Scytonematopsis contorta sp. nov. (Nostocales), a new species from the Hawaiian Islands

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Abstract: *Scytonematopsis* Kiseleva is a poorly known cyanobacterial genus with a widespread, mostly tropical distribution. A new species, *S. contorta,* is described from the Hawaiian Island of Oahu based on morphological and molecular evidence. The new species was found growing on rock in damp aerial habitats proximal to streams and waterfalls. It differs in several regards to the other 14 species, in particular in its pronounced ability to produce a spirally contorted trichome within a single filament. The phylogenetic placement of the genus is uncertain. It is associated with both *Rivularia* and *Calothrix* species in the Rivulariaceae, and is distant from the Scytonemataceae, where it has traditionally been placed. However, the relationship of these taxa is unresolved, as there was very little support for the deeper nodes of the phylogeny.

Key words: 16S–23S ITS, *Calothrix*, Cyanobacteria, Hawaii, Oahu, *Rivularia*, *Scytonema*, Scytonemataceae, *Scytonematopsis*, secondary structure

Introduction

Scytonematopsis Kiseleva (1930) is a heterocytous cyanobacterial genus diagnosed by isopolar growth; false branching (mostly geminate), and tapering towards the apices. Some species taper to clear end cells and some species form akinetes, but these features are exceptional rather than typical. Anagnostidis & Komárek (1989) placed *Scytonematopsis* in the Scytonemataceae based on its isopolar growth. The genus contains many species that were originally described in the genera *Scytonema* (which does not taper) and *Calothrix* (which is heteropolar). *Scytonematopsis* currently encompasses both freshwater and marine taxa. Kiseleva (1930) designated the type species as *S. woronichinii*, which was isolated from rice fields from Turkestan. Since then, thirteen more species have been recognized. These include mostly tropical species, from paddy fields, marine waters, and forest soils. One taxon was described from a lake in the High Tatra Mountains (Kováčik & Komárek 1988), and two thermal taxa were described from Yellowstone National Park (Copeland 1936) and Japan (Emoto & Hirose 1952). Taxonomically invalid synonyms of the genus include *Tildenia* Poljanskij, which is a later homonym of a group of herbs belonging to the Piperaceae, and *Setchelliella* DeToni (1936), which is simply a later synonym of *Scytonematopsis*.

Despite the diversity of species in the genus, *Scytonematopsis* is poorly known. The species are published in a very scattered literature. No synthetic treatment of the genus is available. Only a small (~326 bp) fragment of the 16S rRNA gene is available from one unillustrated, undescribed *Scytonematopsis* growing on buildings in Brazil (Crispim et al. 2006). There is certainly a need for further study on this interesting clade of tapering Nostocales.

During a survey of the freshwater algae of the Hawaiian Islands, two distinct populations of *Scytonematopsis*, comprised of a single new species, have been found in waterfall splash zones on Oahu, Hawaii. This species is distinct from all previously described species attributed to the genus. In this paper, we characterize and describe the species using morphological data and report its phylogenetic placement in the Nostocales using 16S rRNA sequence data and secondary structure of the 16S–23S ITS region. The name we propose is *Scytonematopsis contorta* sp. nova, and we will refer to this taxon by that epithet in the remainder of this paper.
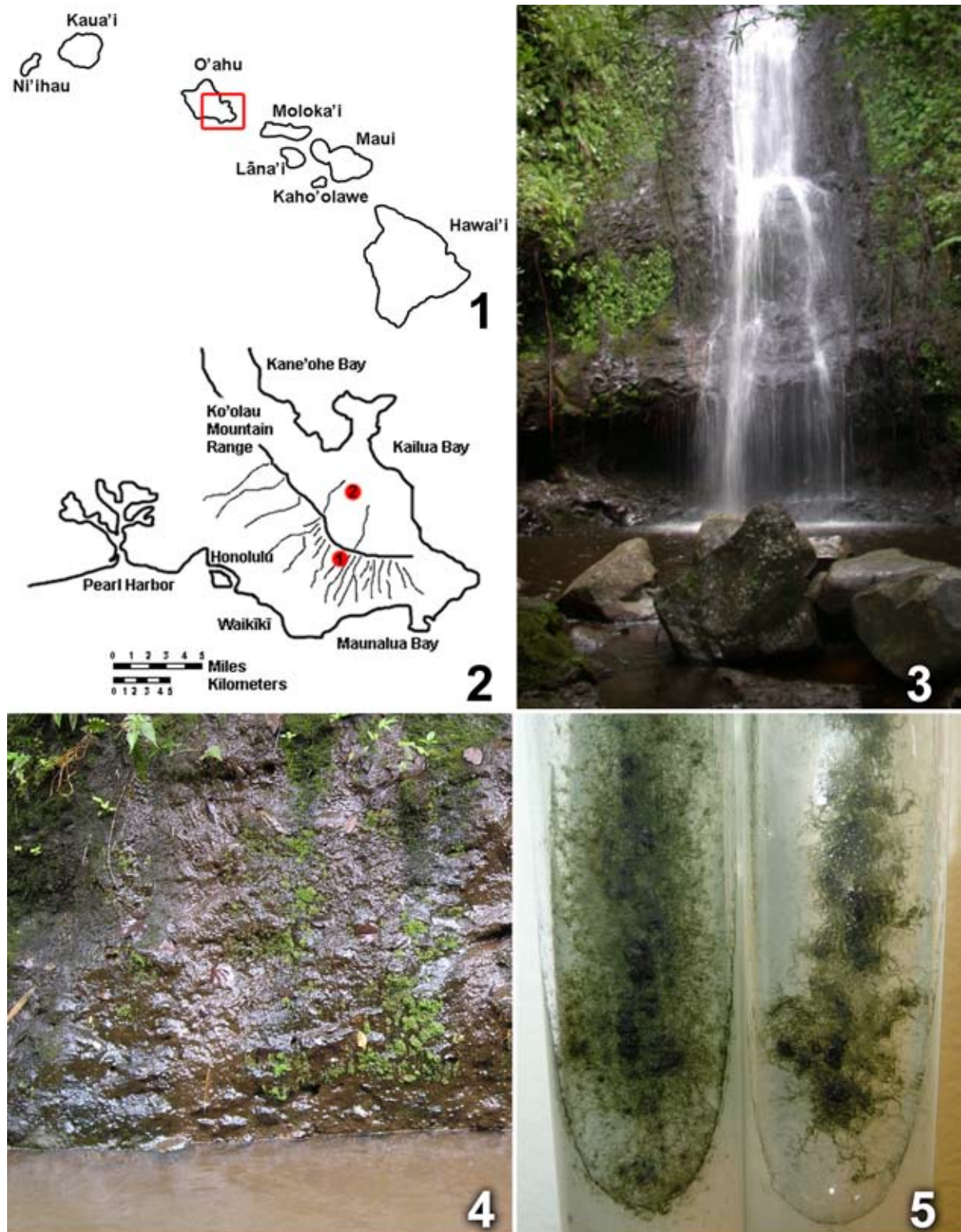
Figs 1–5. Map, site photos, and growth form of *Scytonematopsis contorta*: (1) The Hawaiian Archipelago, southern tip of O'ahu is boxed in red; (2) southeastern region of O'ahu, dots represent locations of sites from which *S. contorta* was isolated; (3) site 04267, waterfall in Palolo Valley; (4) site 04292, wet wall along Maunawili stream; (5) pure cultures of *S. contorta* showing growth form on agar, HA4267 right, HA4292 left.

