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Description of *Stenomitos kolaenensis* and *S. hiloensis sp. nov.* (Leptolyngbyaceae, Cyanobacteria) with an emendation of the genus

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Abstract

Stenomitos is a recently established cyanobacterial genus, some species of which appear to be cryptic. Here we describe two new species in this genus, *Stenomitos kolaensis sp. nov.* isolated from the Al-Fe humic podzols of a boreal forest near Nikel town, Murmansk region, Russia and *S. hiloensis sp. nov.* isolated from a basaltic seep wall on Akeola Road, Hilo, Hawaii, USA. Phylogenetic analyses were conducted on the 16S and 16S-23S ITS rRNA gene regions using Bayesian Inference, and Maximum Likelihood. Phylogenetic analysis of the 16S-23S ITS rRNA region resulted in both *S. kolaensis* and *S. hiloensis* forming separate clades from other *Stenomito*s lineages. Antarctic strains of *Stenomitos frigidus* (previously reported as *"Leptolyngbya frigida"*) show that species to be polyphyletic and in need of revision. The structure of the conserved ITS regions (Box-B, D1-D1′, V2 and V3 helices) provided support for separation of the species, and the p-distances among aligned ITS regions further confirmed that a number of species exist within the genus. *S. kolaensis* and *S. hiloensis* can be distinguished from other described *Stenomitos* species (*S. rutilans* and *S. tremulus*) by their geographical distribution, habitat preference, 16S rRNA phylogeny, and differences in the secondary structure of the 16S-23S ITS region.

Key words: Cyanobacterial taxonomy, Leptolyngbyaceae, 16S rRNA phylogeny, 16S-23S ITS phylogeny, rRNA secondary structure, Hawaii, Russia

Introduction

Extensive recent progress has been made in cyanobacterial taxonomy through the application of 16S rRNA sequencing techniques since the early 2000's (Mai *et al*. 2018). In the case of simple filamentous taxa from the family Leptolyngbyaceae (Synechococcales), this progress has been recently augmented through the separation of three families from Leptolyngbyaceae *sensu lato*: Oculatellaceae, Prochlorotrichaceae, and Trichocoleusaceae (Guiry *et al*. 2018, Mai *et al*. 2018). The generitype of *Leptolyngbya* Anagnostidis & Komárek (1988: 390), *L. boryana* (Gomont) Anagnostidis & Komárek, (1988: 391) was sequenced, allowing definitive characterization of the genus (Johansen *et al*. 2008), and was used to demonstrate the utility of using the 16S-23S ITS secondary structures to define species (Johansen *et al.* 2011). The more narrowly defined Leptolyngbyaceae now includes 15 recently described genera and even more new species (Johansen *et al*. 2008, 2011; Turicchia *et al*. 2009; Taton *et al*. 2011; Dvořák *et al*. 2014, 2015, 2017; Song *et al*. 2015; Vaz *et al*. 2015; Li & Li 2016; Miscoe *et al*. 2016; Jahodářová *et al*. 2017a; Becerra-Absalón *et al*. 2018; Pietrasiak *et al*. 2019; Soares *et al*. 2019; Raabová *et al*. 2019). The other families in the Synechococcales also host many (17 at present) new genera of simple filamentous cyanobacteria (Zammit *et al*. 2012; Sciuto & Moro 2016; Brito *et al*. 2017; Jahodářová *et al*. 2017b; Sciuto *et al*. 2017; Zimba *et al*. 2017; Cellamare *et al*. 2018; Genuário *et al*.

2018); Mai *et al*. 2018; Heidari *et al*. 2018; Zammit 2018; Akagha *et al*. 2019; Chakraborty *et al*. 2019; Konstantinou *et al*. 2019). We anticipate that as many as 50 genera of the simple filamentous cyanobacteria will be described within the Synechococcales in the near future.

Description of cryptic and semi-cryptic genera have been seen in the other well-established clades across the Cyanobacterial phylum. These cases have resulted in the establishment of genera such as *Oxynema* Chatchawan *et al*. (2012: 50) and *Kamptonema* Strunecký *et al*. (2014: 203), *Dapis* Engene, Tronholm & Paul (2018: 443) and *Microseira* (Farlow ex Gomont) McGregor & Sendall (2015: 113), and the newly established cryptic genus *Odorella* Shalygin *et al*. (2019: 511). Based on these instances, we presume that many morphological species within presently unsequenced *Leptolyngbya sensu lato* have the potential to be described as separate genera when molecular techniques are employed. When describing unknown taxa via molecular methods, it is critical to compare their morphology against that of previously described species and genera as these taxa may have already been described morphologically based on the botanical approach. If this can be accomplished it will give a deeper understanding of their evolutionary history and will allow for the incorporation of important morphological and ecological traits into future phylogenetic models.

Cyanophyte research in Russia has a long and rich history (Davydov & Patova 2017). One of the ground-breaking works was Alexander Elenkin's monograph (Elenkin 1947). Similar to Lothar Geitler's work (Geitler 1932), Elenkin discussed important questions related to the biogeography, evolution, and species concepts in Cyanophytes within territories of the former Soviet Union (Elenkin 1947). In 1953 Elenkin's student, Maximilian M. Hollerbakh, published an exhaustive taxonomic key for the Cyanophyta, containing new and updated information collected since Elenkin published his monograph (Hollerbakh 1953). Hollerbakh's key has served as the primary literature for many generations of phycologists in Russia and the former soviet Republics. Language and availability barriers have prevented the distribution of the Russian Cyanophyte literature into Europe and the Americas. For many researchers, Russia remains a "blank spot" on the map. Several groups of Russian phycologists have conducted floristic, taxonomic, and ecological research on individual regions within Russia (Patova *et al*. 2017, Davydov 2018, Shalygin 2012, Shalygina *et al*. 2016, Gaysina & Bohunická 2018). For example, *Hormoscilla pringsheimii* Anagnostidis & Komárek (1988: 425) (Bohunická *et al*. 2015a) and *Roholtiella bashkiriorum* Gaysina & Bohunická in Bohunická *et al*. (2015b: 90) were isolated and characterized using the modern polyphasic approach and phylogenetic species concepts (Johansen & Casamatta 2005). These findings indicate that the Russian cyanobacterial flora is likely much more diverse than previously thought.

In an effort to deepen the knowledge of Russian Cyanophytes, we have collected, isolated, and characterized a simple filamentous taxon from the family Leptolyngbyaceae (Synechococcales). This taxon appears to be a new species of the recently described genus *Stenomitos* Miscoe & J.R. Johansen in Miscoe *et al*. (2016: 84). The present paper describes *Stenomitos kolaensis sp. nov.* based on morphological and ecological differences, and phylogenetic analysis of the 16S rRNA gene and associated 16S-23S ITS region. Morphological characterization of *S. kolaensis* required the emendation of the generic description for *Stenomitos.*

There have been several recent studies using a modern, polyphasic approach to study cyanobacteria present in the Hawaiian Islands, all part of the project initiated by Sherwood *et al*. (2014, 2015). Vaccarino and Johansen described two new heterocytous tapering species from subaerial habitats, *Scytonematopsis contorta* Vaccarino & Johansen (2011: 151–152) and *Brasilonema angustatum* Vaccarino & Johansen (2012: 1181). *Komarekiella atlantica* Hentschke, Johansen & Sant'Anna in Hentschke *et al*. (2017: 180–182) was simultaneously reported from Brazil and Kauai when first described. *Fortiea laiensis* Vaccarino & Johansen in Hauer *et al*. (2014: 1096) was sequenced, characterized, and described from a waterfall on Oahu. *Pleurocapsa fuliginosa* Hauck (1885: 515), the type species of the genus, was neotypified based on a Hawaiian isolate. A putative *Cylindrospermum* Kützing ex Bornet et Flahault (1886 (7): 249) species from Hawaii that falls outside of the *Cylindrospermum* clade and is consequently likely in a new genus was sequenced, characterized, and reported in a paper dealing with that *Cylindrospermum* (Johansen *et al*. 2014). Finally, Miscoe *et al*. (2016) reported 20 cyanobacterial species including 12 new species and four new genera, including *Stenomitos rutilans* Miscoe & Johansen in Miscoe *et al*. (2016: 85). These works demonstrate that the Hawaiian cyanobacterial flora likely contains many species and genera new to science. While undertaking the work on *S. kolaensis*, we discovered a new *Stenomitos* species among our Hawaiian isolates, differing from the generitype both in morphology and molecular phylogeny. This recently characterized strain is also treated in this manuscript, and described as *Stenomitos hiloensis sp. nov*.

