosilinea nodulosa* UTEX 2910, with 99.5–99.4% identity. In the 16S rRNA phylogenetic tree (Fig. 4), *N. chupicuarensis* is the sister taxon to *N. nodulosa* UTEX 2910 and these two species are grouped together in a weakly supported clade in all three analyses performed (posterior probabilities/bootstrap support = 0.68, 76, 70). However, based on the analysis of the 16S-23S ITS region, *N. chupicuarensis* was distant from *Nodosilinea nodulosa* UTEX 2910 (p-distance = 0.0822), being closest to a soil strain from China, *Nodosilinea sp*. CXA007.4 (p-distance = 0.0665) (see Table 1). Members of the same bacterial species have been found to have p-distance <0.03, and the average intraspecies p-distance is ~0.01 (Erwin & Thacker 2008, Osorio-Santos *et al.*

2014, Pietrasiak *et al.* 2014), strongly suggesting that *N. chupicuarensis* is distinct from all other sequenced species (described or undescribed) of *Nodosilinea*. The 16S-23S ITS phylogenetic tree (Fig. 5) shows *N. chupicuarensis* to be phylogenetically separated from the other *Nodosilinea* strains, which fall into one of two supported clades.

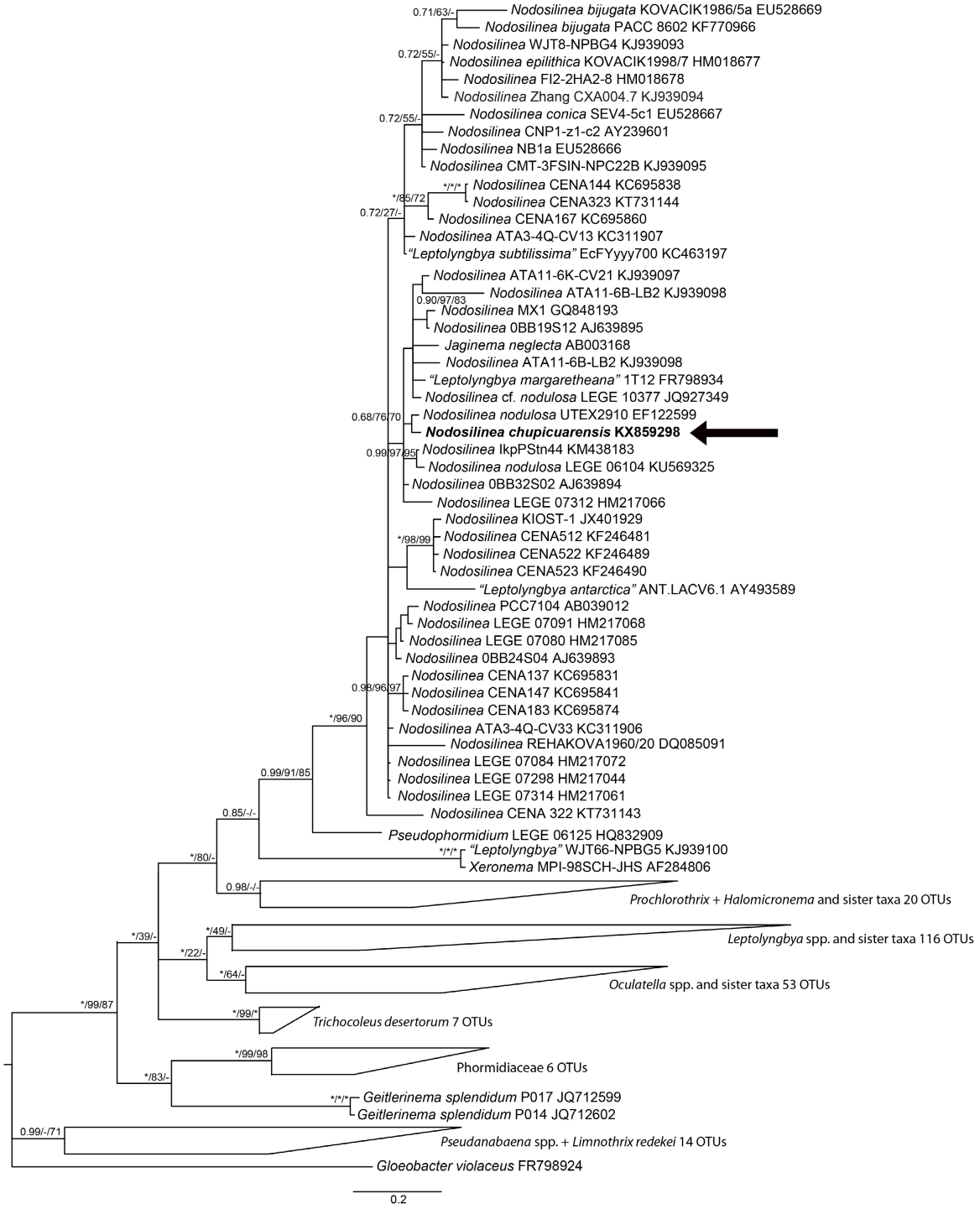

FIGURE 4. Phylogenetic position of Nodosilinea chupicuarensis (denoted with an arrow) in a Bayesian Analysis (285 OTUs, 1222 positions), with support values on nodes representing BA posterior support/ML bootstrap support/MP bootstrap support, respectively. The uncollapsed tree from which this figure was derived appears in supplemental materials.

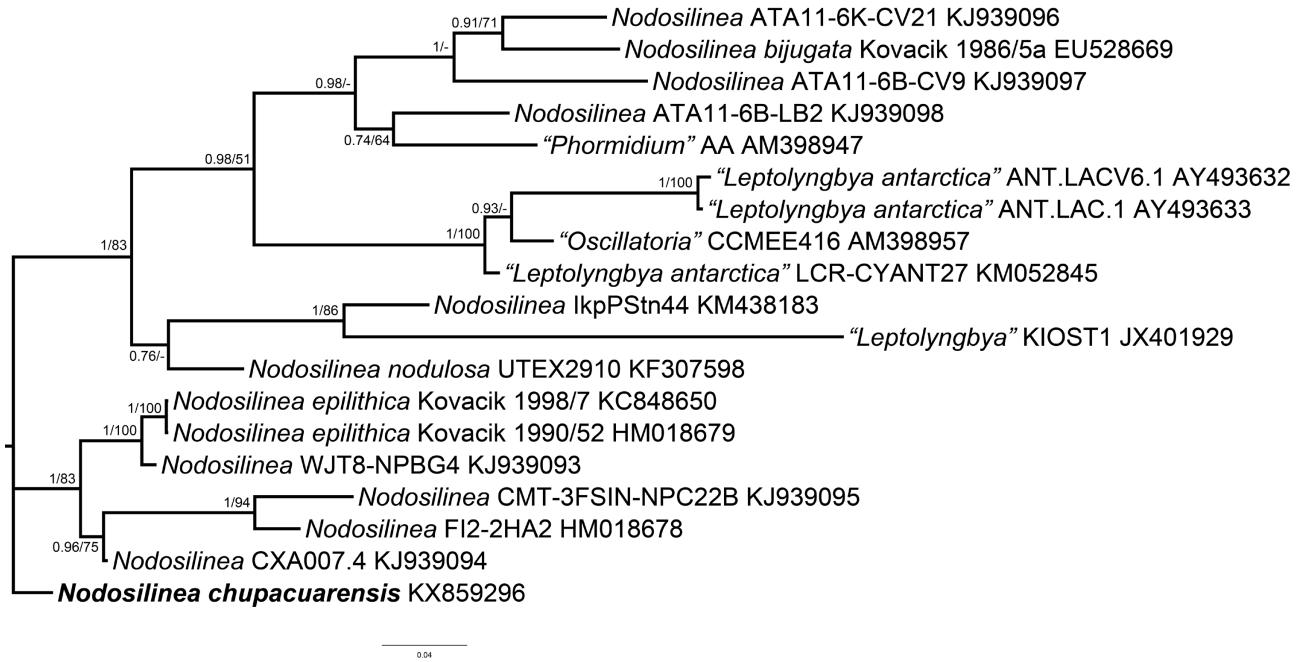

FIGURE 5. Unrooted tree of *Nodosilinea* strains based on Bayesian Analysis of 19 sequences (562 DNA positions, 114 coded indels). Bootstrap values from the parsimony analysis (562 DNA positions, gaps coded as a fifth base) are mapped onto the nodes in which the analyses were in agreement.