Materials and Methods

Samples were collected in July 2009 (dry season). Samples were placed in whirlpak bags or small eppendorf tubes, and then partitioned among collaborators. Environmental samples were vouchered in 2.5% CaCO$_3$–buffered glutaraldehyde and deposited in the Bernice P. Bishop Museum. Fresh material was kept for culturing at John Carroll University. Details of current and progressive environmental and isolate collections can be found online at the Hawaiian Algal Database webpage: http://packrat.stjohn.hawaii.edu/hfwadb/index.html.

Site Descriptions. Sites containing *S. contorta* were located on the southeastern tip of O'ahu (Fig. 1), at the end of the Ko'olau Mountain Range (Fig. 2). The first population was collected from the first major waterfall in Wai'oma'o Stream (the east branch of Palolo Stream) accessed via the Palolo Valley Trail, headed up towards Ka'au Crater (Fig. 3). This site was approximately at 21° 18' 50" N latitude, 157° 46' 44"W longitude, and the environmental accession number was 04267. The sample consisted of mud scraped from the cave–like formation behind the waterfall. The second population was geographically near the first site, but separated by the Ko'olau Ridge in a different watershed. It was collected from a wet wall along Maunawili stream, approximately 50 meters downstream from Maunawili Falls (Fig. 4). The site was approximately at 21° 20' 52"N, 157° 46' 28"W, and the environmental sample was accessioned as 04292.

Laboratory work. Fresh samples were inoculated onto Z8 medium (Carmichael 1986) agar plates and grown under low light (<200 µE) conditions. Colonies were picked after an extended growth period (1–2 months or more) and isolated into pure culture for microscopy and molecular analysis. Both strains were characterized using an Olympus BX60 photomicroscope with high resolution Nomarski DIC optics.

    Approximately 25–30 mg of healthy culture cells were used for DNA extraction using the UltraClean Microbial DNA Isolation Kit from MO BIO Laboratories, Inc (Carlsbad, CA, USA). DNA was eluted into 50 µl of solution MD5 and stored at –20°C. The 16S–23S internal transcribed spacer (ITS) region of the rRNA operon was amplified using primers 1 and 2 (Boyer et al. 2001, 2002). A 25 µL reaction for each strain was run in a C1000 Thermocycler (BIORAD). The PCR cycle was carried out as follows: 95°C for 5 minutes; followed by 35 cycles of: 95°C for 1 minute, 57°C annealing temperature for 45 seconds, and 72°C for 4 minutes; ending in an additional 5 minutes at 72°C and finally an indefinite hold at 4°C. The final concentrations of reagents used were as in Lukešová et al. (2009). PCR products (~1600 bp) were cloned using a Strataclone PCR cloning kit (La Jolla, CA,

USA), which utilized cloning vector pSC–A–amp/kan. Vector DNA was isolated from clones using a QIAprep Spin Miniprep kit from QIAGEN. Plasmids containing inserts were sent to Functional Biosciences (Madison, WI, USA) for sequencing with primers M13 forward, M13 reverse, 3, 5, and 8 (Boyer et al. 2001, 2002). The program Sequencher (v. 4.1) was used to edit and proofread the sequences.