Materials and Methods

Strain isolation and morphology:—Culture of *Stenomitos kolaensis* were isolated from the soils collected near Nikel town (69.3691° N, 29.899° E), sampled on 15 June 2014. Culture is stored in the private collection of Vera Redkina and Regina Shalygina (INEP). The soils present in the sampling site consisted of Al-Fe humic podzols with a pH 5.22 developed on moraine, sandy sediments. Vegetation was primarily composed of shrubs, pine trees and moss-lichen communities. Natural populations of *Stenomitos hiloensis* were isolated from a seep wall on Akeola Road in Hilo on the Big Island of Hawaii in the Hawaiian Islands (19.7035° N, 155.136° W), sampled on 22 May 2010. Hilo has a tropical rainforest climate, never experiencing freezing temperatures. In both instances, cultures were isolated into Z8 media following standard techniques (Carmichael 1986). Morphological characters were described from unialgal cultures using a Zeiss Axioscope (Oberkochen. Germany) microscope equipped with Nomarski DIC optics. Morphometric measurements were taken using AxioVision 4.8 (Oberkochen. Germany).

Molecular methods:—Genomic DNA was isolated using a DNeasy UltraClean Microbial Kit (Cat ID: 12224-50, QIAGEN, Venlo Netherlands). The partial 16S rRNA gene and associated 16S-23S ITS region was amplified as described in Osorio-Santos *et al*. (2014). Obtained amplicons were cloned into the pSC- A-amp/kan plasmid of the StrataClone PCR Cloning kit (La Jolla, California, USA). All plasmids were extracted using the QIAprep Spin Miniprep Kit (Cat ID: 27104, QIAGEN, Venlo Netherlands) and sent for Sanger sequencing at Functional Biosciences (Madison, WI, USA).

Phylogenetic analyses:—Two identical copies of the 16S rRNA gene and associated 16S-23S ITS region from *Stenomitos kolaensis* and four identical copies of the same region for *Stenomitos hiloensis* were assembled using Sequencher 4.9 (Ann Arbor, MI, USA). A total of 187 additional 16S rRNA sequences and eight 16S-23S ITS sequences were downloaded from NCBI GenBank (Clark *et al.* 2016). Our 16S rRNA phylogeny was primarily constructed from taxa within the following families: Pseudanabaenaceae, Prochlorothricaceae, Trichocoleusaceae, Oculatellaceae, and Leptolyngbyaceae. All sequences were aligned in Sina ACT module (Pruesse *et al.* 2012) according to the secondary structure of the 16S molecule. The model for Bayesian Inference (BI) was chosen using jModeltest2 (Darriba *et al.* 2012), which gave prior configuration settings of 010123, with rates set at invgamma. The standard substitution model (GTR+I+G) was also used, and gave identical topologies in the ingroup (Leptolyngbyaceae).

Maximum Likelihood (ML) was run using a GTR+I+G model. BI analyses were performed using Mr. Bayes and the following parameters applied: eight Markov Chain Monte Carlo (MCMC) simulations run for 50 million generations (stop value = 0.01), sampled every 100 generations, and a 25% burn-in (Ronquist *et al.* 2012). ML was calculated in RaxML v.7.2.8 using 1,000 bootstrap replicates (Stamatakis *et al.* 2008). 16S rRNA p-distance were inferred using MegaX (Kumar *et al.* 2018).

Sequences of the 16S-23S ITS region of the operon containing both tRNA genes were aligned using secondary structure (8 distinct strains of *Stenomitos*), with indels coded (0 for gap, 1 for nucleotide), and an unrooted phylogenetic tree was obtained with a BI analysis with 4200 generations discarding the first 25% of samples as burn in, choosing NST=MIXED, and applying the GTR+I+G evolutionary model. Average standard deviation of split frequencies was <0.005 and the average PSRF for this analysis was 1.000. These ITS alignments were analyzed in PAUP using parsimony as the criterion, with gapmode set to newstate, steepest descent off, multrees on, and swap=TBR . We utilized 10,000 nreps for both the heuristic search and the bootstrap analysis. The BI analysis was run using the CIPRES Science Gateway V. 3.3 (Miller *et al.* 2015) with Xsede (Towns *et al.* 2014). The MP analysis was run using PAUP 4.0b10, and bootstrap values were mapped on to the BI tree. P-distances were also determined using the SHOWDIST command in PAUP 4.0b10 (Swofford 2002). Secondary structures of the ITS domains were determined using the program Mfold *Ver. 3.1* (Zuker 2003), and prepared for publication using Adobe Illustrator CS 5.1.

Graphical design:—All figures were created using Photoshop/Illustrator CC (Adobe System Inc., SanJose, CA, USA). Line drawings of *Stenomitos kolaensis* and *S. hiloensis* were made in Photoshop CC using a Wacom Intuos PRO tablet pen tablet (Wacom Europe GmbH, Düsseldorf, Germany).

Results

The original description of *Stenomitos* does not circumscribe the two new species in sheath characteristics, the number of trichomes in a common sheath, the possible formation of necridia, and the existence of isodiametric cells. We emend the description of the genus as follows to accommodate *S. kolaensis* and *S. hiloensis*. *Stenomitos* Miscoe et Johansen (2016) emend.

Filaments without false branching, less than 2.5 μm wide. Sheaths thin, in some species firm, persistent, and swollen, sometimes containing two or rarely more trichomes. Trichomes short to long, with necridia present or absent, evidently constricted in some species. Cells longer than wide, occasionally isodiametric, with parietal thylakoids. Apical cells cylindrical, rounded, or conical. Polar granules forming in stationary growth phase.

***Stenomitos kolaensis* Shalygin, Shalygina et Johansen sp. nov.** (Figs. 1, 3 A,B,C)

Macrocolonies in form of thin, blue-green film growing on agar surface. Filaments long, occasionally coiled or slightly waved, seldom containing two trichomes, with or without sheath, 1.8–2 (4.5) μm wide. Mucilaginous sheaths colorless, soft, hyaline to firm and persistent beyond the apex of the trichome, rarely slightly swollen, clearly visible as wider than the trichome, in older stages mostly firm. Trichomes distinctly constricted at the cross-walls, granulated mostly in the older stages, 1.5–2 μm wide. Cells bright blue-green when young, pale green when old, from isodiametric to elongate, 1.5–2.5 (4) μm long, rarely with polar granules. Apical cells rounded to slightly conical, elongated up to 4.5 μm long. Thylakoids peripheral, necridia present.