ITS secondary structure:—Comparison of secondary structure of 16S-23S conserved domains provides additional confirmation of the phylogenetic similarity among all *Nodosilinea* species presented in this study. While the secondary structure among the D1–D1' helices is identical for six of the strains including *N. chupicuarensis* (Fig. 6 A–F), every helix differs in sequence (Fig. 6 A–H). The Box-B helices were more variable in length, sequence, and structure (Fig. 6 I–P), and could be considered evidence that all eight strains in the comparison set were likely different species. Unlike most other cyanobacterial genera, the V3 helices were unusually conserved. All 19 strains for which complete ITS regions were obtained had V3 helices identical in sequence and structure (Fig. 6 Q).

Physiological traits

Fatty acid profile and optimum pH for growth: It was determined that the three most abundant fatty acids of *N. chupicuarensis* are C18:1, 9*E* (28.16 %), C16:0 (25.07%) and C18:2, 9*E*, 12*E* (13.69%). The complete fatty acid profile is shown in Table 2. The strain was able to grow at pH values of 7, 8, 10 and 12, and the final measured pH value was always 9.5 ± 0.5.

TABLE 2. Fatty acid profile of *Nodosilinea chupicuarensis*

Fatty acid	Content % +/- SD
C14:0	0.31 +/- 0.13
C16:4 (4, 7, 10, 13)	4.01 +/- 0.14
C16:2 (7, 10)	3.50 +/- 0.11
C16:3 (7, 10, 13)	2.98 +/- 0.13
C16:1 (7)	0.87 +/- 0.24
C16:0	25.07 +/- 0.41
C18:3 (6, 9, 12)	6.49 +/- 0.10
C18:4 (8, 11, 14, 17)	1.45 +/- 0.48
C18:2 (9, 12)	13.69 +/- 0.14
C18:3 (9, 12, 15)	6.24 +/- 1.02
C18:1 (9)	28.16 +/- 0.88
C18:1 (9)	6.35 +/- 0.11
C18:0	0.88 +/- 0.13