Phylogenetic Analysis. Phylogenetic analysis was based on a 1123 fragment of the 16S rRNA gene (bp 359–1482). The taxa used included a total of 106 OTUs, with 25 OTUs being novel sequences from Hawaiian freshwater/subarieal strains, 5 OTUs from Utah soil strains, and 76 OTUs from GenBank. Cyanobacteria belonging to the Nostocaceae, Microchaetaceae, Rivulariaceae, Scytonemataceae, and Stigonemataceae were chosen for comparison (encompassing taxa with heterocytes which exhibit no branching, false branching and/or true branching). Three trees were constructed using parsimony, distance, and maximum likelihood analyses in PAUP v.4.02b (Swofford 1998). Parsimony was run using heuristic search settings with simple addition sequence, nearest–neighbor interchange for branch–swapping, and steepest descent. Bootstrapping was conducted with 1000 replicates. Maximum likelihood was run using the general time reversible model, and bootstrapped with 100 replicates. Distance was run using distance measure HKY85, gamma distribution with default shape parameters, nearest–neighbor–interchange branch–swapping algorithm, and steepest descent. Bootstrapping for this tree used 1000 replicates.

    Secondary structures of the 16S internal transcribed spacer (ITS) region were determined for several taxa present in the phylogeny and used for comparison (novel sequences and several from GenBank). These structures were transcribed and folded for all available operons (with and/or without tRNA) using the Mfold web server (Zuker 2003). Images of these structures were drawn using Adobe Illustrator CS3 (ver. 13.0.0).

Results

Scytonematopsis contorta, sp. nov. (Figs 5–17)

Diagnosis: A S. terrestris cellulis multo brevioribus differt. S. shankargarense simile, sed trichomis parum latioribus vagina multo angustiore. A speciebus omnibus generis trichomis dense torsivis et ramificatione geminata profusa differt.
Descriptio: Coloniae griseovirides, non nitentes, effusae a centro, filis paucis erectis a agaro. Fila dense intricata, undulata, trichoma singula, pseudoramificatione geminata frequenti, pseudoramificatione singula rara, 14–30 µm lata. Vagina angusta, hyalina, in luce vivida

fulvescens, non lamellata. Trichomae initio isopolares, apicibus ambabus gradatim angustatis ad extremum obtusum, spirae densae in centro fili formantes, non constrictae vel leviter constrictae ad septa, post fragmentationem heteropolarescentes heterocystis basalibus interdum formantibus, ad trichomam mediam 9–12–(13) μm latae, angustatae gradiatim ad 3–4.0 μm latae, pilo non angustatae. Cellulae nongranulatae vel leviter granulatae, in trichoma media saepe breviorae quam latiorae, impigre cresentes, 1.5–3 (–6.0) μm longae, in parte angustata trichomae plerumque isodiametrae vel longiorae quam latiorae, non dividentes, pigmentosae, 3–6 μm longae. Heterocystae rariorae, terminales vel intercalares, stramineae, globosae, compressae, lunatae vel irregulares, 4–8.5 μm latae, 7.5–11 μm longae. Necridia atroveneta. Akineta in seriebus formantes, irregulare ad regulare connexa, rotundata vel ellipsoidalia, vacuolatescentia, saepe cellulis dividentibus in partibus lunatis duabus, a vagina firma aperta emergentia, 8.5–12 μm lata, 3–10 μm longa.

English Diagnosis: It differs from *S. terrestris* by its much shorter cells. Similar to *S. shankargarensis* but with slightly wider trichomes and a much narrower sheath. Differs from all species in the genus by its dense coiling and profuse geminate branching.

English Description: Colony grayish green, non–shiny, spreading far from center, with few filaments erect from agar. Filaments densely entangled, wavy, with a single trichome per filament, with frequent double false branching, with rare single false branching, 14–30 μm wide. Sheath thin, hyaline, becoming golden brown in high light, not lamellated. Trichomes at first isopolar, both ends gradually tapered to a blunt end, forming dense spirals in center of filament, unconstricted to slightly constricted at the crosswalls, becoming heteropolar following breakage, sometimes developing basal heterocytes following breakage, 9–12–(13) μm wide at midtrichome, tapering very gradually to 3–4.0 μm wide, not tapering to a hair. Cells ungranulated or slightly granulated, in midtrichome frequently shorter than wide, actively growing, 1.5–3 (–6.0) μm long, in tapered region of trichome mostly isodiametric to longer than wide, not dividing, fully pigmented, 3–6 μm long. Heterocytes rare, terminal or intercalary, yellowish, round, compressed, crescent shaped, or irregular, 4–8.5 μm wide, 7.5–11 μm long. Necridia dark bluish–green. Akinetes formed in series, irregularly to regularly connected, round or ellipsoidal, becoming vacuolated, often with a single round cell divided in two halves, emerging from an open, firm sheath, 8.5–12 μm wide, 3–10 μm long.

Type Location: Wai'oma'o Stream, Palolo Valley, O'ahu, Hawai'i, 21° 18' 50" N latitude, 157° 46' 44"W longitude. Subaerial on wet rock walls and waterfall splash zones.

Holotype here designated: *Scytonematopsis contorta* HA4267–MV1 BISH 747428.

