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Abstract

Twelve strains of *Leptolyngbya*-like filaments were isolated from biological soil crust samples from two localities in the Central Region of Mexico. The strains were morphologically distinguished from most Synechococcalean species by the obligate presence of a blackish sheath. Phylogenetic analysis based on 16S rRNA gene sequence placed all strains into a strongly supported single clade sister to *Scytolyngbya*. The genetic identity between our strains and all other Synechococcales, including *Scytolyngbya*, was less than 95%, and the strains were further distinguished by morphology and terrestrial ecology. The conserved domains of the 16S-23S ITS region had secondary structures distinct from all other closely related genera, which included *Scytolyngbya, Stenomitos, Chamaethrix,* and *Pantanalinema.* Based on the combination of morphological, molecular, and ecological evidence, we here describe two species: *Chroakolemma opaca gen. et sp. nov*. and *C. pellucida gen. et sp. nov*. Based on the ability to form blackish sheaths, these two species are morphologically similar to *Leptolyngbya edaphica, Chamaethrix vaginata* and *Trichocoleus badius*. The latter two species have been sequenced and are phylogenetically distant from *Chroakolemma*. *Leptolyngbya edaphica* is a soil species described from Russia and shares other morphological similarities with *Chroakolemma*, including wide sheaths, coiled filaments, pale blue-green trichomes, and constricted cross-walls. We consider these characteristics diagnostic of *Chroakolemma*, and accordingly propose *Chroakolemma edaphica comb. nov.*

Keywords: Chihuahuan Desert, Atexcac, Mezquital Valley, Tehuacan Valley

Introduction

Recently, cyanobacterial classification has undergone rapid revision based upon use of a polyphasic approach to define new taxa and molecular characterization of classical taxa (Komárek *et al*. 2014, Mareš 2017). One of the most problematic groups has been the Leptolyngbyaceae in the Synechococcales, as the genera in this family are morphologically simple and consequently very similar (Komárek 2017). Numerous new genera have been recognized based primarily on molecular phylogenetic evidence with supporting ecological and morphological characterization. These genera include *Halomicronema* Abed, Garcia-Pichel & Hernández-Mariné (2002: 368), *Phormidesmis* Turicchia, Ventura, Komárková & Komárek (2009: 179), *Nodosilinea* Perkerson & Casamatta (2011: 1404), *Plectolyngbya* Taton, Wilmotte, Šmarda, Elster & Komárek (2011: 184), *Haloleptolyngbya* Dadheech, Mahmoud, Kotut & Krienitz (2012:273), *Oculatella* Billi & Albertano in Zammit *et al*. (2012: 351), *Neosynechococcus* Dvořák, Hindák, Hašler, & Hindáková in Dvořák *et al.* (2014: 26), *Alkalinema* Vaz, Genuário, Andreote, Malone, Sant'Anna, Barbiero & Fiore (2015: 302), *Pantanalinema* Vaz, Genuário, Andreote, Malone, Sant'Anna, Barbiero & Fiore (2015: 301), *Scytolyngbya* Song & Li (2015: 74), *Pinocchia* Dvořák, Jahodářová & Hašler in Dvořák *et al*. (2015:115), *Limnolyngbya* Li & Li (2016:480), *Thermoleptolyngbya* Sciuto & Moro (2016:33), *Kovacikia* Miscoe, Pietrasiak & Johansen in Miscoe *et al*. (2016:83), *Stenomitos* Miscoe & Johansen in Miscoe *et al*. (2016:84), *Chamaethrix* Dvořák,

Hašler, Pitelková, Tabáková, Casamatta & Poulíčková (2017:270), *Timaviella* Sciuto, Moschin & Moro (2017:318), *Elainella* Jahodářová, Dvořák & Hašler in Jahodářová *et al*. (2017a:4), *Onodrimia* Jahodářová, Dvořák & Hašler in Jahodářová *et al.* (2017b:30), *Lusitaniella* Brito *et al.* (2017: 25), *Toxifilum* Zimba, Huang, Foley et Linton (2017: 190), and *Myxacorys* Pietrasiak & Johansen in Pietrasiak *et al.* (in review).

The type genus of the Leptolyngbyaceae, *Leptolyngbya* Anagnostidis & Komárek (1988: 390), has actually been very problematic as it has been broadly circumscribed both morphologically and ecologically, and has few synapomorphies that define it. Species in the family Leptolyngbyaceae with thin trichomes (0.5 to 3.5 μm wide), no aerotopes, and consistent production of sheaths have been historically placed in this genus. The members of the genus are variable with regard to degree of constriction at the cross-walls, formation of necridia, formation of false branching, and end cell morphology. The type species, *Leptolyngbya boryana* (Gomont 1899: 36) Anagnostidis & Komárek (1988: 390), is well-defined and a number of strains in this species have been sequenced and their morphology carefully characterized (Johansen *et al.* 2011). Many of the new genera mentioned above were first named *Leptolyngbya* in NCBI GenBank, resulting in a very polyphyletic genus (Casamatta *et al*. 2005, Johansen *et al.* 2008, 2011, Taton *et al*. 2011, Zammit *et al*. 2012, Osorio-Santos *et al*. 2014, Silva *et al*. 2014, Vaz *et al*. 2015). Only after molecular data showed that they were phylogenetically distinct from the clade containing the generitype were they placed in other genera, resulting in taxonomy that more accurately reflects evolutionary history. This group is difficult and revisionary work as well as α-level taxonomy still remains to be conducted.

This manuscript contributes to the α-level taxonomy of the Leptolyngbyaceae. We studied soil biocrust communities from two localities in semi-desert environments from Central México. In these biocrusts we observed filaments clearly belonging to the Leptolyngbyaceae in field samples, and these filaments were subsequently isolated into culture. The populations all possess widened and blackish sheaths, false branching, constricted crosswalls, and cells distinctly longer than wide, features which in combination are sufficient to propose a new genus, *Chroakolemma*. Additionally, the molecular analysis with 16S rRNA sequences and hypothetical secondary structure of ITS 16S-23S confirm the uniqueness of these strains.

Herein, we describe *Chroakolemma gen. nov*. for these distinctive populations, which contains three species, *C. opaca sp. nov*., *C. pellucida sp. nov*., and *C. edaphica comb. nov.*

Material and methods

Sample collection and cultures:—*Chroakolemma* filaments were found inhabiting soil crusts of semi-deserts from Central México in two localities. The first locality was near to Actopan, Hidalgo state within the Mezquital Valley with mean annual precipitation (MAP) of 436 mm, mean annual temperature (MAT) of 16.4°C, with soil type being a Feozem (Mollisol), and a xeric savanna-like scrub vegetation dominated by *Prosopsis* Linnaeus (1767: 68) spp. (Mesquite shrubs and trees) with crassiculate (fleshy stemmed) plants. The second site was around the crater lake of San Luis Atexcac (L.A), Puebla state, with MAP of 372 mmm, MAT of 13.9°C, soil type Feozem, and xeric vegetation dominated by *Yucca* Linnaeus (1753: 47) and other Agavoideae. These localities constitute the southern end of the Chihuahuan Desert and the two sites are separated by basaltic volcanic flows, mountains, and pine-oak forest growing at the higher elevations. We collected surface samples of biocrust in sterile plastic petri dishes 15 cm in diameter, which were then stored in darkness inside envelopes until processing. The samples were dried for preservation and deposited in the collection of Biological Crusts of the Phycological Herbarium of Science Faculty (FCME), housed in the Universidad Nacional Autónoma de México (UNAM) in México City. A subsample of each soil sample was broken up by grinding in a mortar with a pestle, and then dried crushed soil was applied directly to agar plates containing BG11 medium (Allen 1968). When colonies began to grow, they were removed to clean agar plates and allowed to grow. In this way, several strains were isolated from the different samples collected, four in the locality of the Mezquital Valley (686, 701, 702, 708) and three for Atexcac (716, 718, 719). After checking for contaminants, uncontaminated cultures were transferred to liquid media to achieve sufficient biomass for DNA extraction and sequencing. Strains were grown in a culture room at 28°C with continuous light of 20–50 μE. The strains were cryopreserved with 15% glycerol and incorporated into the collection housed in the Universidad Autónoma de Madrid (UAM, Spain).