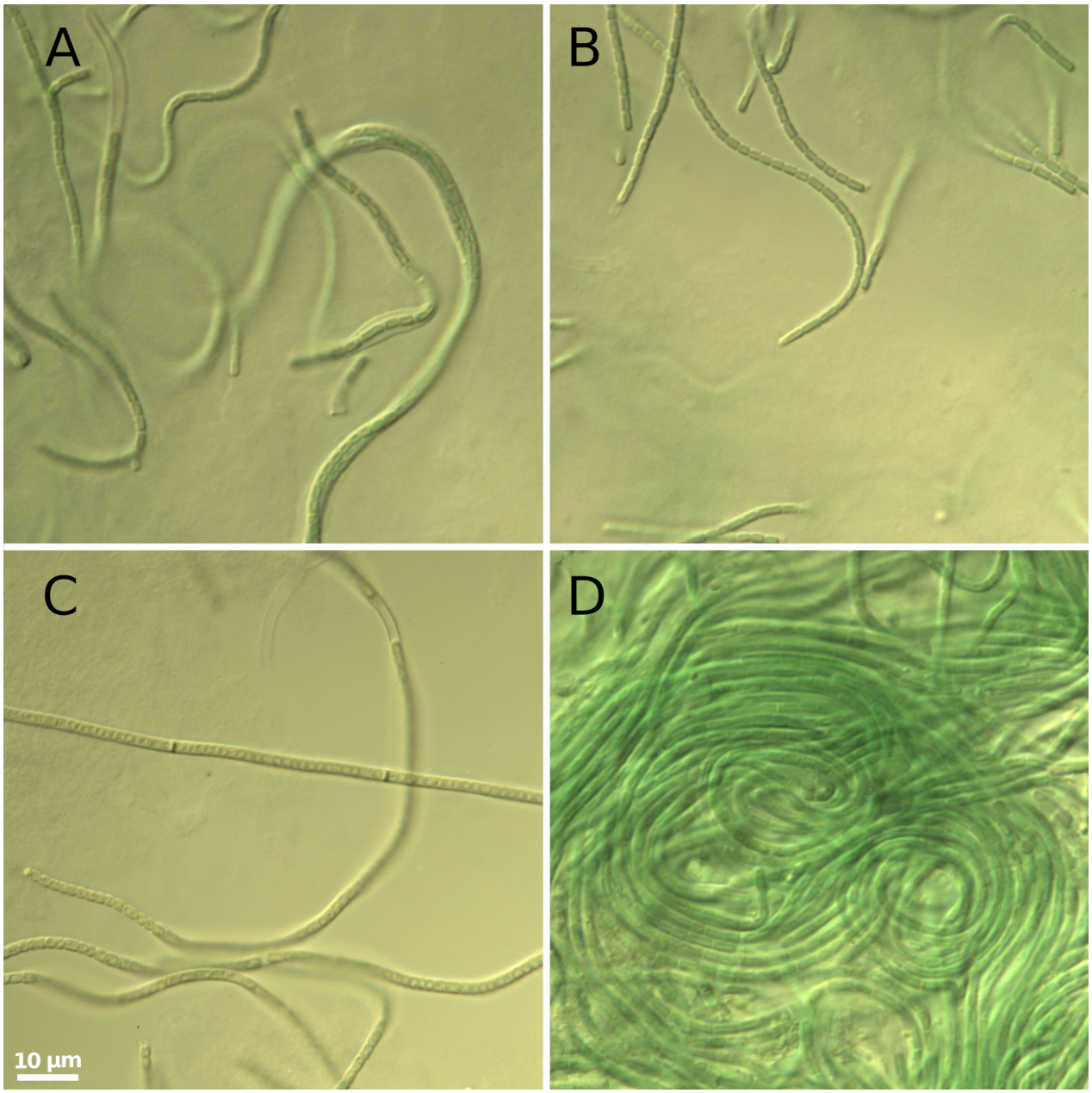

FIGURE 1. Light micrographs of *S. kolaensis*. A. Filaments showing sheath and two trichomes sharing a common sheath. B. Trichomes free of sheath, showing clear constrictions at crosswalls and cells slightly longer than wide. C. Trichome with cells isodiametric to shorter than wide. D. Entangled trichomes. All photos at same magnification, scale = 10 μm.

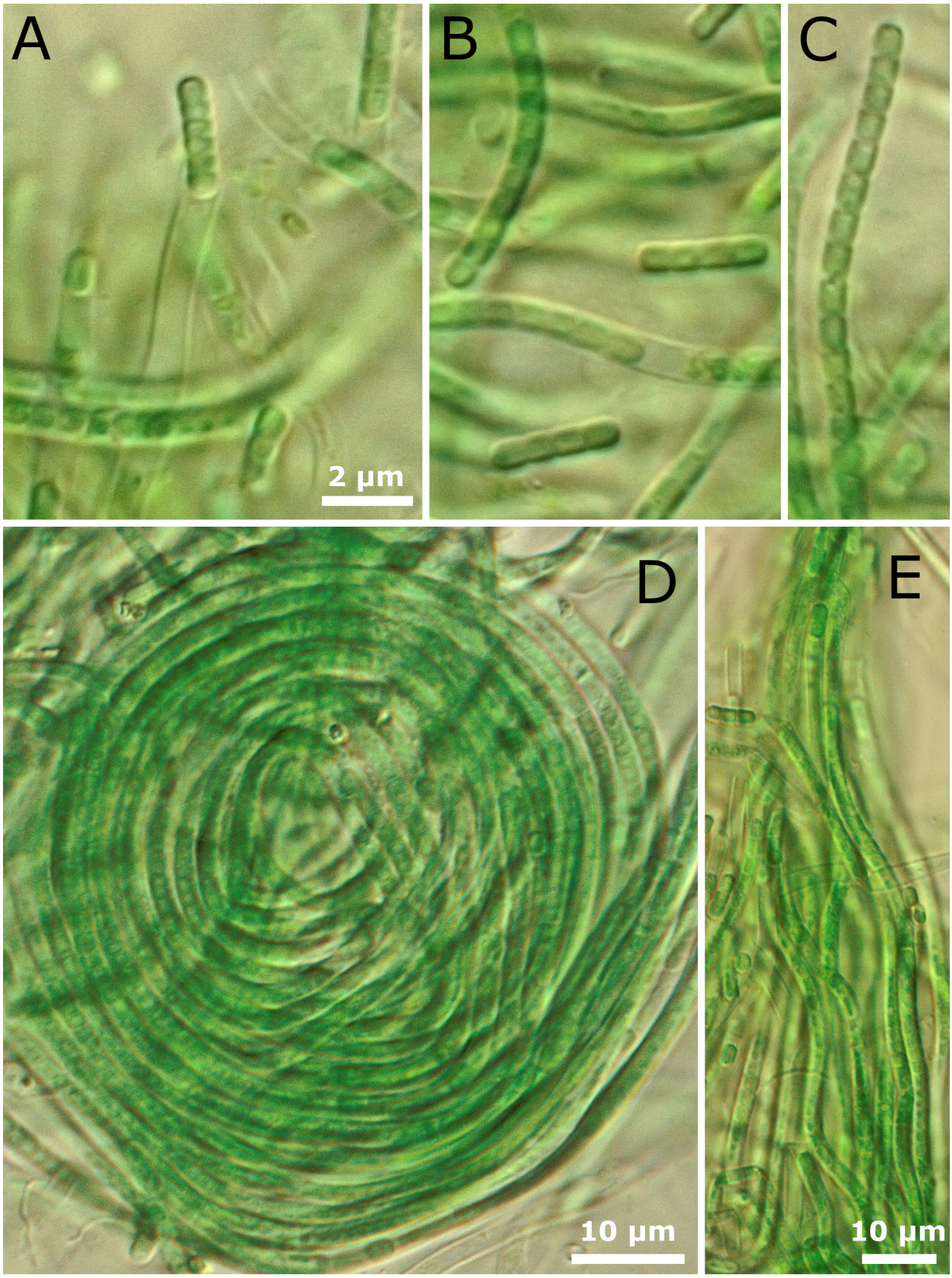

FIGURE 2. Light micrographs of *S. hiloensis*. A, B. Trichomes producing hormogonia and with thin, firm sheaths. C. Trichome lacking sheath, showing clear constrictions at the crosswalls. D, E. Entangled trichomes.

