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RESEARCH ARTICLE

Revision and expansion of the genus *Spirirestis* (Tolypothrichaceae, Cyanobacteria)

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Abstract

Recent phylogenetic analyses of members of the Tolypothrichaceae (Nostocales, Cyanobacteria) based on 16S rRNA gene sequence data have demonstrated that the soil-inhabiting members of the family belong to a clade separate from the aquatic and subaerial members of the family. The soil-inhabiting species clade includes *Spirirestis*, a monophyletic taxon originally defined by its tight spiral coiling. Most of the soil-inhabiting species have been identified in the past as belonging either to *Hassallia* or *Tolypothrix*, which are subaerial and aquatic taxa, respectively. A comprehensive study of the terrestrial Tolypothrichaceae led us to conclude that all terrestrial Tolypothrichaceae should be included in the genus *Spirirestis*, even though most of those isolates lack the spiral coiling diagnostic of the genus. Using a polyphasic approach, we recognize seven distinct clades in *Spirirestis*, which we split into seven species: *S. rafaelensis* (the generitype), *S. californica* comb. nov., *S. pseudoramosissima* comb. nov., *S. lignicolor* sp. nov., *S. williamsae* sp. nov., *S. hydroterrestris* sp. nov., and *S. atacamensis* sp. nov. *Spirirestis rafaelensis* and *S. californica* are represented by multiple isolates, and we postulate that with time and further taxon sampling, some of the strains we included in these two

Abbreviations: BI, Bayesian inference; ESS, estimated sample size; GTR, general time reversible; ITS, internal transcribed spacer; MCMC, Markov chain Monte Carlo; ML, maximum likelihood; MP, maximum parsimony; PCR, polymerase chain reaction; PD, percent dissimilarity (of the sequence of ITS rRNA regions); PS, percent similarity (of 16S rRNA gene sequence); PSRF, potential scale reduction factor.

species may be recognized as additional species. As the study of soil cyanobacteria continues, additional species of *Spirirestis* will likely be discovered and described.

KEYWORDS
16S–23S ITS, biological soil crusts, *Hassallia*, Nostocales, phylogeny, Tolypothrichaceae, *Tolypothrix*

INTRODUCTION

Taxonomic progress in cyanobacteria in the late 20th century had a rough start. A number of microbiologists (Stanier et al., 1978) proposed that cyanobacteria should be described under the *International Code of Nomenclature of Bacteria* (Lapage et al., 1975), and they described a number of new genera that were distinct from classical botanical taxa under that code. Unfortunately, all of these attempts initially resulted in invalid or illegitimate names. *Gloeobacter violaceus* Rippka et al. (1974) was the first of these taxa, later recognized as invalid by Rippka and Cohen-Bazire (1983), and eventually validated Under the ICN by Mareš et al. (2013). *Prochloron didemi* Lewin (1977) followed, based on the earlier taxon *Synechocystis didemi*, which was not properly typified (see Lewin, 1975); this taxon was subsequently validated in Hoffmann and Greuter (1993). *Cyanobacterium stanieri* and *Cyanobium gracile* were described in Rippka and Cohen-Bazire (1983), but due to typification issues, they were invalid. *Cyanobacterium* was later validated by Oren et al. (2022), but *Cyanobium* remains invalid despite a subsequent emendation to the description (Komárek et al., 2020)! *Prochlorothrix hollandica* was carefully characterized, but axenic strains were not obtained, and the type was listed as a strain (Burger-Wiersma et al., 1989), rendering the species invalid. Finally, *Prochlorococcus* was described by Chisholm et al. (1988, 1992), but the genus remained invalid until validated by Komárek et al. (2020). These cases clearly demonstrate the challenges of describing cyanobacteria under the *International Nomenclatural Code for Bacteria* (now the *International Code of Nomenclature for Prokaryotes*). The absence of axenic strains and improper designation of types are a recurring difficulty for microbiologists trying to describe new taxa based on their code of nomenclature.