Paratype here designated: *Scytonematopsis contorta* HA4292–MV4 BISH 747429.

Reference Strain: *Scytonematopsis contorta* HA4267–MV1 (UTEX Culture Collection, Austin, Texas, U.S.A.).

Scytonematopsis contorta has a very distinct ontogeny. Young filaments are isopolar and tapered at both ends (Figs 6–8), similar to the oscillatorialian genus *Ammatoidea* (Komárek & Anagnostidis 2005) and the Pseudanabaenalean species *Tapinothrix clintonii* Bohunická et Johansen (Bohunická et al. this issue). As the filaments mature, the trichomes typically begin to coil within the sheath, creating contortions that cause numerous false branches to occur in close proximity to one another (Figs 6, 10, 12–14). While most branching is geminate, single false branching also occurs (Fig. 11). When single false branches arise, fragmentation can occur, yielding trichomes that appear heteropolar, with a widened base and tapering apex (Figs 9, 11). When terminal heterocytes develop in these heteropolar filaments, they are indistinguishable from *Calothrix* (Fig. 9). However, basal akinetes never develop near these terminal heterocytes, a feature which distinguishes *Scytonematopsis* from *Calothrix*. Indeed, akinetes are very unusual in this taxon as they develop apically in series (Figs 15–17), and have the rather unique characteristic of dividing to produce hemispherical cells (Figs 15, 17). Akinetes are additionally distinguished by their vacuolated appearance (Fig. 17). Because of the different life–cycle stages, one could imagine that field material of *Scytonematopsis* could likely be confused with *Ammatoidea* or *Calothrix,* but if the tapering apices were not ignored it would be unlikely to be confused with *Scytonema* or *Brasilonema* in the Scytonemataceae.

    The tight coiling of the trichome within the middle regions of the sheath appears to be a genetically determined trait characteristic of at least a number of species of *Scytonematopsis*. The feature has been well illustrated for *Tildenia dura* (Kosinskaja 1948), *S. incerta* (Geitler 1933),

and *Scytonematopsis ambigua* (Emoto & Hirose 1952). Geitler (1932, 1933) made mention to the irregularly coiled filaments of both *S. fulginosa* (as *Tildenia fulginosa*), and *S. incerta*, but considered the feature taxonomically unimportant. Because he considered it unimportant, he did not include the feature in the diagnoses of the species he treated. The trait has been illustrated in *Rivularia* species from the Baltic Sea (Sihvonen et al. 2007, fig. 1j), and we suspect that the ability to produce such coiling may be genetic (and therefore taxonomically important), but the degree of expression is likely environmentally plastic. For now, the intensity of the coiling and profuse branching in *S. contorta* distinguishes it from the other species (Table 1).

 Scytonematopsis contorta is distinctive from many of the other species in this genus (Table 1). *S. contorta* is ecotypically distinct in that it occupies quite a different biotope from all other members of this genus. Of the other freshwater members this is the only one located in splash zones near fast flowing water, where the majority of them are from paddy fields, and *S. starmachii* and *S. kashyapi* are from stagnant waters. The rest of the genus is comprised of marine taxa, two thermal taxa (*S. ambigua* and *S. hydnoides*), and one from sedimentary soil (*S. neocaledoniense*). Another species, *S. variabilis* Yoneda *nom. nudum* (Yoneda 1952) was mentioned but not described properly (Umazaki & Watanabe 1994), and therefore has no taxonomic status. In terms of morphology *S. contorta* differs from many of the other species in that the colony is flat and creeping, the trichome is very thin as well as the sheath, and the end cells are pigmented. The cells midtrichome are quite short, and are never nearly as long as several other non–marine species that have a similar trichome width. The trichomes in the regularly–sized midregion are most similar in size to those of *S. shankargarensis* and *S. terrestris*. *S. contorta* differs from *S. shankargarensis* by having more acute tapering, a thinner and non–lamellated sheath, and a slightly thicker trichome; from *S. terrestris* in that the cells are much shorter in length; and from both in that apical cutting (see Pandey & Mitra 1972) was not observed. The akinetes are comparable in appearance to *S. hydnoides* in shape and arrangement, however they were never observed midtrichome in our species.

Phylogenetic Analysis

Seven clones of the rRNA gene of *Scytonematopsis* (3 from HA4267–MV1 and 4 from HA4292–MV4) were sequenced. One clone from each strain of *Scytonematopsis* was identical in the entire sequenced 16S rRNA gene fragment and 16S–23S ITS region. We assume these represent homologous operons in the species. Among the rest of the cloned sequences, 12 nt (dispersed throughout the gene) were non–congruent. None of the operons recovered had either tRNA gene in the ITS region. We conclude that both strains belong to the same species.

 The topology of the parsimony, likelihood and distance analyses were very similar, particularly with reference to *Scytonematopsis, Scytonema, Brasilonema,* and *Calothrix*. We show the parsimony analysis with node support from all three analyses (Fig. 18). Thirty–one OTUs clustered basally in the tree and were mostly comprised of members of the Nostocaceae. We considered these to be our outgroup taxa for all analyses.