Morphological characterization:—Morphological variability was studied in live material. In both the natural populations and the cultured strains the species were analyzed based on morphological and morphometric characteristics, particularly filament and trichome width, sheath morphology and coloration, cell color, cell and apical cell length, presence of granules, and false branching.

The observations were carried out on an Olympus BX 51 photomicroscope equipped with Nomarski DIC (differential interferential contrast) and a DP 12 digital camera, or an Olympus microscope CX 41 and DP 20 digital camera. We used the Sigma scan program to get the species dimensions. The characteristics of our strains were compared to the information in Komárek & Anagnostidis (2005), Dvořák *et al*. (2017), and Song *et al*. (2015).

Molecular characterization:—DNA of each strain was isolated from unialgal cultures using a combination of mechanical and freeze-thaw cycles for rupturing cell walls (Palinska *et al*. 2006). A subsample of each culture was pulverized with 150 ml of PowerBead solution of Kit UltraClean Microbial DNA Isolation Kit (MO-BIO Labs, Carlsbad, CA, USA) in a 0.5 ml Eppendorf vial. The slurry was homogenized and subsequently exposed to five freeze–thaw cycles, alternating immersion in liquid nitrogen and heating to 60 ºC, followed by further mechanical rupture of the sample using a pestle. The 16S rRNA gene with 16S-23S ITS region were amplified by polymerase chain reaction (PCR) using primer 27 Forward (Wilmotte *et al*. 1993) and primer 23S reverse (Lepére *et al*. 2000). The amplification reaction volume was 25 µl and contained 1 or 2 µl DNA, 200µM dNTP, 1µg bovine serum albumin, 10 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 µl 10x polymerase buffer, 5 µl 5x Eppendorf Taqmaster PCR enhancer and 0.75 U Ultratools DNA polymerase (Biotools). The amplified products were run in a Perkin Elmer Gene Amp PCR System 2400 Thermocycler, using the PCR cycle described by Gkelis *et al*. (2005). All PCR products were visualized on a 1% agarose gel and the amplification products were purified with Wizard® SV Gel and PCR Clean-Up System, Promega. The purification products were cloned onto the pGEM®-T Easy Vector plasmid of the pGEM®-T Easy Vector System I, Promega. The clones were isolated using Wizard® Plus SV Minipreps DNA purification System, Promega. Plasmids containing inserts were submitted for bidirectional sequencing using primers T7, SP6 and 684 forward (Mateo *et al.* 2011) to Centro Nacional de Investigación Oncológica (CNIO) in Madrid, Spain. Fragments were assembled using the software PhyDE-1 v0.9971 (Müller *et al.* 2010). The sequences obtained were deposited in the NCBI GenBank database with the accession numbers: Mezquital Valley MF68583–MF685888 and Atexcac MF685889–MF685894.

Alignment and phylogenetic analysis:—The sequences obtained were compared with sequence information available in the National Center for Biotechnology Information (NCBI) database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and the most related sequences were selected. Additional sequences from GenBank were selected based on morphological classification criteria, such that sequences from several Synechococcalean families could be represented, including many of the recently described genera in the order. The taxa used in the 16S rRNA gene phylogenetic analyses included a total of 278 OTUs with *Gloeobacter violaceus* Rippka, J.B. Waterbury & Cohen-Bazire (1974: 436) as outgroup. The multiple-sequence alignment was performed using ClustalW (Thompson *et al*. 1994) and the alignment was later visually checked and corrected using PhyDE-1 v0.9971 (Müller *et al*. 2010). Percent identity among strains based on both the 16S rRNA gene sequence and ITS sequence were calculated using p-distance as derived with the SHOWDIST command in PAUP 4.0 beta version 10.

Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP) analyses were performed using partial 16S rRNA gene sequences containing a maximum of 1065 characters including nucleotides and indels. Bayesian inference was conducted with MrBayes XSDE V3.2.6 (Ronquist *et al.* 2012) through the CIPRES Science Gateway, applying the GTR+G+I model of nucleotide substitutions. A total of 28 million generations were run. The BI analysis had an estimated sample size (ESS) exceeding 300 for all parameters (average ESS ranging 652–13,923), well above the average of 200 typically accepted as sufficient by phylogeneticists (Drummond *et al*. 2006). The final average standard deviation of split frequencies was <0.009. The potential scale reduction factor (PSRF) value for all the estimated parameters in the BI analysis was 1.00, indicating that convergence of the MCMC chains was statistically achieved (Gelman & Rubin 1992). The ML analysis with rapid bootstrapping was conducted using RAxML-HPC v.8 on XSDE V8.2.10 (Stamatakis 2014), also through the CIPRES Science Gateway, applying the GTR+G+ I model of nucleotide substitutions, with 1000 bootstrap iterations. MP analysis was conducted in PAUP version 4.0 beta version 10, with a heuristic search using nreps = 1000, swap = TBR, steepest = no, multrees = no, with 1000 bootstrap iterations subsequently run. Bootstrap values for both the ML and MP analyses were mapped on to the BI analysis tree, the phylogenies figured in this publication. The full uncompressed tree is shown in supplemental materials.

The hypothetical secondary structures of conserved domains in the 16S-23S ITS region, including D1-D1', Box B and V3 helices, were derived using M-fold (Zuker 2003) and re-drawn in Adobe Illustrator. Separate structures for operons with two tRNA genes and no tRNA genes were determined. ITS structures of other close taxa were determined and reported for comparative purposes.

Results

Chroakolemma Becerra-Absalón & Johansen, *gen*. *nov*.

Diagnosis:—Genetically most similar to *Scytolyngbya,* from which it differs in the coloration and width in the sheath material and in the presence of coiled filaments. Morphologically similar to *Chamaethrix*, from which it differs by the obligately blackish coloration of the sheaths. It also differs notably in the secondary structure of the conserved domains of the 16S-23S ITS region and is phylogenetically distinct based on 16S rRNA gene sequence.

Description:—Solitary or intricated filaments, entangled with other filamentous cyanobacterial species forming biocrusts or biofilms on soil surfaces. In culture on agar-solidified media, filaments forming thin and compact biofilms, intricated, sometimes coiled, with rare single false branching. Sheaths firm, firstly thin and colorless, soon becoming thick and colored, open at the end, with one trichome per sheath. Trichomes isopolar, fine, cylindrical, sometimes tapering at ends, straight or coiled, constricted at the crosswalls. Cells isodiametric or longer than wide, with homogenous content, without aerotopes, with rare granules, pale blue-green. Apical cell conical or rounded. Reproduction by hormogonia, produced both by simple fragmentation and through formation of necridial cells.

Type species:—*Chroakolemma opaca* Becerra-Absalón & Johansen.

Occurrence:—All three species currently in this genus occur in soils.

Etymology:—*Chroiakos* (Gr.) = colored; lemma (Gr.) = sheath, referring to the distinctive colored sheath.