In contrast, the description of cyanobacteria following the *International Code of Nomenclature for algae, fungi, and plants* has had a long history with the polyphasic approach to cyanobacterial taxonomy being the currently accepted standard approach. The first validly described new cyanobacterial genus and species using a polyphasic approach, including morphology, ecology, and molecular phylogeny, was *Spirirestis rafaelensis* (Flechtner et al., 2002). The description was made following the requirements of the *International Code of Botanical Nomenclature* (Saint Louis Code; Greuter et al., 2000). The genus exhibited very distinct morphology—a regularly spirally coiled heterocytous filament capable of single false branching. This coiling was observed in both cultured and uncultured natural material. This species was restricted to well-developed biological soil crusts in the arid climate of the San Rafael Swell, a geological formation in the Colorado Plateau. Microscopic observations and sequence data closely matching *S. rafaelensis* were subsequently reported for other sites in the Colorado Plateau (Yaeger et al., 2007, 2012). A *S. rafaelensis* strain was also isolated by a member of our research group from Joshua Tree National Park (strain WJT71-NPBG6), sharing identical morphology and high genetic similarities to the San Rafael Swell strains (SRS6, SRS70). Based on the 16S rRNA gene phylogeny, *S. rafaelensis* was placed in the family Microchaetaceae. The coiled form of the genus was never observed by us again.

The Microchaetaceae was reassessed by Hauer et al. (2014), who reviewed the history of the family and proposed the establishment of two new families, the Tolypothrichaceae and the Godleyaceae, reserving the Microchaetaceae for the type species, the marine *Microchaete grisea*. They placed *Tolypothrix, Hassallia, Rexia, Spirirestis*, and *Coleodesmium* in the Tolypothrichaceae and *Godleya* and *Toxopsis* in the Godleyaceae. Earlier, Sant'Anna et al. (2010) placed *Streptostemon* in the Microchaetaceae, but this taxon has since been considered part of the Tolypothrichaceae. Since then, three additional genera have been described in the Tolypothrichaceae: *Dactylothamnos* (Komárek et al., 2015), *Dapisostemon* (Hentschke et al., 2016), and *Kryptousia* (Alvarenga et al., 2017). All sequenced genera currently in the Tolypothrichaceae show high percent identity (>94.5%) based on 16S rRNA gene sequences.

As part of our study of heterocytous cyanobacteria from soils, we noted that the terrestrial Tolypothrichaceae strains formed a clade separate from the aquatic and wet subaerial strains. Furthermore, *Tolypothrix, Hassallia, Coleodesmium*, and *Kryptousia* were all polyphyletic based on the strains assigned to each, indicating that revision within this family was still needed. The soil clade contained *Spirirestis* as well as strains assigned to *Tolypothrix* and *Hassallia*.

Twenty-two years after the description of *Spirirestis*, we are pleased to return to this genus. In this manuscript, we revise *Spirirestis*, emend the description of the type species, introduce two new combinations, and describe five species new to science.

MATERIALS AND METHODS

A total of 30 strains belonging to the Tolypothrichaceae were isolated through several independent efforts by a diverse group of researchers over a period spanning 2 decades. In all cases, soils were diluted in liquid cyanobacterial media (Z8 and $BG11_0$) and then spread on enrichment plates containing agar-solidified media, following the methods of Jusko and Johansen (2024). Unialgal colonies were picked and transferred into liquid media after 4–8 weeks, and, upon achieving sufficient growth, were transferred to agar-solidified slants. Details on the origin of these strains are in the Supporting Information (Table S1). Cultures are maintained in the Algal Culture Collection at John Carroll University, University Heights, Ohio, United States, except for KH22-PS, which is maintained in the Cyanobacterial Culture Collection at Banaras Hindu University, Varanasi, India. Herbarium vouchers of immobilized, air-dried material obtained from cultured isolates were prepared and deposited in several curated herbaria (see Table S2).