 Based on the taxon sampling available at present, the Scytonemataceae, Rivulariaceae, and Stigonemataceae were not well resolved. The Stigonemataceae formed a fairly monophyletic clade, except for three OTU's including two *Calothrix* species and *Chlorogloeopsis* (Fig. 18). The Scytonemataceae *sensu stricto* (Fig. 18, clade A) included seven *Scytonema* OTU's and eight *Brasilonema* OTU's. *Scytonematopsis contorta* formed a clade with two *Petalonema* strains (Fig. 18, clade B)*,* but was separated from the Scytonemataceae by a group of Rivulariaceae (*Calothrix* and *Rivularia*, Fig. 18, clade C). Microchaetaceae was sister to the clade containing all of these clades. Three additional Rivulariaceae (*Gloeotrichia* and *Calothrix*) occur in a basal position to the branching Nostocales. *Calothrix* was the most clearly polyphyletic genus, occurring in four distinct clades. It seems apparent that the characteristic of tapering trichomes has likely arisen more than once in the Nostocales, as taxa with that trait do not form a monophyletic group.

 Node support was weak along the spine of the phylogenetic analyses. Only the family Stigonemataceae had strong support. The familial relationships of all other taxa are in doubt. While the two populations of *Scytonematopsis* are certainly the same species, the placement of this genus in the Scytonemataceae is unsupported. Even if the Rivulariaceae and Scytonemataceae

Figs 6–17. Light micrographs of *Scytonematopsis contorta*: (6) growth habit, showing dense coiling trichomes with subsequent profuse branching, scale 50 µm; (7, 8) isopolar trichomes tapered to both ends, scale 50 µm; (9–17) scale bar 20 µm; (9) heteropolar "*Calothrix*–like" appearance; (10, 13, 14) profuse coiling and geminate branching; (11) rare single false branching; (12) geminate false branching; (15,16) formation and release of spores from sheath; (17) vacuolization of spores.

Table 1. *Scytonematopsis* – Currently accepted and possibly related species [(Ref) References/Authors: (1) Kováčik & Komárek 1988; (2) Copeland 1936; (3) Tilden 1910; (4) Umezaki & M. Watanabe 1994; (5) Emoto & Hirose 1952; (6) Akiyama 1977; (7) Geitler 1933; (8) this paper; (9) Pandey 1965; (10) Varma 1974; (11) Geitler & Ruttner 1935; (12) Couté, Tell & Thérézien 1999; (13) Kiseleva 1930; (14) Desikachary 1959; (15) (Mertens) C. Agardh ex Bornet et Flahault 1886; (16) Harvey ex Setchell et Gardner 1919; (17) Bornet & Flahault 1886; (18) Kosinskaja 1948].

Species Name	Filament width (µm)	Trichome width (µm)	Habitat/Described From	Diagnostic characters	Ref.
Marine					
S. crustacea Syn *Calothrix*	12–20(40)	8–15	Widespread Distribution	Colorless end cells. Resting cells in series, long–cylindrical, smooth.	1
S. fulginosa [Sensu Tilden]	20–46–(70)	10–20	Coast of Hawaii, USA	Wide–spread lamellate sheath, coiling noted by Geitler.	2,3
S. pilosa Syn *Calothrix* Syn *Setchelliella*	10–40	10–20	Widespread Distribution	Black filaments erect on coast rocks.	4
Non–Marine					
S. ambigua	9–12–(15)	5–8	Thermal species, Japan	Colorless end cells, coiling in filaments	5,6
S. calotrichoides	6–8(11)	(2)6–7	Tropcial inland waters	Colorless end cells. Branching almost always single.	7
S. contorta	(6)14–30	(3)9–12–(13)	Tropical, Waterfall splash zones on Hawaii	Profuse coiling and double false branching, non–lamellate sheath, round or ellipsoidal spores sometimes vacoulized	8
S. ghazipurensis	(7)14–27	(5)10–17	Tropical, Paddy field, India	Apical cutting present, wide sheaths.	9
S. hydnoides	13–22	(2.5–3.5)5–10	Thermal, from Yellowstone National Park, USA	Wide–flaring lamellated sheath ends, colorless end cells, and extremely long heterocytes (20–90 µm), resting cells in series.	2
S. incerta	(3–6)7–9(10)	(2–3)6	Tropical (Geitler), and from Paddy Fields, India	Small amount of coiling noted by Geitler.	7,10
S. kashyapi Syn *Spleaopogon*	4.5(7)	(4)6	Tropical, on *Ceratophyllum* in stagnant pond, Benaras, India	No apical cutting, single false branching, longer than wide cells (2–4x) throughout the length of the filament, thick and smooth walled spores in chains.	11
S. neocaledoniense	5–9	2–5	Tropical–Terrestrial, forest soils of New Caledonia	Diagnosed by its sharp apex. Has longer than wide cells (2–3x) throughout the length of the filament.	12
S. shankargarhensis	(11–)12–16(19)	(5–7)9–12(–13)	Tropical, paddy field, near Allahabad, India	Apical cutting present. Compared to *S. terrestris*, heterocysts longer, less frequent branching.	10
S. starmachii	8–9.3–18.6–21	5–7–9.3–11.6	High Elevation Lake, High Tatra Mountains	Colorless end cells, *Ammatoidea* and *Calothrix* like stages, controlled reproduction of heterocytes, double false branching.	1
S. terrestris	(6–7)10–13(–14)	(3.5–6)8–11	Tropical/paddy field, near Allahabad, India	Apical cutting present. Compared to *S. shankargarensis*, Heterocytes short, more frequent branching.	10
S. woronichinii	(8–)9–10(10–5)	(4) 6.5–8	Tropical, paddy field in Turkestan	The type species. Apical cutting present. The spores are quite long (10–19 µm).	13,14
Unclear Taxa					
Calothrix pulvinata	15–18	8–12	Marine, widespread Distribution	Colony erect, fasciculated. The cells are ½ to ⅓ long as wide. Very abruptly tapered.	15
Calothrix vivipara	12–24	9–15	Marine, widespread distribution	Double false branching reported.	16,17
Tildenia dura	23–34.5–(98.8)	(8)–9.2–14.3	Marine, Gulf of Mexico and California Coast	Limestone encrusted colony, wide spreading lamellate sheath, coiling in filaments	18