Chroakolemma opaca Becerra-Absalón & Johansen, *sp*. *nov*. (Fig. 1, a–j)

Diagnosis:—This species is morphologically similar to *C. pellucida* but differs in its possession of an opaque sheath, which obscures visibility of the trichome, wider trichomes, and longer cells. It differs from *C. edaphica* in presence the blackish sheath, the longer cells, and occurrence in drier habitat (semi-desert).

Description:—Solitary or intricated filaments entangled with other filamentous cyanobacteria species forming biocrust. In culture the thallus is a thin and compact biofilm. Filaments intricated, sometimes coiled, 2.1–5.8 µm wide, with rare single false branching, with hormogonia often developing from the false branches that break but remain attached by sheath material to the parent filament. Sheaths firm, firstly thin and colorless, soon becoming thick, blackish, opaque, obscuring the trichome, open at the ends, with one trichome per sheath. Trichomes isopolar, fine, cylindrical, sometimes tapering at ends, constricted at cross walls, 1–3.8 µm wide. Cells distinctly longer than wide, sometimes isodiametric, pale blue-green, with homogenous content, lacking aerotopes, rarely granulated, 1.6–6.9 µm long. Apical cell conical, sometimes rounded. Reproduction by hormogonia, produced both by simple fragmentation and through formation of necridial cells.

Habitat:—Aerophytic, forming surface biocrust with other species from semi-desert soils.

Type Locality:—Ampliación La Peña, in Mezquital Valley, Hidalgo.

Holotype here designated:—MEXICO. Hidalgo state, Ampliación La Peña, in Mezquital Valley, elevation 2010 m A.S.L., 20° 16’ 02.9” N, 98° 54’ 57.5” W, collected 17 October 2014. *Collector number* FCME CB18!, Dry environmental soil sample deposited in Bio-crust collection from the herbarium of Facultad de Ciencias, FCME, UNAM. Also, in part, reference strain 701 preserved in 4% formaldehyde deposited in FCME.

Reference Strain:—701, Laboratorio de Ficología, Departamento de Biología Comparada, UNAM.

Etymology:—*opaca* (L): opaque, referring to the opaque mature sheath which obscures visibility of the trichome.

Chroakolemma pellucida Becerra-Absalón & Johansen, *sp*. *nov*. (Fig. 1, k–u)

Diagnosis:—Similar to *C. opaca*, but with sheath transparent even when mature and colored, with more frequent false branching, and shorter cells. Secondary structure of the Box-B and V3 helices of the ITS region also different between the two species.

Description:—Solitary or intricated filaments entangled with other filamentous cyanobacteria species forming biocrust. In culture the thallus is a thin and compact biofilm. Filaments intricated, sometimes coiled, 2.7–5.7 µm wide, with rare single false branching, with hormogonia often developing from the false branches that break but remain attached by sheath material to the parent filament. Sheaths firm, firstly thin and colorless, later thick, blackish, but translucent, frequently open at the ends, with one trichome per sheath. Trichomes isopolar, fine, cylindrical, constricted at cross walls, 1–3.2 µm wide. Cells distinctly longer than wide, sometimes isodiametric, pale blue-green, with homogenous

content, lacking aerotopes, rarely granulated, 1.8–4.4 µm long. Apical cell cylindrical and rounded, sometimes obtusely conical. Reproduction by hormogonia, produced both by simple fragmentation and through formation of necridial cells.

Habitat:—Aerophytic, in biocrusts with other species from semi-desert soils.

Type Locality:—Outside of the cone of the volcanic lake, Atexcac, Puebla.

Holotype here designated:—MEXICO. Puebla state, outside of the cone of the volcanic lake, Atexcac, elevation 2046 m A.S.L., 19°20' 13" N, 97°21' 19" W, collected 8 March 2014. *Collector number* FCME CB1!, Dry environmental soil sample deposited in Bio-crust collection of the herbarium Facultad de Ciencias, FCME, UNAM. Also, in part, reference strain 719 preserved in 4% formaldehyde deposited in FCME.
(19°20' 13" N, 97°21' 19" W, elevation 2046 m A.S.L., collected 8 March 2014 and 26 Sept. 2014)

Reference Strain:—719, Laboratorio de Ficología, Departamento de Biología Comparada, UNAM.

Etymology:—*pellucida* (L): transparent, referring to the transparent nature of mature sheaths.

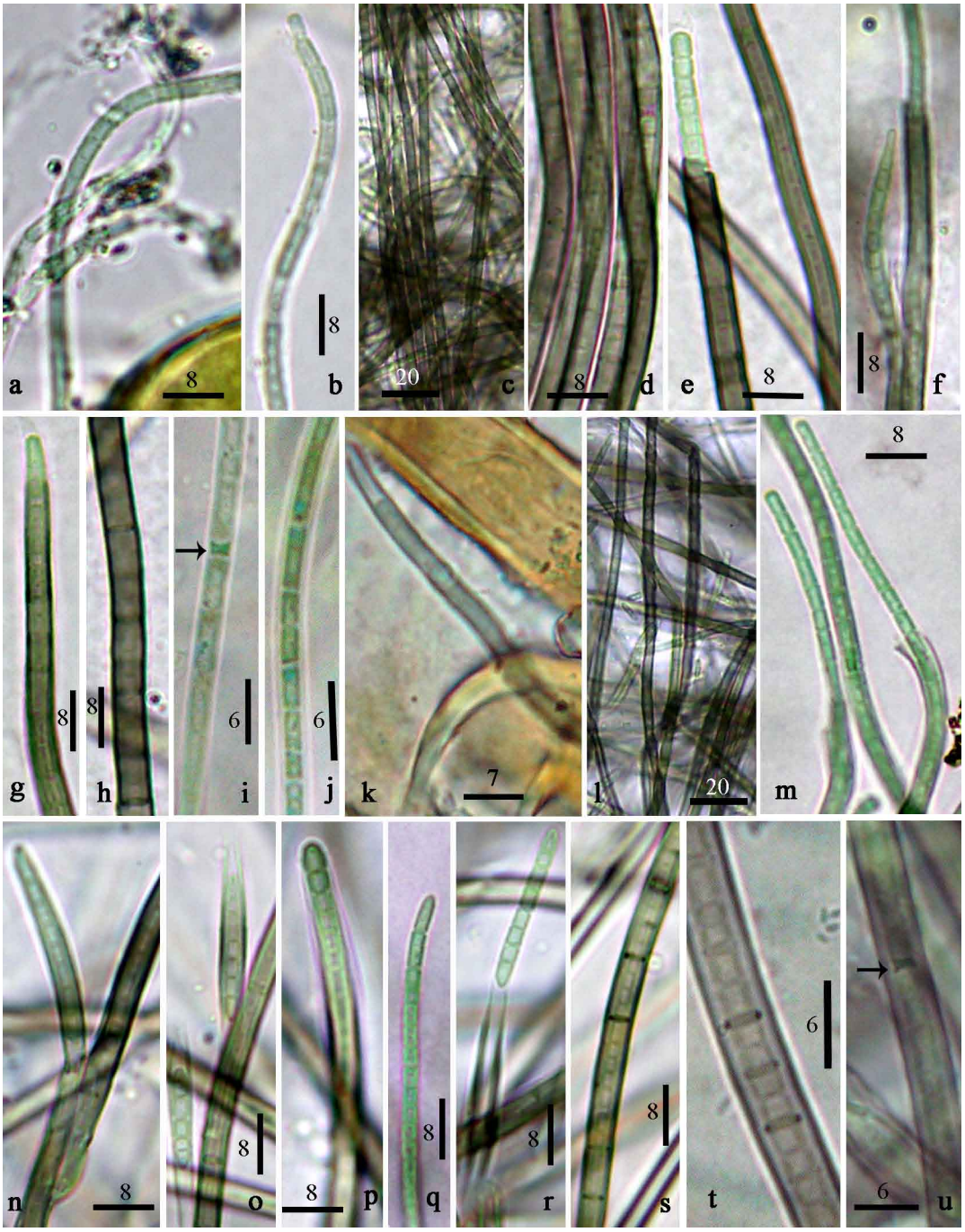

FIGURE 1. Micrographs of *Chroakolemma* spp. a–j) *Chroakolemma opaca*. a–b) from field sample; c–j) in culture; c–e) filament appearence; f) false branching; g) apical cell; h) sheath; i–j) vegetative and necridial cells (black arrow); k–u) *Chroakolemma pellucida*. k) from field sample; l–u) in culture; l–m) filament appearence; n–o) false branching; p–q) apical cell; r) hormogonium; s) sheath; t–u) vegetative and necridial cells. The number adjacent to each scale represents the length of the scale bar in µm.