Sequencing of the strains also occurred over an extended period of time but followed fairly standard and similar methods. Genomic DNA was extracted from strains using Qiagen DNeasy Powersoil Pro Kits (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene and the 16S-23S rRNA internal transcribed spacer (ITS) region using primers VRF1R and VRF2F (Flechtner et al., 2002; adapted from Wilmotte et al., 1993). The reaction mixture contained 1 μL of each primer at 0.01 mM, 12.5 μL of LongAmp™ Taq 2× Master Mix (NEB, Ipswitch MA), 1 μL template DNA (50 ng · mL^{-1}), and 9.5 μL nuclease free water. Using this mixture, PCR was performed with 35 cycles of denaturation (94°C for 45 s), annealing (at 57°C for 45 s), extension (72°C for 135 s), and a final extension at 72°C for 5 min. Polymerase chain reaction products were inserted into the *lac*Z gene of plasmid pSC-A-amp.kan and cloned into StrataClone (Agilent, Santa Clara, CA) competent *Escherichia coli* cells via heat shock following the manufacturer's protocol. Transformed *E. coli* cells were plated on agar-solidified LB-ampicillin plates with 40 μL X-Gal, and three properly transformed colonies were picked via blue-white screening. Plasmid DNA was isolated with Qiagen QIAprep Miniprep kits following the manufacturer's protocol. Insertions were confirmed by *Eco*R1 restriction enzyme digestion, followed by visualization on 1% TBE agarose gels. Two or three clones of each strain were sent to Functional Biosciences, Inc. (Madison, WI, United States) for Sanger sequencing. Primers M13 forward, M13 reverse and internal primers VRF5 (5′-TGT ACA CAC CGG CCC GTC-3′), VRF7 (5′-AAT GGG ATT AGA TAC CCC AGT AGT C-3′), and VFR8 (5′-AAG GAG GTG ATC CAG CCA CA-3′; Nübel et al., 1997; Wilmotte et al., 1993) were used to obtain partial overlapping sequences. Sequences were error-proofed using Chromas software (v. 2.6.6) and assembled into contigs by alignment with ClustalW (Larkin et al., 2007). When possible, two or three clones were used to construct consensus sequences.

The 16S rRNA gene alignment was analyzed using Bayesian inference (BI) and maximum likelihood (ML) methods using the CIPRES Science Gateway (Miller et al., 2010). Posterior probabilities and bootstrap support values were mapped to the nodes on the BI analysis. Methods for these analyses followed those reported in Jusko and Johansen (2024) with the following exceptions: 93 sequences of Tolypothrichaceae, including aquatic, terrestrial, and subaerial strains were aligned with an outgroup of 13 Nostocales taxa outside of the Tolypothrichaceae. The length of sequences in the alignment varied from 1161 to 1469 nucleotides. The BI analysis was run for 61.36 million generations until the stop value of 0.01 was achieved, with the first 25% of samples discarded as burn-in. The ML analysis was run on the same alignment with 1000 bootstrap iterations. The BI analysis yielded a mean estimated sample size (ESS) exceeding 3100 (range: 3166–15,943) for all parameters, significantly higher than the average of 100 accepted as sufficient (Drummond et al., 2006). The average standard deviation of split frequencies was ≤0.01. The potential scale reduction factor (PSRF) value for all parameters in the BI analysis was 1.00, indicating convergence of the Markov chain Monte Carlo chains was achieved (Gelman & Rubin, 1992). The 16S-23S ITS rRNA region sequences were aligned to run BI analysis and maximum parsimony (MP) analyses following methods in Jusko and Johansen (2024). The alignment was restricted to sequences from 38 strains identified to belong to the soil-dwelling clade in the 16S rRNA gene analyses, with a maximum sequence length of 543 nucleotides. The phylogenetic analyses used the GTR + G + I model for both the BI and the ML analyses.

Percent identities among 16S rRNA gene sequences and percent dissimilarities among 16S-23S ITS rRNA region sequences were determined using the SHOWDIST command in PAUP (Swofford, 1998). The positions of the D1–D1′, Box-B, and V2 and V3 helices were identified based on their conserved basal clamps. These clamps for San Nicolas Island (SNI) strains were determined by creating multiple alignments of closely related taxa with ClustalW (Larkin et al., 2007) with taxa of known helix locations serving as reference points. Secondary structures were predicted via Mfold

(Zuker, 2003) on the UNAFold Web Server (http://www.unafold.org). Drawing mode was set to untangle with loop fix and all other settings were set to default. Structures derived from Mfold were post-edited using Adobe Illustrator. Herbarium abbreviations follow the online Index Herbariorum (Thiers, 2025).

RESULTS

Taxonomy

Seven taxa are recognized below, the generitype, *Spirirestis rafaelensis*, along with two new combinations and four new species. Taxa were identified using a polyphasic approach employing morphology, ecology, 16S rRNA gene phylogeny and percent similarity (PS), 16S-23S ITS rRNA region phylogeny, percent dissimilarity, and secondary structure of conserved domains. We provide both microphotographs (Figures 1–7) and drawings (Figures 8 and 9) below in accordance with the *International Code of Nomenclature for Algae, Fungi, and Plants* (Turland et al., 2018). Diagnostic features based on morphological, molecular, and ecological characteristics are noted below in the taxonomic descriptions of all species. Molecular justification of species together with general results on morphology follow the formal descriptions.

Spirirestis rafaelensis Flechtner *et* J.R.Johans. *in* Flechtner et al., 2002. Figure 1.