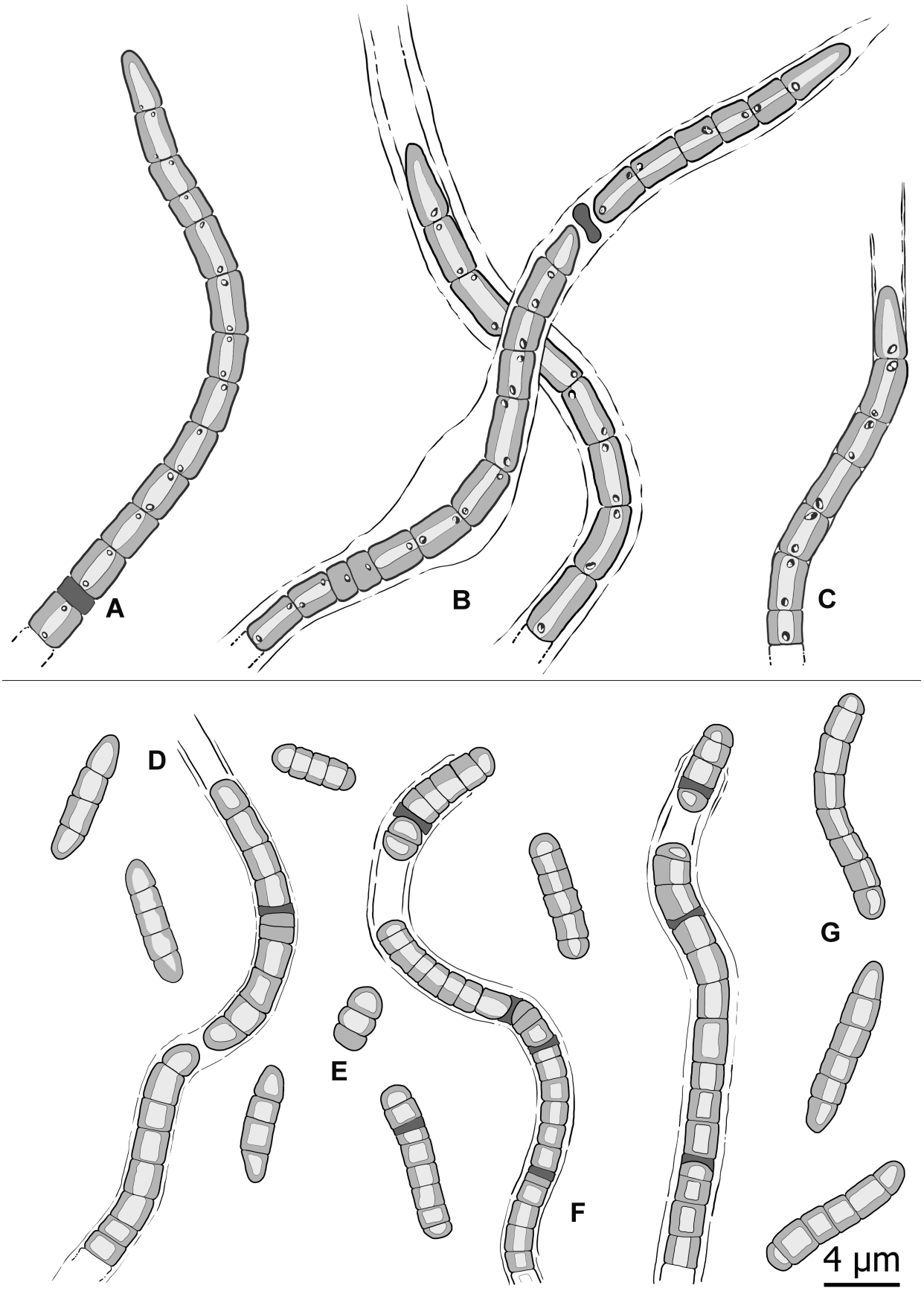

FIGURE 3. Line drawings of *Stenomitos* taxa. A-C. *Stenomitos kolaensis*. D-G. *Stenomitos hiloensis*.

Holotype here designated: dried specimen deposited into herbarium of Polar-Alpine Botanical Garden-Institute, Kirovsk, Murmansk Province, Russia, under following accession number: KPABG(C):4182.

Type locality: Five km north-west from Nikel town (69.3691° N, 29.899° E), Al-Fe humic podzol soil in a young boreal forest composed of coniferous and deciduous trees, Kola Peninsula, Russia, sampled 15 July 2014 by Regina Shalygina.

Etymology: named for its locality – Kola Peninsula, Russia.

NCBI GenBank accession number: KU175690.

***Stenomitos hiloensis* Johansen, Gargass et Shalygin sp. nov.** (Figs. 2, 3D-G)

Filaments long to short, arranged in parallel or spiral, with or without sheaths, 2 μm wide. Sheath firm, colorless, thin, usually visible only during or following hormogonia formation. Trichomes isopolar, unbranched, untapered, clearly constricted near the transverse cell walls, 0.7–1.8 μm wide. Cells isodiametric, blue-green, not granulated, 0.5–1.2 μm long. Apical cells rounded, same size as regular cells. Hormogonia short, 2-8 celled usually with appressed cells. Necridia frequent. Thylakoids parietal.

Holotype here designated: dried specimen deposited into herbarium of Bernice Pauahi Bishop Museum, Honolulu, Hawaii, USA under following accession number: BISH 776187.

Type locality: Seep wall on Akeola Road (19.7035°N, 155.136°W) in the tropical rain forest, Hilo, Hawaii, sampled 22 May 2010 by Rex Lowe, Pat Kociolek and Melissa Vaccarino.

Reference strain: HA6792-KK3 (isolated into culture by Katie Kavulic), deposited in the Cyanobacterial Culture Collection at John Carroll University.

Etymology: Pertaining to the city of Hilo.

NCBI GenBank Accession number: MN152980.

Comparison with other taxa:—Up until this manuscript, *Stenomitos* contained only three species: *S. rutilans* Miscoe *et* J.R. Johansen *in* Miscoe *et al.* (2016: 85)*, S. tremulus* (J.R. Johansen *et* Casamatta *in* Casamatta *et al*. 2005: 420) Miscoe *et* J.R. Johansen *in* Miscoe *et al.* (2016: 86), and *S. frigidus* (Fritsch 1912: 31) Miscoe *et* J.R. Johansen *in* Miscoe *et al*. (2016: 86). *Stenomitos kolaensis* and *S. rutilans* are easily distinguished under the microscope via differences in coloration: blue-green versus red-brown, respectively, as well as differences in diameter and length of the cells, and the occurrence of necridia in *S. kolaensis* (Table 1). It is unlikely that geographical distribution for the *S. kolaensis* from the near Arctic will overlap with Hawaiian taxa in tropical rainforest climate. *S. tremulus* was isolated from a pond in Bylot Island, Nunavut, Canada at a latitude very similar to the site in the Kola Peninsula from which *S. kolaensis* originates. *S. kolaensis* was isolated from soils, has wider trichomes and shorter cells than *S. tremulus*, and shares only 97.75% genetic identity based on the 16S rRNA gene; it is consequently easily separated from that species.

S. hiloensis is ecologically similar to *S. rutilans*, but shows clear phylogenetic and morphological separation from *S. rutilans*. *S. hiloensis* has blue-green cells in contrast to red-brown in *S. rutilans,* additionally filament were little bit wider. *S. hiloensis* is thinner and has shorter cells than *S. kolaensis* (Table 1.).

TABLE 1. Morphological comparison among named *Stenomitos* species. Information on previously described taxa obtained from Miscoe *et al*. (2016), Casamatta *et al*. (2005), and Komárek & Anagnostidis (2005).

Characteristics/Species	*S. kolaensis*	*S. hiloensis*	*S. rutilans*	*S. tremulus*	*S. frigidus*
Color of trichome	Blue-green	Blue-green	Red-brown	Blue-green	Blue-green
Width of the trichome	1.5–2 μm	0.7–1.8 (2) μm	0.8–1.2 μm	1.0 μm	0.8–1.2 (1.8) μm
Length of cells	1.5–2.5 (4.5) μm	0.5–1.2 μm	2.8–4.8 μm	3–7 μm	1.0–2.4 μm
Constricted at CW	+	+	–	–	+
Sheath	+	+	– (+)	+	– (+ diffluent)
Necridia	+	+	–	–	–
Locality/habitat	Russian subarctic/ soil	Hawaii/Seep wall	Hawaii/Cave wall	Northwest Territories/ benthos of pond	Antarctica/benthos of streams and lakes

The numerous Antarctic strains ascribed to *Stenomitos frigidus* (=*Leptolyngbya frigida* (Fritsch 1912: 31) Anagnostidis & Komárek (1988: 391)) represent three different species, which are clearly phylogenetically and ecologically separated from the other species in the genus and from each other. One of these is likely equivalent to Fritsch's original taxon. The CANT/CAU and WJT strains are all isolated from arid soils, and undoubtedly represent an undescribed species. A detailed study of these *"L. frigida"* strains, with morphological observations and further characterization of their ITS regions, would certainly uncover further species-level diversity in the genus. Revisiting *S. tremulus* and characterizing its ITS region would also be helpful in the revisionary work that is needed.