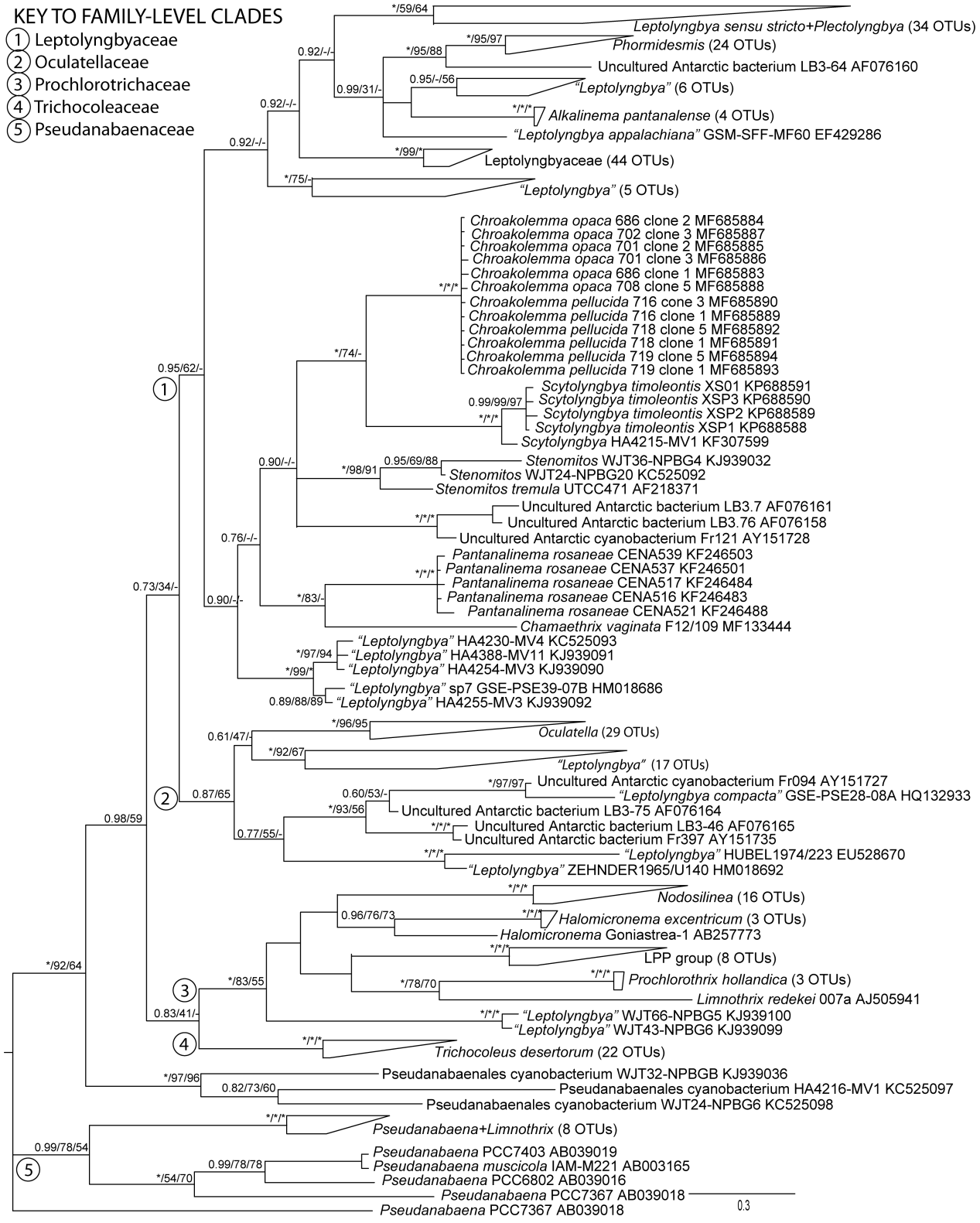

FIGURE 2. Bayesian Inference analysis of 277 Synechococcalean OTUs plus *Gloeobacter violaceus* as outgroup, based on a 1065 nucleotide alignment of partial rRNA gene sequences. Support values are BI posterior probability/ML bootstrap/MP bootstrap, with * meaning a posterior probability of 1.00 or bootstrap value of 100%, with – meaning a bootstrap support value less than 50%. Taxa names in quotation marks, e.g. *"Leptolyngbya"* are, in our opinion, incorrectly named sequences or taxa requiring revision. The type species for *Leptolyngbya*, *L. boryana*, is contained within the collapsed clade at the top of the phylogeny. We followed Mai *et al.* (2018) for designation of families. Collapsed nodes can be seen expanded in Fig. S2.

Chroakolemma edaphica (Elenkin) Becerra-Absalón & Johansen *comb. nov.*

Basionym:—*Plectonema puteale* f. *edaphica* Elenkin (1949:1782).

Synonyms:—*Lyngbya lagerheimii* f. *edaphica* Elenkin (1949: 1577), *Leptolyngbya edaphica* (Elenkin) Anagnostidis et Komárek (2001: 366).

Morphological comparison with other species:—The populations and strains studied were morphologically most similar to *Chamaethrix vaginata* Dvořák, Hašler, Pitelková, Tabáková, Casamatta & Poulíčková (2017:270). The strains of *Chroakolemma* have sheaths similar to *Chamaethrix* when they are thick and blackish. This character is obligate in *Chroakolemma*, in which mature filaments always have colored sheaths, whereas in *Chamaethrix* sheaths only sometimes are brown or blackish (Table 1). This blackish pigment differs in coloration from both gloeocapsin and scytonemin which are known to have variable expression (Storme *et al.* 2015). Another morphological difference between these two genera is that false branches in *Chroakolemma* are typically single, only being formed by the adhesion of hormogonia to the sheath of the filaments, whereas in *Chamaethrix* false branches are geminate (Table 1). *Chamaethrix* is phylogenetically distant from *Chroakolemma*, so morphological similarities are analogous characters not indicating shared ancestry. *Scytolyngbya* is the phylogenetically closest genus to *Chroakolemma* (Fig. 2). *Scytolyngbya* also possesses colored sheaths, but the sheaths have a fairly unique reddish tinged yellow-brown color and are firm and rigid (Table 1). Our strains of *Chroakolemma* also have thinner filaments than *Scytolyngbya* (Table 1).

TABLE 1. Comparison of some general and specific characteristics among the three species of *Chroakolemma, Chamaethrix vaginata* and *Scytolyngbya timoleontis* Song, Jiang & Li (2015: 74). Characteristics of previously described species were extracted from Komárek & Anagnostidis (2005), Dvořák *et al*. (2017) and Song *et al*. (2015), respectively.