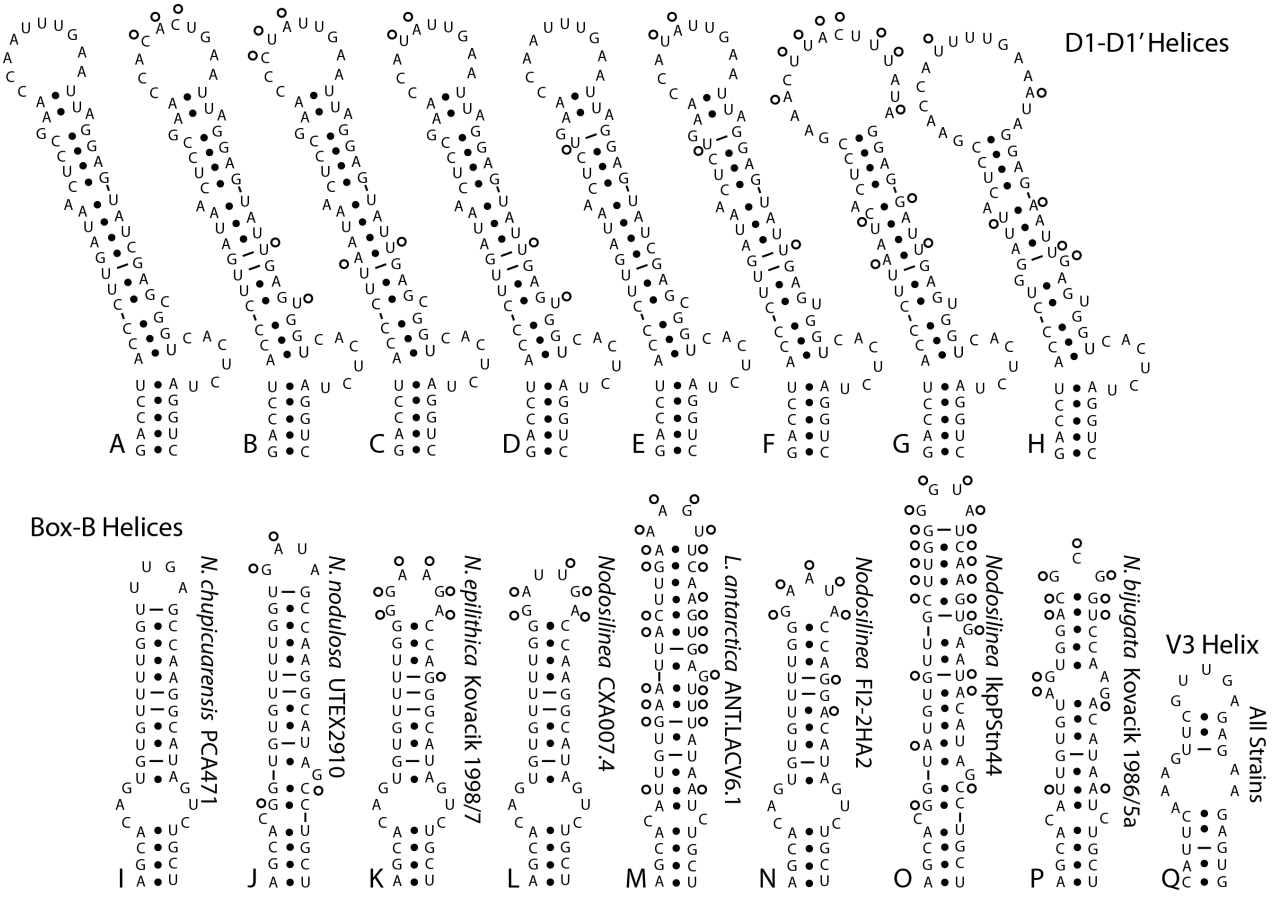

FIGURE 6. Secondary structure of conserved domains of the 16S-23S ITS regions for 8 representative strains of *Nodosilinea*. A–H: D1-D1' helices; I–P: Box-B helices; Q: V3 helix (all 19 strains). Strain labels in the bottom row apply to the D1-D1' helices in the first row. Bases differing from those in *N. chupacuarensis* are indicated by hollow circles next to the differing bases.

Discussion

The Leptolyngbyaceae is a large and relatively well-characterized family. The 16S rRNA gene has been sequenced for generitypes of the genera *Leptolyngbya*, *Plectolyngbya* Taton, Wilmotte, Marda, Elster *et* Komárek *in* Taton *et al*. (2011: 184), *Haloleptolyngbya* Dadheech, Mahmoud, Kotut *et* Krienitz (2012: 273), *Halomicronema* Abed, Garcia-Pichel *et* Hernández-Mariné (2002: 368), *Alkalinema* Vaz *et al.* (2015: 302), *Pantanalinema* Vaz *et al.*, (2015: 301), *Kovacekia* Miscoe, Pietrasiak *et* Johansen *in* Miscoe *et al.*, (2016: 83), *Nodosilinea*, *Neosynechococcus* Dvořák *et al.* (2014: 26), *Oculatella* Zammit, Billi *et* Albertano (2012: 352), *Phormidesmis* Turicchia *et al.* (2009: 179) and *Prochlorothrix* Burger-Wiersma, Stal et Mur (1989: 256). Additionally, non-type species in *Tapinothrix* Sauvageau (1893: 123), *Romeria* Koczwara *in* Geitler (1932: 915), and *Trichocoleus* Anagnostidis (2001: 369) have been sequenced (Bohunická *et al.* 2011, Lopes *et al*. 2012, Mühlsteinová *et al.* 2014). Nevertheless, the classification of many specimens based on morphological traits is difficult due to the fact that some of these taxa are morphologically poorly distinguishable and phenotypically plastic, making identification of incompletely characterised field material problematic (Komárek *et al.* 2014).