Fig. 18. Parsimony tree of phylogenetically close taxa in the Microchaetaceae, Scytonemataceae, and Stigonemataceae, with representative Nostocaceae as outgroup taxa. Clades of related taxa distant from *Scytonematopsis* were collapsed. Bootstrap values at supported nodes (> 50%) are given for parsimony, maximum likelihood, and distance, respectively. An asterisk equals a value of 100. Scale number of evolutionary steps.

were combined into a single family, they would not form a supported monophyletic taxon (Fig. 18).

The *Rivularia* clade (containing two *Calothrix* species) had high bootstrap support in all analyses, and was sister to *Calothrix*. The *Calothrix* clade consisted of three supported clusters having very high bootstrap support (≥ 98% in all three analyses, not shown). The *Brasilonema* and *Scytonema* clades occured as sister taxa. The

Scytonema clade formed two distinct clusters with high bootstrap support (≥ 99% in all three analyses, not shown). *Brasilonema* formed a monophyletic cluster. This grouping was supported in each analysis, with highest bootstrap support using distance (100%). The Stigonemataceae was made up of two supported distinct clusters, the *Fisherella/Hapalosiphon* (node support 87/79/99) and thermal *Mastigocladus* (node support 100/98/100) clades (not shown).

Table 2. Percent similarity matrix for 14 strains of comparison using a 1123 nucleotide partial sequence of the 16S rRNA gene [Strain access numbers: (1) HA4292–MV4–p2, HQ847559; (2) HA4267–MV1–p3, HQ847558; (3) ANT.GENTNER2.8, AY493624; (4) HA4277–MV1–p6G, HQ847568; (5) AF334697; (6) HA4186–MV5–p10AB, HQ847580; (7) BECID14, AM230671; (8) HAF2–B2–c1–p11A, HQ847553; (9) AF236651; (10) PCC 7110, AM709637; (11) HA4187–MV1–p1H, HQ847567; (12) SRS6, AF334692; (13) PYH6, AM230703; (14) CM1-VF14, AY577537].

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Scytonematopsis contorta*	–												
2. *Scytonematopsis contorta*	99.55	–											
3. *Petalonema* sp.	92.96	93.06	–										
4. *Petalonema* sp.	92.16	92.06	95.31	–									
5. *Calothrix parietina*	91.20	91.21	91.12	90.40	–								
6. *Calothrix* sp.	91.97	91.98	91.98	91.52	97.56	–							
7. *Rivularia* sp.	92.32	92.15	92.96	92.32	90.75	91.24	–						
8. *Scyotnema* sp.	89.90	89.62	90.89	90.43	89.76	89.71	90.87	–					
9. *Scytonema hyalinum*	91.49	91.59	93.86	93.76	91.65	91.41	92.21	91.22	–				
10. *Scytonema hofmanii*	91.38	91.67	93.95	93.48	90.45	91.12	90.57	89.95	93.21	–			
11. *Brasilonema* sp.	92.16	92.43	94.15	94.15	91.85	91.80	93.23	92.25	94.67	93.58	–		
12. *Spirirestis rafaelensis*	92.97	92.69	95.50	94.69	91.31	91.79	93.31	90.00	92.51	92.77	92.44	–	
13. *Gloeotrichia echinulata*	91.50	91.42	93.34	93.15	90.58	91.97	92.68	89.52	92.14	90.87	92.07	94.77	–
14. *Nostoc desertorum*	91.61	91.33	94.87	93.15	89.58	90.34	90.96	89.27	91.96	92.23	91.45	94.61	93.61

Secondary structures

Secondary structures including the D1–D1' helix, Box–B helix, and V3 helix were determined for several available operons of representative strains included in the phylogenetic analysis. Visual comparison of these structures often showed a high degree of variability between genera, as well as significant variability between operons within single strains.

The D1–D1' helix was highly variable among the different genera reported, with all genera forming distinctive structures (Figs 19–26). Past studies of the secondary structure of ITS regions have demonstrated that the basal portion of the D1–D1' helix is typically conserved in closely related taxa (Řeháková et al. 2007; Johansen et al. 2008; Siegesmund et al. 2008; Lukešová et al. 2009). This group of Nostocales was unusual in that none of the structures were identical. All *Scytonematopsis contorta* D1–D1' helices were the same, and had a 6 bp helix subtending the unilateral bulge (Fig. 19). The structurally most similar helix was found in *Nostoc desertorum* (Fig. 20), a taxon quite distantly related to *Scytonematopsis* based on phylogenetic analysis (Fig. 18). The most distinctive and different helices could be found in *Brasilonema* (Figs 24–24) and *Petalonema* (Fig. 36). While common motifs have been identified for *Nostoc* (Řeháková et al. 2007; Lukešová et al. 2009) and the Microchaetaceae (Casamatta et al. 2006), the Scytonemataceae and Rivulariaceae

as presently defined do not appear to have such common motifs. In one instance, we show the marked variability between operons from the same strain, *Brasilonema* HA4187–MV1 (Figs 24, 25).