	Chroakolemma opaca	Chroakolemma pellucida	*Chroakolemma edaphica*	*Chamaethrix vaginata*	*Scytolyngbya timoleontis*
Thallus	In field samples Solitary filaments, in culture a thin, dirty blue-green to blackish film	In field samples Solitary filaments, in culture a thin, dirty blue-green to blackish film	Solitary filaments or thick, compact, dirty blue-green up to blackish mats	Green, Blue-green or blackish in fine mats	Pale blue-green to yellow-brown
Filaments	Intricate, sometimes coiled	Intricate, sometimes coiled	Irregularly coiled, intricate	Long, straght, bent to undulated	Bent, entangled
False branching	Rare, single branches, from hormogonia	Frequently, single branches, from hormogonia	Very rare	Occasionally, double as in *Scytonema*	Frequently, single branches, from hormogonia. Narrower than filaments
Filament width (μm)	2.1–5.8($\bar{x}$ = 3.1)	2.7–5.7($\bar{x}$ = 4.4)	2.5–5	4.15	6–8.4 ($\bar{x}$ = 7.2)
Sheaths	At first thin, colourless, later thick, blackish, opaque	At first thin, colourless, later thick, blackish, transparent	At first thin, colourless, later thick, slightly to intensely brownish or blackish	Colorless, firm, thin or thick seldom intensely violet to dark violet, brownish to black	At first thin, colourless, later yellow-brown, widened and lamellated
Color of trichomes	Pale blue-green	Pale blue-green	Pale blue-green	Blue-green	Not apparent
Trichome width (μm)	1–3.8($\bar{x}$ = 2)	1–3.2($\bar{x}$ = 1.7)	1.7–2.5 (3)	2.8	1.9–2.6 ($\bar{x}$ = 2.3)
Crosswalls	Constricted	Constricted	Constricted	Unconstricted or slightly constricted	Constricted

TABLE 1. (Continued)

	Chroakolemma opaca	Chroakolemma pellucida	*Chroakolemma edaphica*	*Chamaethrix vaginata*	*Scytolyngbya timoleontis*
Cell form	Distinctly longer than wide, non-granular	Isodiametric to longer than wide, non-granular	Isodiametric or distinctly longer than wide	Longer than wide, often granulated	Longer than wide, cylindrical, non-granular
Cell length (μm)	1.6–6.9($\bar{x}$ = 3)	1.8–4.4($\bar{x}$ = 3)	(.9)1.5–3	3.74	3.5–10.8(32.1), $\bar{x}$ = 6.6
Occurrence	Aerophytic, formed biocrust with other species from semidesert soils	Aerophytic, formed biocrust with other species from semidesert soils	Soil or subaerophytic species, from Rusian soils also from frescoes in Roman hypogea	Soil crust	Epilithic, in a water treatment facility, with Fe and Mn enrichment

Chroakolemma opaca, *C. pellucida*, and *C. edaphica* had similar characteristics, such as the blackish sheath, coiled filaments, pale blue-green trichomes and constricted crosswalls, but they differ in appearance of the sheath, width of filaments and trichomes, and cell length (Table 1). The diagnostic difference between *C. opaca* and *C. pellucida* is that in first the sheath is opaque, while in second it is translucent.

C. opaca had longer cells and wider trichomes than the other species. *C. edaphica* had the smallest measurements in all characteristics, including width of filaments and trichomes and length of cells (Table 1).

Molecular Evidence for Taxonomic Classification:—Phylogenetic analysis showed *Chroakolemma* to be sister to *Scytolyngbya* and a previously unidentified species from Hawaii that likely belongs in *Scytolyngbya* (Fig. 2). Both *Chroakolemma* and *Scytolyngbya* belong to a large number of Leptolyngbyaceae separate from the clade containing *Oculatella* and its relatives and the clade containing *Nodosilinea* and its relatives.

The genetic identity among *Chroakolemma*, *Scytolyngbya*, *Stenomitos, Chamaethrix* and *Pantanalinema* is less than 95%, suggesting that *Chroakolemma* is evolutionarily distant from all the phylogenetically closest taxa (Table 2).

TABLE 2. Percent similarity of strains based upon p-distance analysis of the 16S rRNA gene sequence data (percent similarity = 100 × (1-p)). *Chroakolemma* comparisons are in bold font. Sequences followed by B were from operons with both tRNA genes, those followed by N were from operons lacking tRNA genes.

	1	2	3	4	5	6	7	8	9	10	11
1. *Chroakolemma opaca* 701B											
2. *Chroakolemma opaca* 701N	99.9										
3. *Chroakolemma pellucida* 719B	**100**	**99.9**									
4. *Chroakolemma pellucida* 719N	**100**	**99.9**	**100**								
5. *Scytolyngbya timoleontis* B	94.8	94.7	94.8	94.8							
6. *Pantanalinema rosaneae* N	94.1	94.0	94.1	94.1	93.5						
7. *Stenomitos rutilans* B	94.2	94.2	94.2	94.2	93.6	94.3					
8. *Chamaethrix vaginata* B	93.7	93.6	93.7	92.5	92.7	90.8	90.0				
9. *Phormidesmis priestleyi* B	93.3	93.2	93.3	93.3	92.4	91.2	92.5	91.9			
10. *Alkalinema pantalense* B	93.5	93.4	93.5	93.5	91.6	91.8	92.0	93.7	93.0		
11. *Oculatella subterranea* B	92.3	92.2	92.3	92.3	92.8	92.9	92.6	89.9	90.3	91.0	
12. *Trichocoleus desertorum* B	91.9	91.8	91.9	91.9	92.1	93.4	92.1	89.9	90.4	90.1	91.4

Chroakolemma has two distinct ribosomal operons, one with both tRNA genes and the other lacking tRNA genes, an arrangement found in most Leptolyngbyaceae. The D1-D1' helix differs between operons of *C. opaca* and *C. pellucida,* but is almost identical between species within operon type (Fig. 3 A–D). *Chroakolemma* is distinctly different in the D1-D1' helix structure from all closely related Leptolyngbyaceae (Fig. 3 E–H). The Box-B helices in *Chroakolemma* resembled those in other Leptolyngbyaceae in the basal part of the helix, but differed in length, sequence, and structure of the terminal loop (Fig. 3, I–P). Furthermore, the Box-B helix in the Mezquital population (*C. opaca*) was the same in both operons, whereas they differed between operons in the Atexcac population (*C. pellucida*). All three Box-B helices observed in *Chroakolemma* were distinctly different (Fig. 3, I–K).