The 16S gene sequence of *Nodosilinea chupicuarensis* has high similarity (99.5%) with *Nodosilinea nodulosa* UTEX 2910. According to the original guidelines of Stackebrandt & Goebel (1994), the cut-off recommended for recognition of bacterial species was 97%. The cut-off was raised to 98.7% more recently by Stackebrandt & Ebers (2006), a level later confirmed by Yarza *et al.* (2014). Above this level, they recommend that DNA-DNA hybridization studies should be conducted to determine species. Below this level, they consider strains to belong to separate species. Consequently, *N. chupicuarensis* cannot be separated from *N. nodulosa* based on the 16S rRNA similarity criterion.

However, the similarity of 16S rRNA gene sequence has been found insufficient for establishment of a number of closely related species of cyanobacteria (Boyer *et al.* 2001, Siegesmund *et al.* 2008, Osorio-Santos *et al.* 2014).

The analysis of the more variable 16S-23S internal transcribed spacer (ITS), including secondary structure, length of conserved domains, p-distance, and phylogenetic analysis, is a better tool for separation of species, and has been validated in a number of studies (Řeháková *et al.* 2007, Johansen *et al.* 2011, Perkerson *et al.* 2011, Vaccarino & Johansen 2012, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014; Bohunická *et al.* 2015, Miscoe *et al.* 2016, Sciuto & Moro 2016, Berrendero Gómez *et al.* 2016). The 16S-23S ITS p-distance between *N. chupicuarensis* and *N. nodulosa* is 0.082 (Table 1), which we take as strong evidence that our strain is separate from that taxon. We also consider the fact that *N. nodulosa* was isolated from marine plankton in the South China Sea Li & Brand (2007) while *N. chupicuarensis* was isolated from archaeological stone monuments experiencing extensive periods of heat, insolation, and dehydration additional strong evidence that these are separate lineages. Finally, the morphology of the filaments, particularly the regular spiralling and rare production of very loose nodules, is evidence that our species does not belong to any described species. We concluded that the strain herein described is not *N. nodulosa* or any other described species of *Nodosilinea* Perkerson *et al.* (2011). The ecological niche in which *N. chupicuarensis* develops is a poorly studied microenvironment and, as shown here, these subaerial biofilms can be a source of undescribed species. Subaerial microorganisms that grow on stone monument biofilms are usually subjected to a manual removal process, but it would be important to characterize them before eradicating them. This work shows that, before the effort to eradicate subaerial biofilms, it is desirable to describe its microorganisms.

Morphology in Leptolyngbyaceae is often not very informative. In many cases, when a phyletic change first occurs (e.g. a physiological change) it may result only in cryptic phenotypic expression. These evolutionary species are very difficult to diagnose morphologically (Osorio-Santos *et al.* 2014, see fig. 69). Nevertheless, some morphological characteristics may help to diagnose *N. chupicuarensis*, such as the presence of large spirally coiled trichomes and multiseriated regions inside the filaments, when the strain grows under low light intensity. Furthermore, some physiological traits may help to more accurately diagnose this species, such as the optimum pH of growth and the production of exopolysaccharides. This species is clearly an alkaliphile, and this characteristic may be an adaptation to the calcareous substrata on which it naturally grows. The fatty acid profile did not contribute diagnostic information in this study, as there is not a robust fatty acid profile database of Leptolyngbyaceae species for comparison purposes. Nevertheless, these data may become of greater value as more profiles become available. It has been reported that one strain of *Leptolyngbya* sp. presents a similar fatty acid profile (Sahu *et al.* 2013), considering only the most abundant fatty acids (C18:1, 9*E* and C16:0), which could be a characteristic of this group. Yet, the strongest evidence supporting the description of the strain as a new species is the sequence of the 16S-23S ITS region. Based on the dissimilarity in the ITS regions of the *Nodosilinea* sequenced thus far, it is very likely that more species in this genus will be described in the near future.