The Box–B helix for *S. contorta* was also distinct (Figs 27–34). Although it had the identical basal structure and sequence (a 4 bp helix), it was longer than all other Box–B helices and had a different apical sequence (Fig. 27). The two operons in *Brasilonema* shared identical Box–B helices (Figs 32, 33). All other Box–B helices were different from one another.

Because *S. contorta* lacked tRNA genes in all recovered ITS regions, it had no V2 helix. The V3 helix, however, was present in all taxa. *S. contorta* clearly had the longest V3 helix of any species examined (Figs 35–42). There was insufficient similarity in any of these helices to make inferences about phylogenetic relationships.

An examination of the percent similarity (100*(1–P)) revealed a similarity of 99.55–100.00% between the two populations of *S. contorta* (Table 2), with most of the variability attributable to different operons. The next most similar strains based on this simple metric were *Petalonema* (92.96–93.06%) and *Spirirestis* (92.69–92.97%). All other representative taxa in Table 2 were less than 92.5% similar to both populations (Table 2), and indeed were not similar
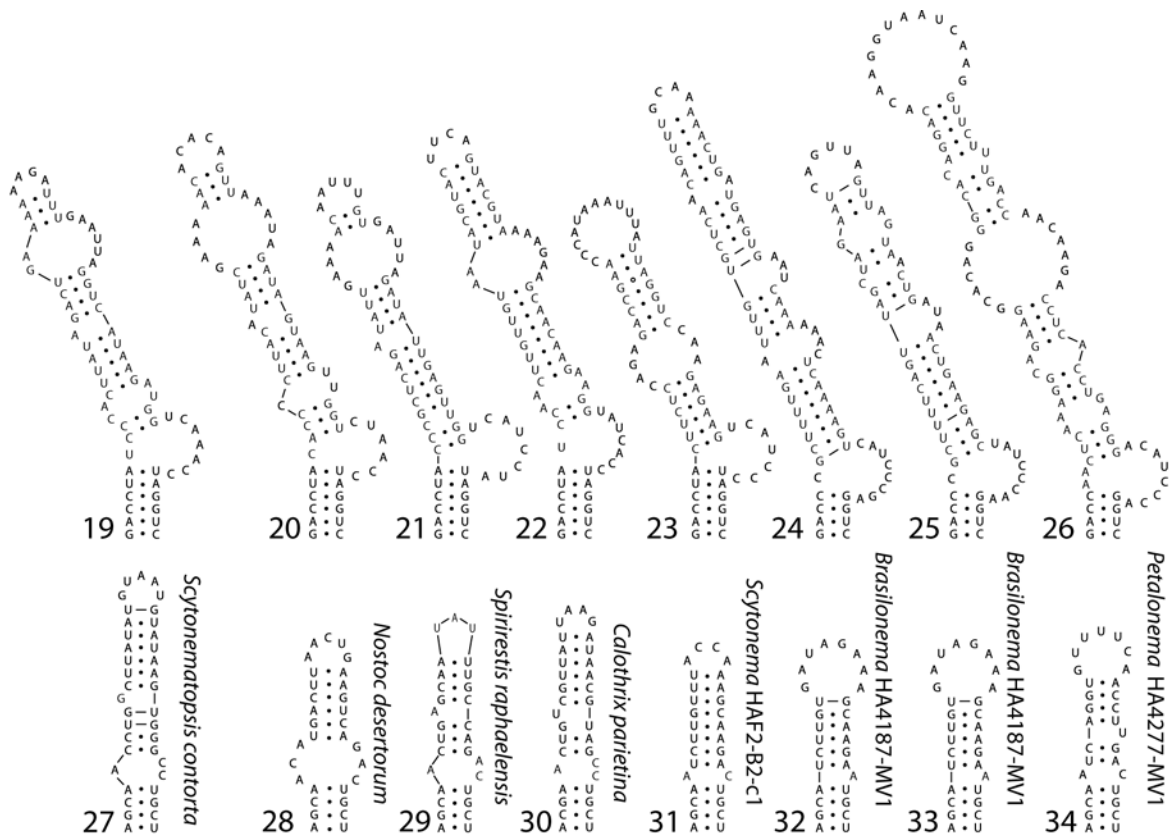
Figs 19–34. Secondary structure of ITS regions in phylogenetically close taxa: (19–26) D1–D1' helices; (27–34) Box–B helices. Structures from the same taxa and operon are aligned vertically. Taxon accessions listed in Table 2.

to each other at all. The deep genetic divergence in these taxa suggests that our taxon sampling of the group is insufficient, and we will need many more taxa before the phylogeny of the tapering Nostocales can be well resolved.