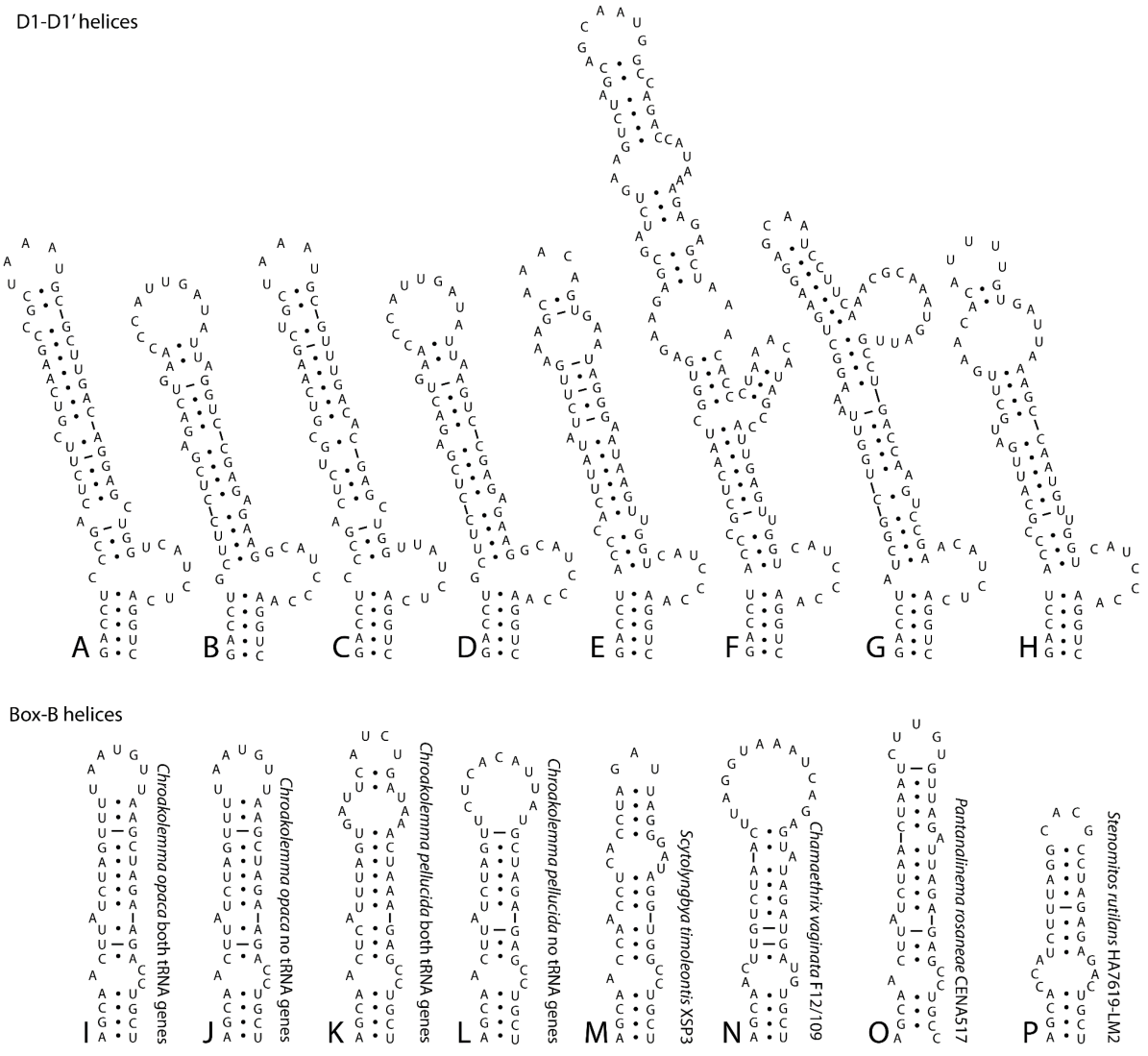

FIGURE 3. Hypothetical secondary structures for two semi-conserved domains in the 16S-23 ITS region in *Chroakolemma* species and phylogenetically closest taxa. Strain labels to right of Box-B helices apply to both structures in a vertical column. A–H) D1-D1' helices. I–J) Box-B helices.

Most of the V3 helices in selected Leptolyngbyaceae were similar in their basal regions, but soon diverged in the main part of the helix, such that all were different except for the two species of *Chroakolemma* (Fig. 4 A–E). The two species of *Chroakolemma* did not vary based on operon (operons within species had identical V3 sequence), and differed by only one nucleotide substitution in the terminal loop, but this substitution did not affect secondary structure (Fig. 5A).

The length of conserved domains in the 16S-23S ITS also contributed evidence that *Chroakolemma* was a separate evolutionary lineage from other Leptolyngbyaceae (Table 3). The following recognizable domains had consistent lengths within both operons of both species that differed from the domain lengths for all other close genera: leader (8 nt), D1-D1' helix (61 nt), D4+spacer (24 nt), V3 (38 nt), and spacer+D5+spacer (25 nt). In those operons with both

tRNA genes, the V2 helix (20 nt) and spacer preceding the Box-B helix (45 nt) were similarly distinct (Table 3). The two species of *Chroakolemma* were highly similar in lengths of conserved domains, only differing in the Box-B helices for the operons with no tRNA genes.

The final evidence for the separation of *C. opaca* from *C. pellucida* lies in the dissimilarity between homologous ITS sequences of the two species. We had several strains of both species, and within both taxa percent dissimilarities were 0.00–1.75% when comparisons were made within homolgous operons, but between the two species the percent dissimilarities were >7.5% for operons with both tRNA genes and >8.5% for operons lacking tRNA genes. When separate phylogenetic analyses were run on the two ITS alignments, the two species clades showed reciprocal monophyly (Fig. S1), a criterion that Erwin and Thacker (2008) considered significant in their decision to recognize cryptic species in *Synechococcus spongiarum* Usher, Toze, Fromont, Kuo et Sutton (2004: 183).

TABLE 3. Nucleotide lengths for conserved domains in the 16S-23S ITS region of *Chroakolemma* spp., closest relatives (*Scytolyngbya, Pantanalinema, Stenomitos,* and *Chamaethrix*), and more distantly related Leptolyngbyaceae. For operons lacking tRNA genes, the D3 to spacer preceding the Box B helix forms a single domain.

Strain	Leader	D1-D1' helix	Spacer+D2+Spacer	D3+spacer	tRNA ^{Ile} gene	Spacer+V2+spacer	rRNA^{Ala} gene	Spacer	Box-B helix	Spacer	Box A	D4+spacer	V3 helix	Spacer+D5+Spacer
Chroakolemma opaca Both	8	61	34	12	74	20	73	45	41	15	11	24	38	25
Chroakolemma pellucida Both	8	61	34	12	74	20	73	45	41	18	11	24	38	25
Chroakolemma opaca None	8	61	36	132					41	15	11	24	38	25
Chroakolemma pellucida None	8	61	36	132					44	18	11	24	38	25
Scytolyngbya timoleontis Both	7	64	42	11	74	24	73	44	40	15	11	30	66	51
Panatanalinema rosaneae 1	6	82	36	137					44	15	23	23	39	28
Panatanalinema rosaneae 2	6	81	34	135					44	15	11	23	40	28
Stenomitos rutilans	7	65	34	12	74	9	73	51	34	17	11	29	66	30
Phormidesmis WJT66-NPBG16	7	73	40	12	74	25	73	47	48	17	11	29	46	39
Alkalinema pantanalense	7	64	39	17	74	46	73	37	48	18	11	28	32	29
Oculatella subterránea	7	62	37	11	74	91	73	37	34	15	11	32	39	21
Trichocoleus desertorum	7	62	41	13	74	24	73	14	36	17	11	23	47	28

Discussion

Chroakolemma is a monophyletic taxon diagnosable from the other members of Leptolyngbyaceae. In all phylogenetic analyses, the *Chroakolemma* clade was strongly supported (Fig. 2, Fig. S2). *Chroakolemma* is sister to *Scytolyngbya*, a relationship with strong support in the BA and ML analyses (Fig. 2). Given this close relationship, the question that must be asked is: Should our species be placed in a new genus or in the existing genus *Scytolyngbya*? The p-distance analysis of the 16S rRNA gene to all close relatives shows that it is less than 95% identical to all other genera (Table 2), strong evidence that it is a distinct genus from all of these genera. In other taxonomic studies of cyanobacteria (Nübel *et al*. 2000, Abed *et al*. 2002, Fiore *et al*. 2007, Řeháková *et al* 2007, Perkerson *et al*. 2010, Siegesmund *et al*. 2008) new cyanobacterial genera were erected based on similar evidence.