Conclusions

The genus *Nodosilinea* was described less than a decade ago, but with this publication it already has five species, including a marine taxon, a freshwater taxon, and three soil taxa. Many sequences in molecular databases (e.g. NCBI nucleotide) belong to the *Nodosilinea* clade, as illustrated in the phylogenetic analysis. Herein, we describe a subaerial species morphologically distinct from all other described taxa, but with the number of genetically distinct members of the genus, we recognize that this genus undoubtedly harbors cryptic species. If all possible species were described, the morphological boundaries between them would undoubtedly blur. What to do taxonomically with these numerous cryptic lineages is a conundrum facing many taxonomists working with character-poor taxa (most microalgae) in an age when molecular data are becoming increasingly available.

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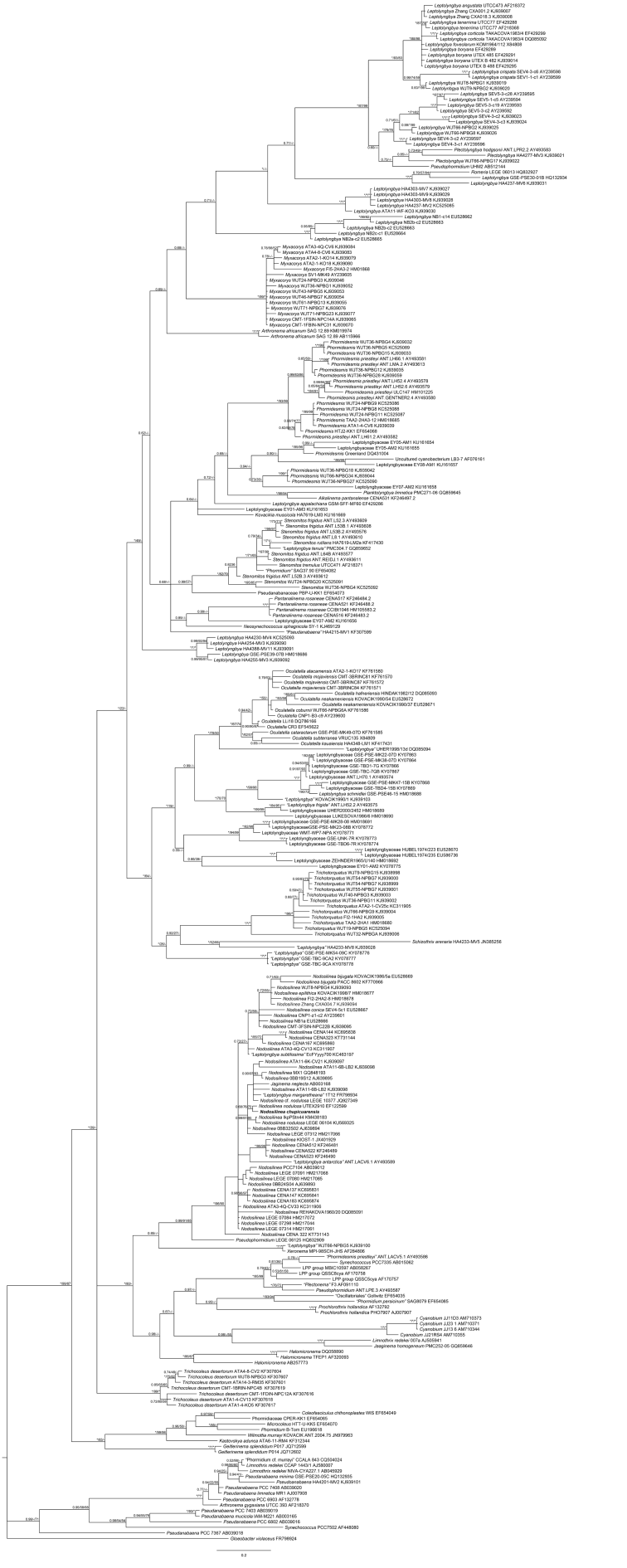
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