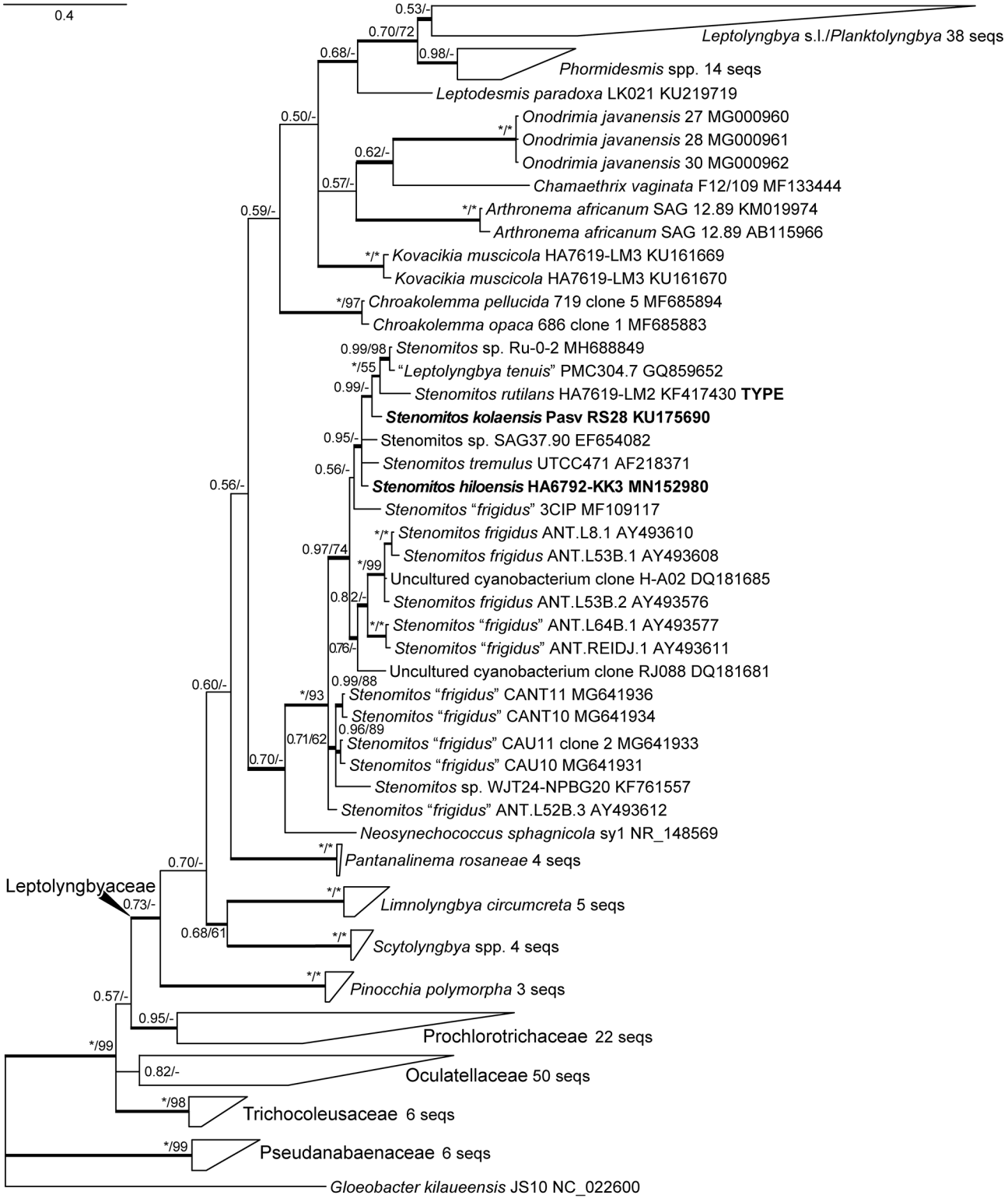

FIGURE 4. Bayesian Inference analysis based on 16S rRNA sequence data with maximum likelihood bootstrap support values mapped on to nodes. Heavy bold lines report nodes that were represented in both BI analysis and ML analysis.

Phylogeny:—All branches on the 16S rRNA phylogenetic tree clustered into five major families within Synechococcales (Fig. 4). Asterisks (*) indicate maximum support values, hyphens (-) indicate support values ≤ 50, support values are given in parentheses after each clade in the following order: BI/ML. Clades referring to the following families Pseudanabaenaceae (*/99), Prochlorothricaceae (0.95/-), and Trichocoleusaceae (*/98), had high support values, at least in the BI analysis. The clade containing the Leptolyngbyaceae was unsupported at the family level (0.73/-), possibly because of the unstable position of the *Pantanalinema*, *Pinnochia,* and *Limnolyngbya*/*Scytolyngbya* nodes. The Oculatellaceae was only weakly supported in our analyses (0.82/-). The unstable groups in the Leptolyngbyaceae did not affect the position of the genus *Stenomitos* within the family in any of our analyses. Additionally, the "AGC" triad in Helix 23 and the "A:U" pairing in the apical portion of Helix 27, both in the 16S rRNA molecule, were found in *Stenomitos,* supporting the affiliation of that genus within the Leptolyngbyaceae (see Mai *et al*. 2018). The *Stenomitos* clade was highly supported (*/93; Fig. 4). The *Stenomitos* clade consisted of three Antarctic clusters (Fig. 4: ANT and CANT labeled taxa), Mojave Desert species (Fig. 4: WJT24-NPBG20) and additional taxa from a wide range of geographic locations (Hawaii, Russia, subarctic, arid Europe). All species and strains within the *Stenomitos* clade possessed >97% genetic identity based on 16S rRNA gene sequence (Table 2), supporting recognition of a single genus. Many strains, including those assigned specific species epithets had genetic identities ≥98.7%, the proposed threshold for species recognition within prokaryotes (Yarza *et al.* 2014). The sister taxon to *Stenomitos*, *Neosynechococcus sphagnicola* Dvořák *et al*. (2014: 26), was also above the recognized generic threshold of 94.5% identity for all *Stenomitos* strains. Consequently, 16S genetic identity alone does not provide clear evidence that these the *Stenomitos* species are separate taxa, or that the genus *Stenomitos* is separate from *Neosynechococcus*. The phylogeny based on the 16S rRNA gene region showed *S. kolaensis* and *S. hiloensis* to be members of the subclade containing *S. rutilans* and *S. tremulus*. *Leptolyngbya frigida* (Fig. 4), which we designate *S. "frigidus"* due to its uncertain placement in that species is polyphyletic and appears to represent at least three different species. The ITS phylogeny indicated that the *Stenomitos* taxa for which we have ITS sequence separate into two clades. *S. kolaensis* is more closely related to *S. rutilans*, while *S. hilosensis* is in the clade containing several *S. frigidus* (Fig. 5).

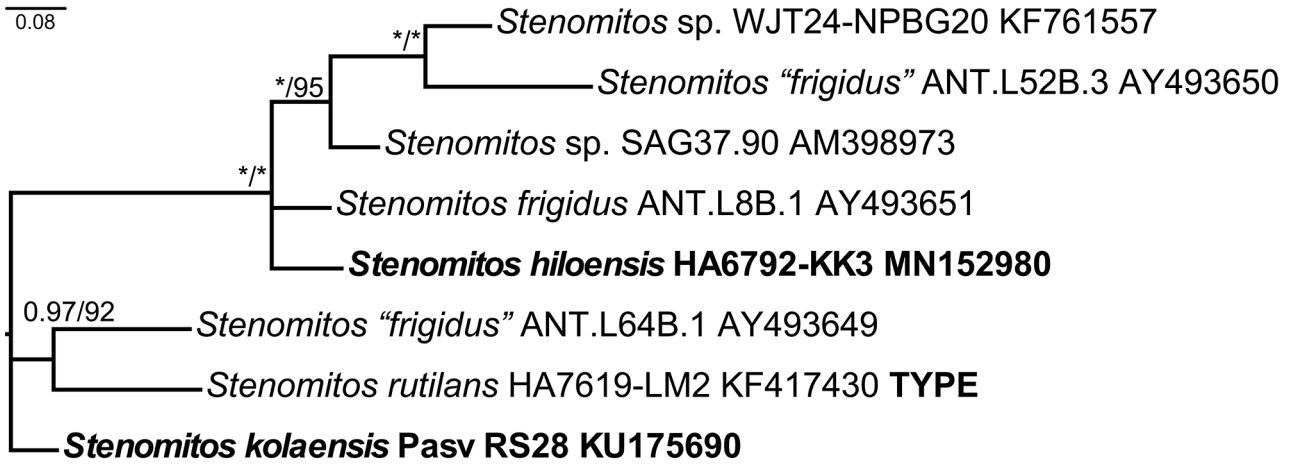

FIGURE 5. Bayesian Inference analysis based on 16S-23S ITS sequence data with maximum parsimony bootstrap values mapped on to nodes.

Secondary structures of 16S–23S ITS:— ITS analysis included a comparison of the conserved domains (D1-D1′, Box-B, V2 and V3 regions) of eight strains of *Stenomitos* (Figs 6, 7). The structure of the D1-D1′ helices was found to be highly conserved across all eight strains, with the exception of a desert soil crust strain, *Stenomitos* sp. WJT24-NPBG20, which had an altered structure in the terminus of the helix due to several nucleotide substitutions (Fig. 6 H). Although the D1-D1′ helix structure was conserved, there were significant differences in the actual sequences in this helix, particularly in the 28 nucleotides forming the terminus of the helix. No two D1-D1′ helices were identical in sequence, but some pairs differed only in a single nucleotide. The V2 helix situated between the two tRNA genes was highly divergent among strains, and consisted of either a long helix (Fig. 6 J, L-N, P) or a very short helix that may not even form (Fig. 6 I, K, O). The long helices were quite different in sequence, length, and structure. The short helices differed in sequence but not structure. No two V2 helices were identical in sequence.