Discussion

Our strains certainly fit the current description of *Scytonematopsis* in terms of morphology. The geminate false branching, coiling within filaments, isopolarity, and tapering towards the ends clearly affiliate *S. contorta* with the freshwater species in the genus. However, the higher level taxonomy is uncertain. The node containing our species was well supported, but its position in the tree was not. Furthermore, it was more proximal to the Rivulariaceae than the Scytonemataceae. It is very clear that heteropolar tapering taxa such as *Rivularia* and *Calothrix* have a confused taxonomy. *Calothrix* is especially problematic, falling into at least four different clades in our tree. Others have determined *Calothrix* to be present in 4–5 clades as well (Sihvonen et al. 2007; Berrendero et al.

2008). While we do not have definitive evidence, it appears that *Scytonematopsis* actually belongs in the Rivulariaceae.

It seems very likely that *Scytonematopsis* is polyphyletic. Given the wide ecological range in which species occur (tropical marine, tropical paddy soils, tropical aerial habitats, temperate freshwater, thermal springs, etc.) it seems likely that there may at least be a freshwater/marine dichotomy. We need to have greater taxon sampling in the genus to establish the monophyly or polyphyly of the genus. The Rivulariaceae are in need of revision, and not all recent work has contributed to that revision. The type species for *Calothrix* is marine: *Calothrix confervicola* Agardh ex Bornet et Flahault (1886), while the type species for *Rivularia* is freshwater: *Rivularia dura* Roth ex Bornet et Flahault (1886). It seems unfortunate to us that Rippka et al. (2001) decided to place all marine strains of tapering cyanobacteria in *Rivularia*, and all freshwater strains in *Calothrix*. This decision contradicts the principle of taxonomic priority and ignores nomenclatural rules. If *Scytonematopsis* were included in the Rivulariaceae, narrowly defined it
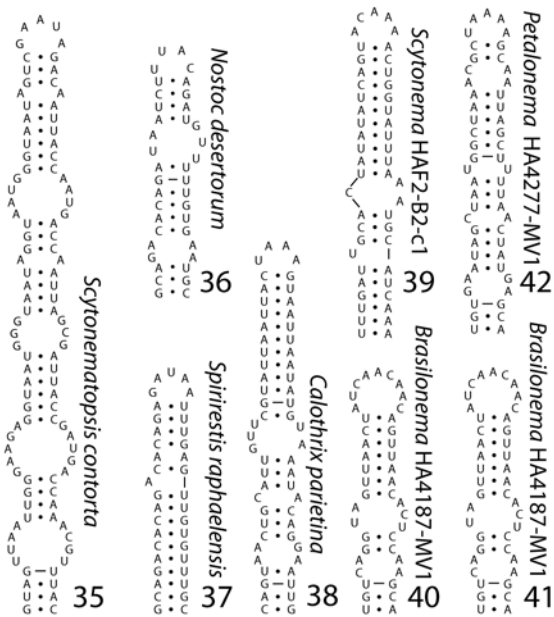
Figs 35–41. V3 structures from 16S–23S ITS regions in phylogenetically close taxa. Taxon accessions listed in Table 2.

would include isopolar freshwater taxa. *Calothrix* should be represented by the heteropolar marine taxa, and *Rivularia* should contain the heteropolar freshwater taxa. Additional freshwater *Calothrix*–like taxa would need diagnosis and one or more new genera. BERRENDERO et al. (2008) noted that one clade of freshwater tapering species in their study belonged in a new genus, but they did not erect the taxon in that paper.

One unusual finding was the phylogenetic placement of *Fortiea* in the Nostocaceae rather than the Microchaetaceae where it is typically placed (KOMÁREK & ANAGNOSTIDIS 1989). This rare and poorly studied genus is characterized by unbranched heteropolar filaments which have a basal heterocyte and are widened at the opposite end. We do not feel we have enough taxon sampling in this group to come to a definitive conclusion regarding the familial taxonomy of this genus, but suspect that the Microchaetaceae narrowly defined includes only taxa capable of at least some false branching. Further study in this genus is planned for future work, as we have isolated two strains from Hawaii in this genus.

We were surprised by the variability in the secondary structures of the 16S–23S ITS region. This high level of variability indicates that the representatives in the combined Scytonemataceae and Rivulariaceae are actually deeply divergent. Some taxa, such as *Brasilonema* species, had highly similar secondary structures (data not

shown). However, putative sister taxa, such as *Scytonematopsis* and *Petalonema*, were quite distinct, indicating a long evolutionary history since their last common ancestor. It is also unsettling to consider placement of *Petalonema* in the Rivulariaceae based on its placement in the phylogeny in this paper.

In previous papers, we have found a high level of conservation in the secondary structure of the D1–D1' helix and Box–B helix among Pseudanabaenales (JOHANSEN et al. 2008; JOHANSEN et al. 2011; BOHUNICKÁ et al., this issue), the Phormidiales (SIEGESMUND et al. 2008), and selected Nostocales (ŘEHÁKOVÁ et al. 2007; LUKEŠOVÁ et al. 2009). We suspect that with more taxon sampling, we would begin to see similar patterns in the Rivulariaceae and Scytonemataceae. It is critical in this work to try to obtain the same operons between taxa (distinguishable by the presence or absence of tRNA genes). We tried to obtain an operon for *Scytonematopsis* with both tRNA genes, but did not recover one in the seven sequences we cloned. It may be that this genus does not have operons with tRNA genes, or it could have a mutation in the primer region that prevents us from obtaining that particular operon.

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