We also analyzed secondary structure of the 16S-23S ITS region, which recently has been suggested as a character useful for α-level taxonomy (Boyer *et al*. 2001, Johansen & Casamatta 2005, Siegesmund *et al*. 2008, Johansen *et al*. 2011, Osorio-Santos *et al*. 2014). The length of conserved domains in the 16S-23S ITS and structure of the D1-D1', Box-B, and V3 helices in *Chroakolemma* were distinctly different from the four phylogenetically closest genera (*Scytolyngbya*, *Stenomitos, Chamaethrix,* and *Pantanalinema*) in the Leptolyngbyaceae (Figs. 3, 4). These differences also provide evidence that *Chroakolemma* is an evolutionary lineage separate from other Leptolyngbyaceae, including *Scytolyngbya.*

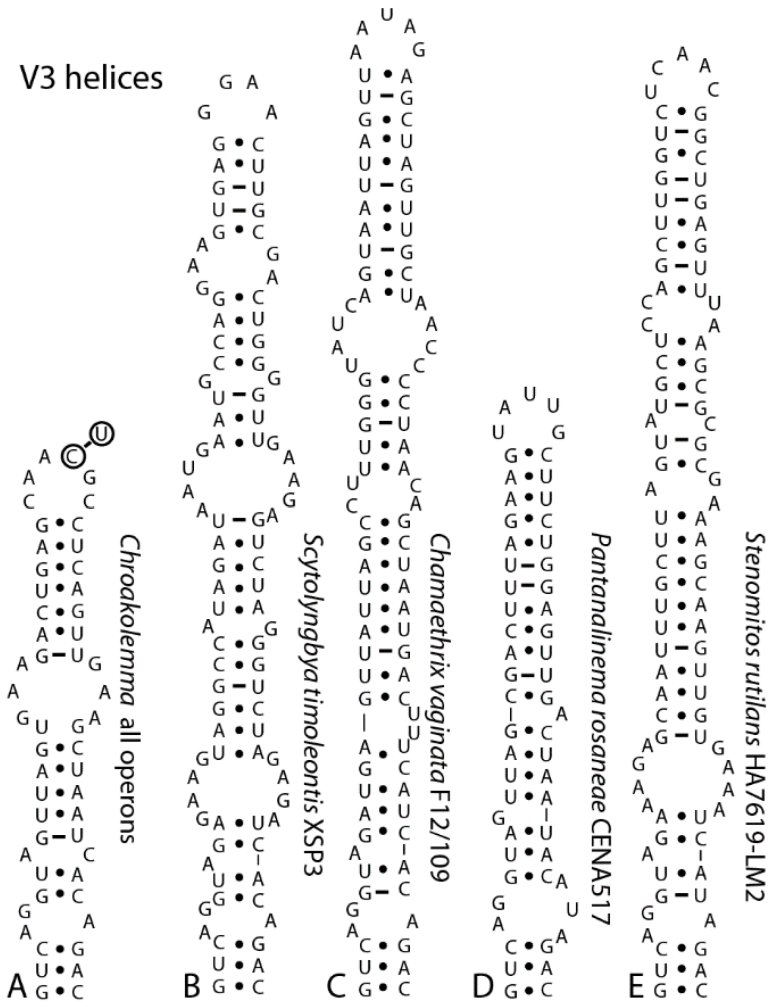

FIGURE 4. Hypothetical secondary structures of V3 helices in the 16S-23 ITS region in *Chroakolemma* species and phylogenetically closest taxa. Strain labels are to the right of the V3 helices.

In addition to the molecular evidence, our *Chroakolemma* strains presented morphological characteristics recognizable both in field and culture populations, such as the presence of wide blackish sheaths, coiled filaments, pale blue-green trichomes, and constricted cross-walls. These characteristics appear to be diagnostic for the genus, and separate the species in the genus from species in the phylogenetically close genera *Scytolyngbya, Chamaethrix, Pantanalinema,* and *Stenomitos*. The very distantly related taxon, *Trichocoleus badius* (Johansen & Lowe in Johansen *et al.* 2008: 26) Mühlsteinová, Johansen & Pietrasiak (2014: 248), possesses blackish sheaths and irregularly coiled and bent filaments, but has much more irregular sheaths and is ecologically distinct as well (Johansen *et al*. 2008).

Schizothrix heufleri Grunow ex Gomont (1892: 325) also is capable of producing blackish blue-green to blackish violet sheaths and has cell dimensions having overlap with *Chroakolemma* species. Its ecology is quite different, being subaeorophytic on wet rocks and in waterfalls in temperate climates. Unforunately, neither the type species of *Schizothrix, S. fuscescens* Kützing ex Gomont (1892 324), nor *S. heufleri* have yet been sequenced. *Schizothrix* is a heterogenous genus (Komárek & Anagnostidis 2005), and therefore we are very reluctant to consider *Chroakolemma* to be a species complex within *Schizothrix* in the absence of phylogenetic evidence. Furthermore, *Chroakolemma* has open sheaths in contrast to the closed sheaths of *Schizothrix*. It is possible that if *S. heufleri* is sequenced and found to be phylogenetically similar to *Chroakolemma* that it will be transferred in the future into *Chroakolemma*. We consider it unlikely that *S. fuscescens* and *S. heufleri* are in the same genus. *S. arenaria* Gomont (1892: 312) has been sequenced, and it is very distant from *Chroakolemma* (<88% gene identity based on the 16S rRNA gene sequence).

Therefore, we conclude that all the evidence analyzed in this study indicates that our strains belong to a new genus, which we call *Chroakolemma*. The strains from the two localities show minor morphological differences, such as the opaqueness of the sheath and longer cells in *C. opaca.* The phylogenetic analysis based on the 16S rRNA gene sequence could not resolve the two species (Fig. 2), an observation made in the description of other cryptic or semi-cryptic species

(Perkerson *et al*. 2011, Osorio-Santos *et al*. 2014, Johansen *et al*. 2014). The primary evidence for recognition of the two Mexican species of *Chroakolemma* lies in the high sequence dissimilarity of the ITS region sequence between homologous operons. As a rule, strains/sequences within the same species will have percent dissimilarities averaging ~1.0% and in all pairwise comparisons be less than ~3.0%, while different species will have percent dissimilarities >4.0% and demonstrate reciprocal monophyly (Erwin & Thacker 2008). We had percent dissimilarities of >7.5% in the operons with both tRNA genes, and >8.5% for operons lacking tRNA genes. Later workers have confirmed this criterion (Osorio-Santos *et al*. 2014, Pietrasiak *et al*. 2014, Johansen *et al.* 2017, Shalygin *et al.* 2017, Vázquez-Martínez *et al.* 2018, González-Resendiz *et al.* 2018, Mai *et al.* 2018) but specify that different populations >7.0% have strong evidence for being separate species, but when ranges fall between 4–7%, supporting evidence is needed to recognize separate species. A clear discontinuity between percent dissimilarity of within species and between species is needed, combined with reciprocal monophyly. Our species of *Chroakolemma* meet all these requirements. Based on the combined morphological and molecular evidence, we conclude that populations represented by our isolates from two different geographic areas (Mezquital Valley and Atexcac) are different species.