TABLE 2. Genetic identity values based on 16S rRNA gene sequence for *Stenomitos* strains. For strains ≥99.7% identity, only one strain in the set is represented. These strain sets are: (ANT.L8.1+ANTL53B.1+ANTL53B.2+H-A02), (CANT10+CANT11), (CAU10+CAU11), and (ANT.REIDJ.1+ANT.L64B.1). Strain pairs with genetic identity ≤98.7% (considered clear evidence of different species) are in gray highlighting.

	S. kolaensis Pasv RS28	*S. tremulus* UTCC471	*S. hiloensis* HA6792-KK3	*S.* species SAG 37.90	*S. tenuis* PMC304-7	*S. rutilans* HA7619-LM2	*S.* species WJT24-NPBG20	*S. frigidus* ANT.L52B.3	*S. frigidus* CANT10	*S. frigidus* CAU10	*S. frigidus* ANT.L8.1	*S. frigidus* ANT.REIDJ.1	*S.*cf. *frigidus* 3CIP	Unc. *Stenomitos* RJ088
Stenomitos kolaensis Pasv RS28														
Stenomitos tremulus UTCC471	99.0													
Stenomitos hiloensis HA6792-KK3	99.2	99.3												
Stenomitos species SAG 37.90	99.1	99.0	99.2											
Stenomitos tenuis PMC304-7	99.1	98.5	99.0	99.1										
Stenomitos rutilans HA7619-LM2	98.8	98.4	98.3	98.3	98.6									
Stenomitos species WJT24-NPBG20	97.2	97.4	97.1	97.3	97.2	97.7								
Stenomitos frigidus ANT.L52B.3	98.3	98.4	98.5	98.8	98.2	97.7	98.3							
Stenomitos frigidus CANT10	98.3	98.2	98.3	98.3	98.2	97.7	98.3	99.3						
Stenomitos frigidus CAU10	98.1	98.3	98.4	98.4	98.2	97.6	98.3	99.4	99.5					
Stenomitos frigidus ANT.L8.1	98.2	98.5	98.4	98.8	98.5	98.2	97.0	98.3	98.0	98.1				
Stenomitos frigidus ANT.REIDJ.1	98.4	98.6	98.7	98.9	98.6	98.3	97.2	98.5	98.3	98.3	98.8			
Stenomitos cf. *frigidus* 3CIP	98.9	98.8	99.3	99.2	99.0	98.1	97.3	98.7	98.4	98.6	98.6	98.8		
Uncultured *Stenomitos* RJ088	98.3	98.3	98.8	98.5	98.5	97.5	96.8	98.0	97.9	97.9	98.4	98.3	98.8	
Neosynechococcus sphagnicola sy1	95.4	95.8	95.9	95.8	95.8	95.6	95.5	96.7	96.4	96.5	96.6	96.3	96.0	96.3

The Box-B helices of all *Stenomitos* strains differed in sequence. Most also differed in structure, although three were almost identical (Fig. 7 A, D, E). The basal clamp was 3 bp longer than illustrated for most other Box-B helices. As these extra bases always were able to pair in *Stenomitos* we assumed that *in vivo* they also pair, and have shown them (Fig. 7 A-H). The V3 helices displayed a high degree of divergence in terms of structure and sequence (Fig. 7 J-O). Of the examined sequences, only the basal clamp had a clear consensus sequence (5′-UGUCAGGUAGA—UCAYAGACA-3′).

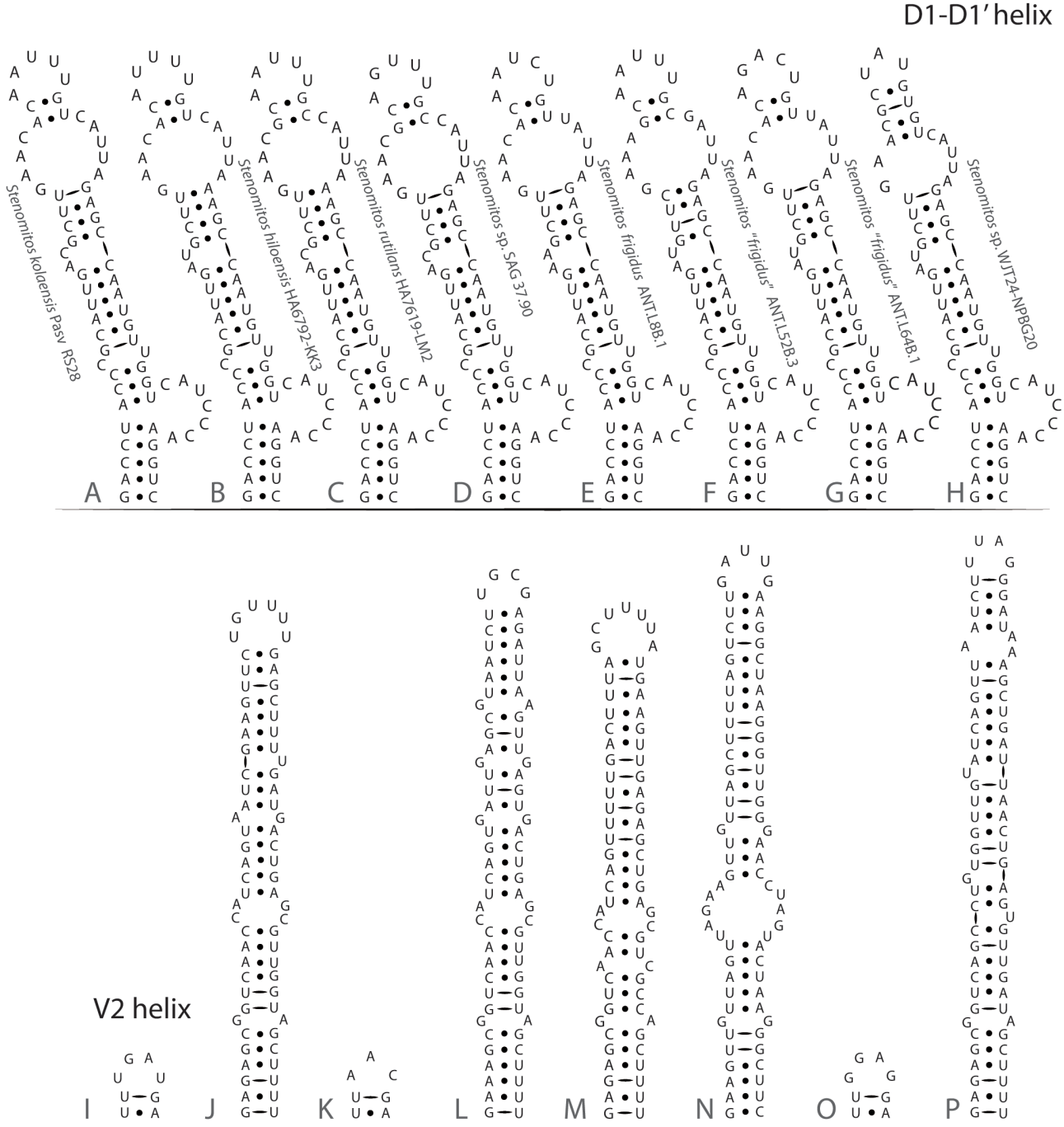

FIGURE 6. Secondary structures of conserved helices of the 16S-23S ITS region. A-H. D1-D1′ helix. I-P. V2 helix. Strain labels for A-H also apply to V2 helices in same column as D1-D1′ helices.

Both *S. kolaensis* and *S. hiloensis* have ITS sequences and structures that are distinct from each other and the other known strains of *Stenomitos*. These differences support the description of our new species. Stronger support for recognition of our species and putative new species not described in this work exists in the percent dissimilarity of the aligned 16S-23S ITS sequences for *Stenomitos* strains (Table 3). According to several studies (Erwin & Thacker 2008, Osorio-Santos *et al*. 2014, Pietrasiak *et al*. 2014 , Becerra-Absalon *et al*. 2018, González-Resendiz *et al*. 2018, Mai *et al*. 2018, Pietrasiak *et al*. 2019), greater than 7% dissimilarity in orthologous ITS regions can be considered strong evidence that strain pairs belong to separate species. Most of the *Stenomitos* strains appear to be distinct species as pairwise comparisons are >10%, with the exception of four Antarctic strains identified as *S. frigidus* that show 0.0% dissimilarity (Table 3).

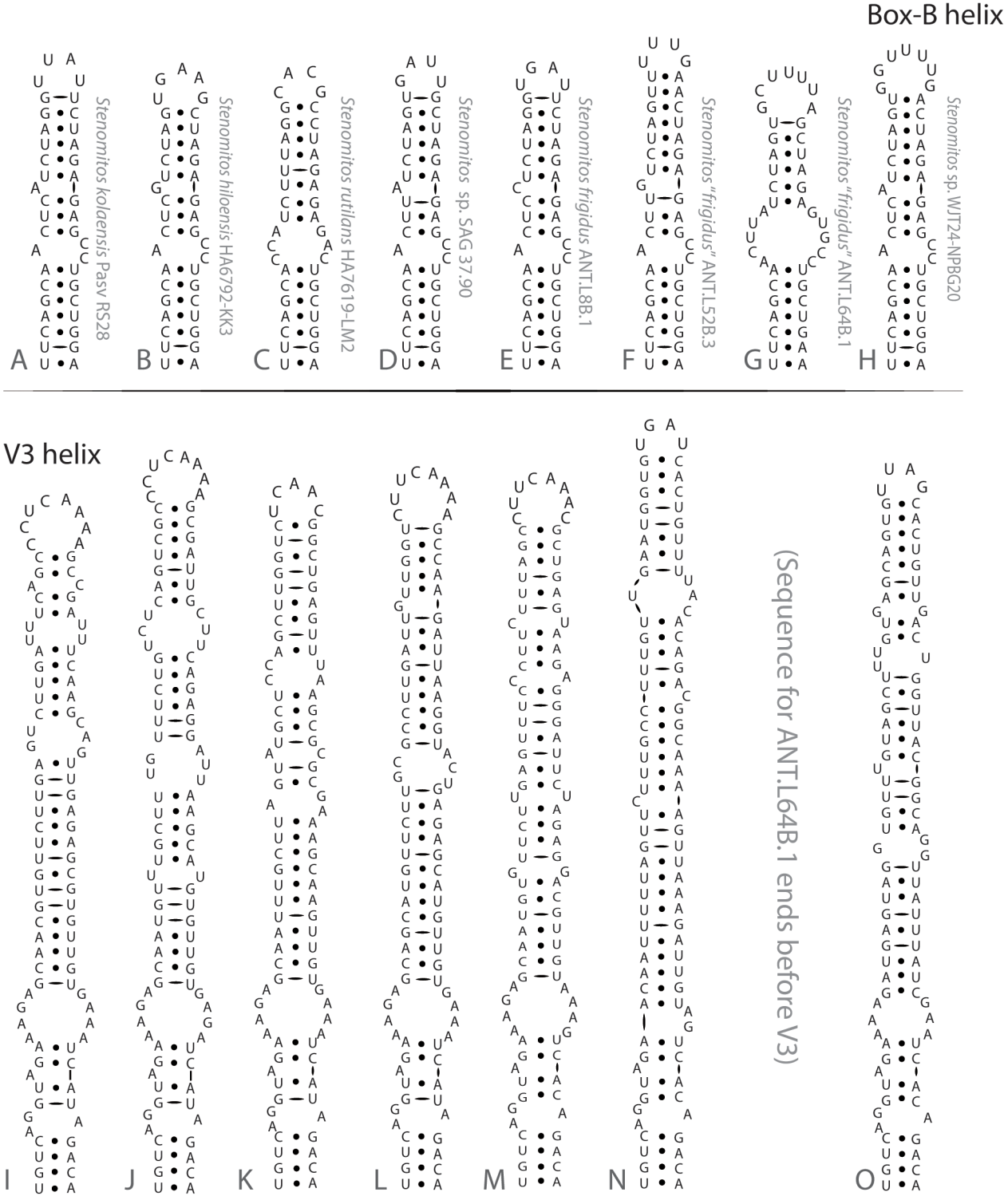

FIGURE 7. Secondary structures of conserved helices of the 16S-23S ITS region. A-H. Box-B helix. I-O. V3 helix. Strain labels for A-H also apply to V3 helices in same column as Box-B helices.

TABLE 3. Percent dissimilarity of aligned 16S-23S ITS regions (operons with two tRNA genes) for *Stenomitos* strains. Strain pairs with percent dissimilarity ≥7.0% (considered clear evidence of different species) are in gray highlighting.

	S. kolaensis Pasv RS28	*S. hiloensis* HA6792-KK3	*S. rutilans* HA7619-LM2	*S.* species SAG37.90	*S. frigidus* ANT.L8.1	*S. frigidus* ANT.L53B.1	*S. frigidus* ANT.L53B.2	*S. frigidus* ANT.L52.3	*S. "frigidus"* ANT.L52B.1	*S. "frigidus"* ANT.L64B.1
Stenomitos kolaensis Pasv RS28										
Stenomitos hiloensis HA6792-KK3	10.6									
Stenomitos rutilans HA7619-LM2	15.4	16.1								
Stenomitos sp. SAG37.90	12.7	16.8	13.3							
Stenomitos frigidus ANT.L8.1	11.1	14.9	15.7	16.7						
Stenomitos frigidus ANT.L53B.1	11.1	14.9	15.7	16.7	0					
Stenomitos frigidus ANT.L53B.2	11.1	14.9	15.7	16.7	0	0				
Stenomitos frigidus ANT.L52.3	11.1	14.9	15.7	16.7	0	0	0			
Stenomitos "frigidus" ANT.L52B.3	21.4	24.6	21.6	21.5	23.2	23.2	23.2	23.2		
Stenomitos "frigidus" ANT.L64B.1	18.6	18.8	19.8	16.6	17.9	17.9	17.9	17.9	14.6	
Stenomitos sp. WJT24-NPBG20	18.8	20.3	19.9	18.7	20.9	20.9	20.9	20.9	18.9	13.3

Discussion

Stenomitos kolaensis (Leptolyngbyaceae) was found to be morphologically more similar to *Tildeniella nuda* Mai, Johansen et Bohunická (Mai *et al*. 2018: 42) – Oculatellaceae, than to the generitype *Stenomitos rutilans,* highlighting the cryptic nature of these genera. The same morphological pattern could be recognized in *S. rutilans,* which was similar to *Pseudanabaena rosea* (Skuja 1956: 66), Anagnostidis (2001: 360) and *Drouetiella lurida* Mai *et al.* (2018: 28).

The substantial differences in morphology that are present within individual genera has already been noted in the cyanobacterial literature (Komárek *et al*. 2014, Shalygin *et al*. 2017, González- Resendiz *et al*. 2018, Mai *et al*. 2018). As an example of that, the recently described taxon *Phyllonema ansata* González-Resendiz, León-Tejera & Johansen (2018: 641) possesses isopolar, untapered filaments that are more similar to *Petalonema incrustans* Komárek (2012: 143) than to the generitype of *Phyllonema, P. avenicicola* Alvarenga *et al*. (2016: 695)*,* which possesses heteropolar tapering filaments (Alvarenga *et al*. 2016, González-Resendiz *et al*. 2018). An additional problem with genus level morphology is that different taxa within a genus may display large variations in filament width (Mai *et al*. 2018, Chakraborty *et al*. 2018). For instance, *Oxynema aestuarii* Chakraborty & Mukherjee (2018: 37) possesses filaments

which are 2.2 μm wide in contrast to the generitype *O. thaianum* Chatchawan *et al*. (2012: 50), which has much wider filaments at 7.9 μm (Chatchawan *et al*. 2012, Chakraborty *et al*. 2018). Similar variation in filament width among species was detected in a number of genera from the family Oculatellaceae (Mai *et al*. 2018). Differences observed in the filament width within genera would place members of those taxa into different families if the traditional taxonomic boundaries were followed (Komárek & Anagnostidis 2005). Further research utilizing increased greater taxon sampling and extensive morphological observations of cultures is needed for this group. Given that the morphology of species within a single "molecular" genus may vary substantially, we anticipate that further emendations of existing taxa will be published in the future.