Both species are in the same type of semi-desert environment and with similar climates, and thus do not appear to be separated by habitat. The localities from which our species originated belong to the same desert system (Chihuahuan Desert), and the distance between them is relatively little, approximately 256 kilometers. As soil species, one would expect these desiccation-resistant taxa to disperse fairly easily by wind. The collection localities are separated by a cordillera that crosses the center of Mexico called the *Eje Neovolcanico Transversal* or Trans-Mexican Volcanic Belt. This mountain range represents 20 million years of volcanic activity and presently forms a transverse barrier that runs east to west between the Pacific and Atlantic Oceans. The cordillera acts as a high elevation barrier between the Mezquital Valley to the north and Atexac Valley to the south. Pine-oak forests occur throughout the range. This forested area lacks biological soil crusts and forms a barrier to dispersal of plant and animal species. Atexcac occurs in the southern portion of the Chihuahuan Desert, known as the Tehuacan zone. The Tehuacan is home to many endemic species of plants and animals across the tree of life (Rzedowski, 2006). It is not surprising that cyanobacterial endemism also occurs.

Chroakolemma opaca and *C. pellucida* are new species. Only one previously described species with similar characteristics appears in the literature: *Leptolyngbya edaphica* (Komárek & Anagnostidis 2005). This species differs in the width of filaments and trichomes and the length of cells, but possesses the blackish sheath and was described from soils. It also bears a morphological resemblance to *Chamaethrix vaginata, Schizothrix heufleri,* and *Trichocoleus badius*, but these species have different ecological preferences (tropical soils that never freeze for *Chroakolemma*, and wet rocks in temperate climate for the rest). The combination of morphological and ecological criteria suggest that *L. edaphica* belongs to *Chroakolemma*, and we have acted on that evidence in creating a new combination for this unsequenced species. According to Komárek & Anagnostidis (2005), *C. edaphica* (as *L. edaphica*) has been reported from very different habitats, including aerophytic environments such as soils in temperate places (Russia) and frescoes in Roman hypogea, as well as in the neuston of aquatic environments. It is possible that *C. edaphica* as reported in the literature is a complex of different species in different genera, but only further collection and sequencing of this taxon will clarify its taxonomic status and biogeographic distribution.

Conclusion

In the last 15 years there has been extensive taxonomic discovery within the simple filamentous forms in the Synechococcales. This is the 19th new genus in this group to be published, and we know of plans by several others to soon publish an additional nine genera not mentioned in our account. Consequently, the filamentous Synechococcalean taxa are becoming one of the most well established groups of cyanobacterial genera, with all of the recent taxa being characterized using a polyphasic approach. This is somewhat surprising given the paucity of morphological characters in the order. These taxa are morphologically simple, but genetically very divergent, and we expect that continued discovery of genera will occur in the near future. The description of new genera also demonstrates the need for more fine-scaled taxonomy. In several instances, once genera are named, new species within these genera are subsequently discovered. This is demonstrated by *Oculatella*, which now has a total of 10 species (Zammit *et al*. in 2012, Osorio-Santos *et al*. 2014, Miscoe *et al*. 2016, Vinogradova *et al*. 2017), *Nodosilinea,* which now has five species (Perkerson *et al.* 2011, Vázquez-Martínez 2018), and *Scytolyngbya* and *Plectolyngbya*, both of which have undescribed species as evidenced in our phylogenetic analysis (see Fig. 2, Fig. S2). All of the *"Leptolyngbya"* sequences in our extended

phylogeny outside of *Leptolyngbya sensu stricto* represent as-yet undescribed genera and species (Fig. S2), and the need for taxonomic work in this interesting and divergent order has certainly not been met, but will provide years of opportunity for algal taxonomists.

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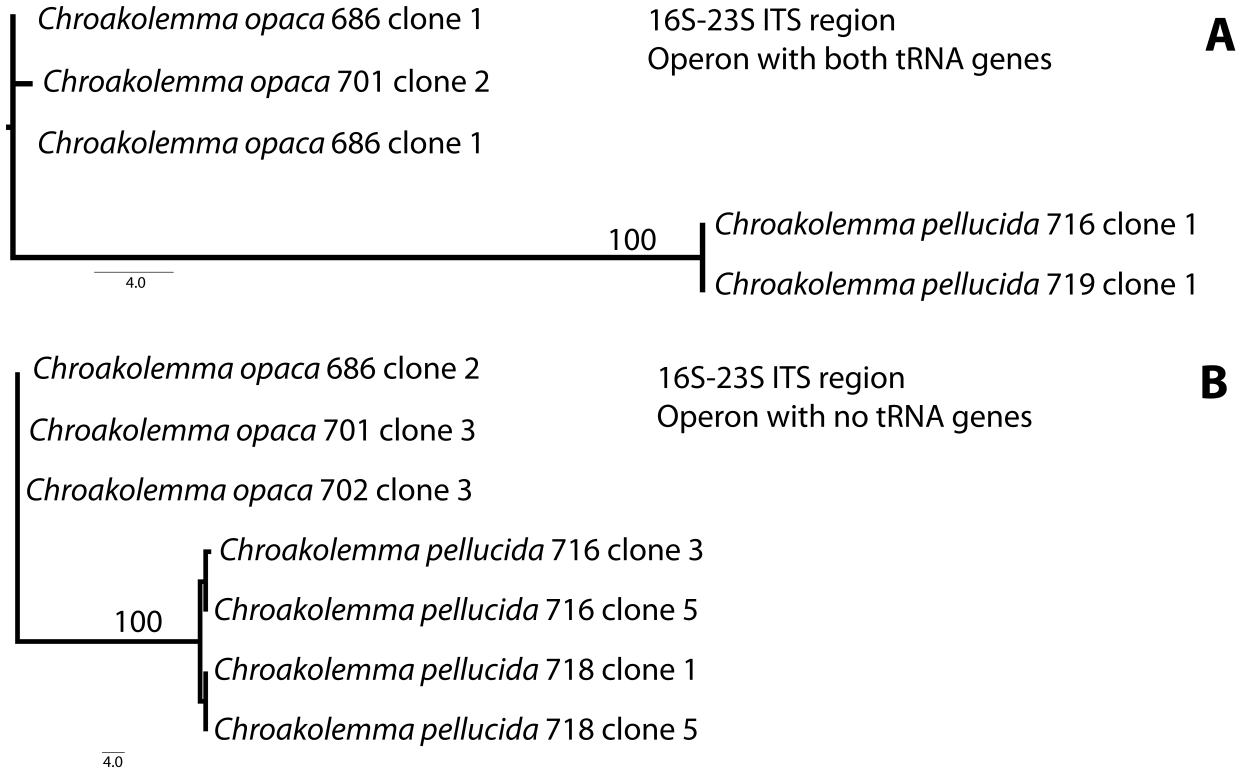

FIGURE S1. Maximum parsimony analysis of the 16S-23S ITS regions. Analysis conducted in PAUP with multrees=yes, swap=TBR, steepest=no, nreps=10,000, bootstraps with 10,000 nreps, indels counted as fifth base. A. Operon with both $tRNA^{Ile}$ and $tRNA^{Ala}$. B. Operon with no tRNA genes.

FIGURE S2. Bayesian Inference analysis of 277 Synechococcalean OTUs plus *Gloeobacter violaceus* as outgroup, based on a 1065 nucleotide alignment of partial rRNA gene sequences. Support values are BI posterior probability/ML bootstrap/MP bootstrap, with * meaning a posterior probability of 1.00 or bootstrap value of 100%, with – meaning a bootstrap support value less than 50%. Taxa names in quotation marks, e.g. *"Leptolyngbya"* are, in our opinion, incorrectly named sequences or taxa requiring revision. We followed Mai *et al.* (2018) for designation of family-level clades.