Questions on cyanobacterial biogeography are highly debated in the literature (Bahl *et al.* 2011, Namsaraev *et al.* 2010, Ribeiro *et al.* 2018). Based on the traditional botanical approach and 16S rRNA sequence data, it has been proposed that some isolated geographic locations, such as Svalbard and the Hawai'ian archipelago, have higher proportions of endemic cyanobacterial taxa (Komárek *et al*. 2012, Miscoe *et al*. 2016). This idea has been challenged based on a meta-genomic analysis that demonstrated the presence of widely distributed cyanobacterial taxa in Svalbard (Pushkareva *et al.* 2018). However next-generation sequencing approaches have significant problems, such as utilization of a shortened region of 16S rRNA gene and poor nomenclatural practices in existing taxonomic libraries (Edgar 2018). In the near future, a curated cyanobacterial reference library utilizing the sequences of type species will be needed to solve the present issues of cyanobacterial taxonomy. We agree that some geographic locations possess endemic cyanobacterial species. However, such regions cannot remain static over the course of geological time. It has been suggested that algae, including cyanobacteria, can be transported via atmospheric currents or by highly motile organisms such as birds and mammals (Kristiansen 1996). New data indicates the presence of cyanobacteria in the guts of some birds, such as Greylag geese (Wang *et al.* 2019) . The viability of cyanobacterial cells passed through avian digestive tracts is questionable, however, research indicates that some cyanobacterial taxa may survive ingestion (Atkinson 1972). Recent taxonomic findings by Osorio-Santos *et al*. (2014) suggests occurrence of "evolutionary species" (= cryptic taxa) of the genus *Oculatella* in the process of speciation. Such cryptic taxa display diagnostic differences in ITS sequences, but not in morphology. Further divergence leads to the development of distinct morphological autapomorphies that facilitate the description of an "uncontested species". For instance, *O. kazantipica* Vinogradova & Mikhailyuk in Vinogradova *et al*. (2017: 518) has much wider filaments in the older stages in comparison with any other species of *Oculatella*.

Based on the high taxonomic resolution of our 16S-23S ITS rRNA phylogeny, we propose that *Stenomitos rutilans* possesses a phylogenetic position close to the most recent common ancestor (MRCA) of *S. kolaensis*. One hypothesis for this is that geographic dispersion of the MRCAs of *Stenomitos rutilans* and *S. kolaensis* from Hawaii to Russia was precipitated through dispersal by birds migrating via the West Pacific Flyway. The absence of prevailing winds connecting the Hawaiian archipelago with north-western, subarctic Russia supports this idea. How these ancestral populations were dispersed further west is unclear, but may have been facilitated by the East Asian-Australian and Central Asian-Indian Flyways. The presence of members of *Stenomitos* in Alaska (USA) and Chukotka (Russia) would support this hypothesis. However, testing this hypothesis is outside the scope of the current paper and should be tested in the future using a meta-genomic approach.

Analysis of the secondary structure of the ITS region is an essential tool in the differentiation of closely related species of cyanobacteria (Boyer *et al*. 2001, Casamatta *et al*. 2006, Komárková *et al*. 2013, Kilgore *et al*. 2018). The taxonomic importance of ITS analysis is especially important when dealing with toxic and potentially toxic cyanobacterial lineages (Aguilera *et al*. 2018, Sant'Anna *et al*. 2019). Recently, ITS analysis was used in the transfer of the long-established genus *Cylindrospermopsis* Seenayya & Subba Raju (1972: 54) to the genus *Raphidiopsis* Aguilera *et al*. (2018: 144). Undoubtedly, such work should include analysis of the genes responsible for toxin production and how these genes are expressed (Ngwa *et al.* 2012) in addition to the characterization of the ITS region. Shalygin *et al* (2017) demonstrated that sequential differences in the ITS region within one species of *Cyanomargarita* Shalygin, Shalygina & J.R. Johansen (2017: 769) were less than those between distinct species.

Additionally, large mutations may occur in the ITS region outside of the D1-D1′, V2, and Box-B regions (Shalygin *et al*. 2017, 2019). These results suggest unequal mutation rates within the ITS region. Analysis of the ITS region of *Stenomitos* demonstrated structural and sequential similarities at the genus level, particularly in the D1-D1′ and Box-B helices. Surprisingly, the Box-B helix of *S. kolaensis* was more similar to that of *O. cataractarum* than to other representatives of *Stenomitos* (cf. Osorio-Santos *et al*. 2014). However, in *Stenomitos* the D1-D1′ and V3 helices were quite different in comparison with those helices found in *Oculatella*. Further research is required to investigate the origin of similarities in the secondary structures of these distantly related taxa.

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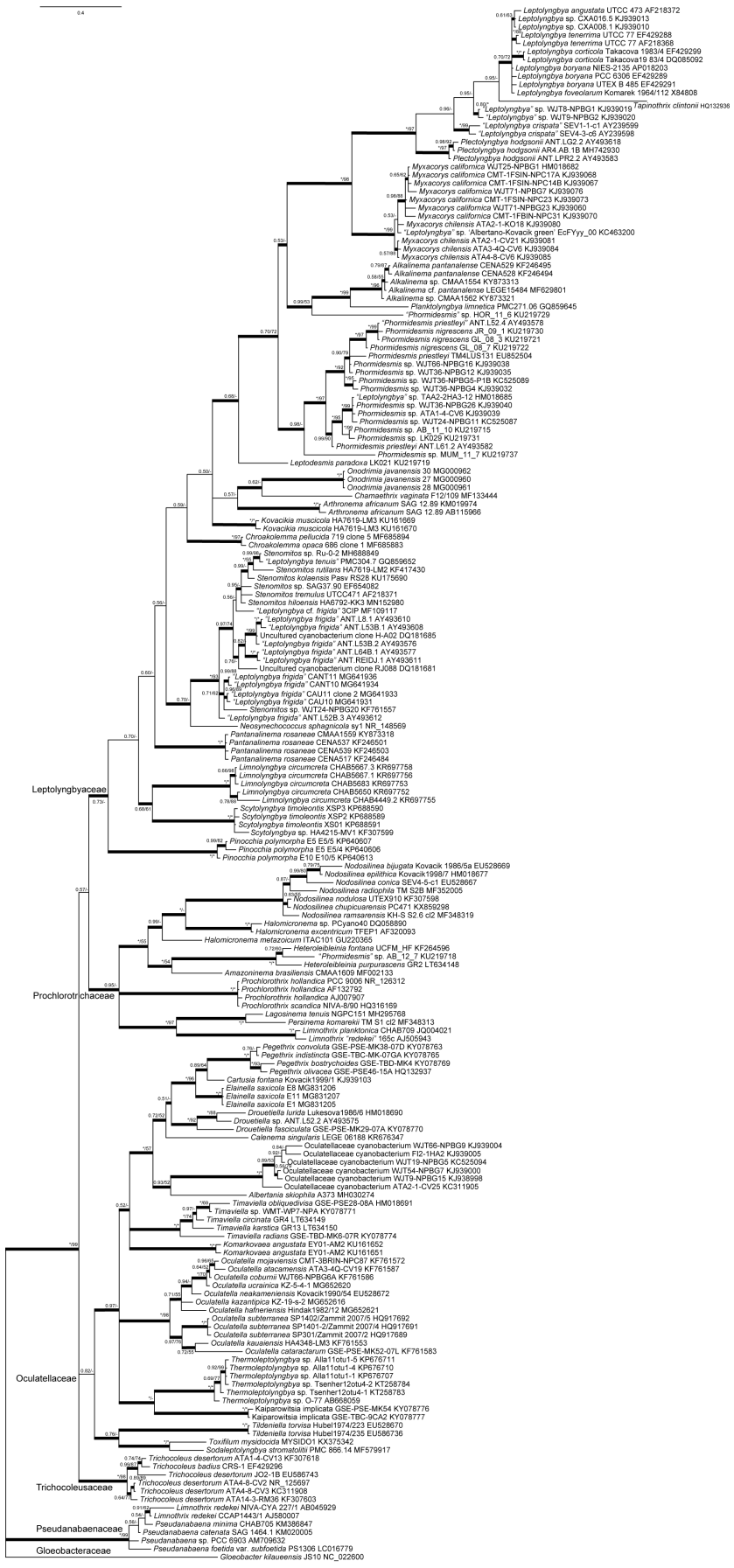

FIGURE S1. Uncollapsed Bayesian Inference analysis based on 16S rRNA sequence data with maximum likelihood bootstrap support values mapped on to nodes. Heavy bold lines report nodes that were represented in both BI analysis and ML analysis.