Emended description: Filaments facultatively forming tight, regular spirals which can be seen to taper slightly in young cultures, also sinuous, or straight in older cultures as well as in strains not from the type locality, with uncommon single false branching and rare double false branching, with false branching usually distant from the heterocytes, 6–20 μm in diameter, with spirals when formed 16–28 μm in diameter at their widest point, tapered end of spirals about 75%–80% of the maximum diameter; distance between regular spirals 8–12(–16) μm. Sheath firm, usually colorless, occasionally golden to brown, thin in young cultures becoming thicker and showing parallel lamellations or transverse striations in older cultures, 1–5 μm thick. Trichomes unconstricted to distinctly constricted at the crosswalls, more commonly and more strongly constricted in older cultures and in loosely coiled or extended filaments, 5.5–16.0 μm in diameter. Cells blue-green to olive green, mostly shorter than wide, occasionally quadratic, 2.0–11 μm long, without aerotopes, cell contents sometimes granular, especially in older cultures. Heterocytes both intercalary and basal within filaments, mostly single but occasionally double in intercalary position, rarely triple, spherical to oval, sometimes appressed on one side in basal position, light brown, 5–15 μm in diameter, 3–20 μm long. Necridia present, bow-shaped. Hormogonia short 80–310 μm long.

Type locality: nearly inaccessible perched shelf located below the Wedge Overlook area of the San Rafael Swell, Emery County, Utah (39.1011° N, 110.7452° W; Flechtner et al., 2002)

Habitat: well-developed biological soil crusts in Utah and California in the United States and China

Reference strain: SRS70 (from which the holotype was prepared, see Flechtner et al., 2002)

Additional strains: CM1-HA08, CM1-HA09, CM1-HA10, CM1-HA11, CMT-1BRIN-NPC13, CMT-1BRIN-NPC34, CMT-1SZIN-NPC9, CMT-1SZIN-NPC20, CMT-2BRIN-HLNPC9, CMT-3SWIN-NPC18, CNP3-B3-C04, CXA 109-3-BZ, EM2-HA1, FI5-MK38, SRS6, UB1-KK1, UFS-BI-NPMV-1A2-F06, WJT2-NPBG8, WJT71-NPBG6

Taxonomic notes: *Spirirestis rafaelensis* is the type species of the genus, originally described based upon the strong coiling of trichomes observed in strains isolated from the San Rafael Swell, Utah (SRS6, SRS70). Coiling was also observed in strains WJT71-NPBG6 and LQ-10, but it has not been documented in any other strains. This species occurs in soils of inland hot and cold deserts in Utah, California, and China, primarily in undisturbed sites with well-developed blackened algal crusts.

Spirirestis californica (J.R.Johans. *et* Flechtner) J.R.Johans. *et* B.Jusko *comb. nov*. Figures 2, 8a.

Basionym: *Hassallia californica* J.R.Johans. *et* Flechtner *in* Flechtner et al., 2008, *Western North American Naturalist* 68: p. 417, figs 59, 60, 73.

Emended description: Filaments in exponential growth phase straight, short, singly false branched with branches nearly perpendicular to the main filament, with very thin sheath, 8–11 μm wide, becoming wider later in life cycle due to increased mucilage production, 10–20 μm wide. Sheath colorless, thin and firm in exponential growth phase, becoming thicker, lamellated and fibrous in stationary phase, often encasing the trichome apices. Trichomes straight, constricted at crosswalls, 8.0–8.5 μm wide in exponential phase, becoming 5–14 μm wide with age. Cells deep blue-green, centroplasm not distinguishable from chromoplasm, shorter than wide in exponential phase, 1.6–4.0 μm long, becoming 2–8 μm long later in life cycle. Heterocytes rare in complete medium, common in nitrogen free medium, intercalary or terminal, unipolar, hemispherical to globose, 6.6–13 μm wide, 3.6–12 μm long. Necridia rarely present, vestigial, not bow shaped. Hormogonia 12–60 μm long.

Epitype here designated: SBBG 238546! Voucher prepared from reference strain, SNI-TA17-ML2, isolated from poorly developed biological soil crust, upslope from spring origin site, on cliff face with shallow soil pockets. Collected by J. R. Johansen with W. F. Hoyer and K. E. Hasenstab-Lehman. 33.232864° N, 119.517807° W. Sampled 25 May 2021, San Nicolas Island, California, United States. Designated as an

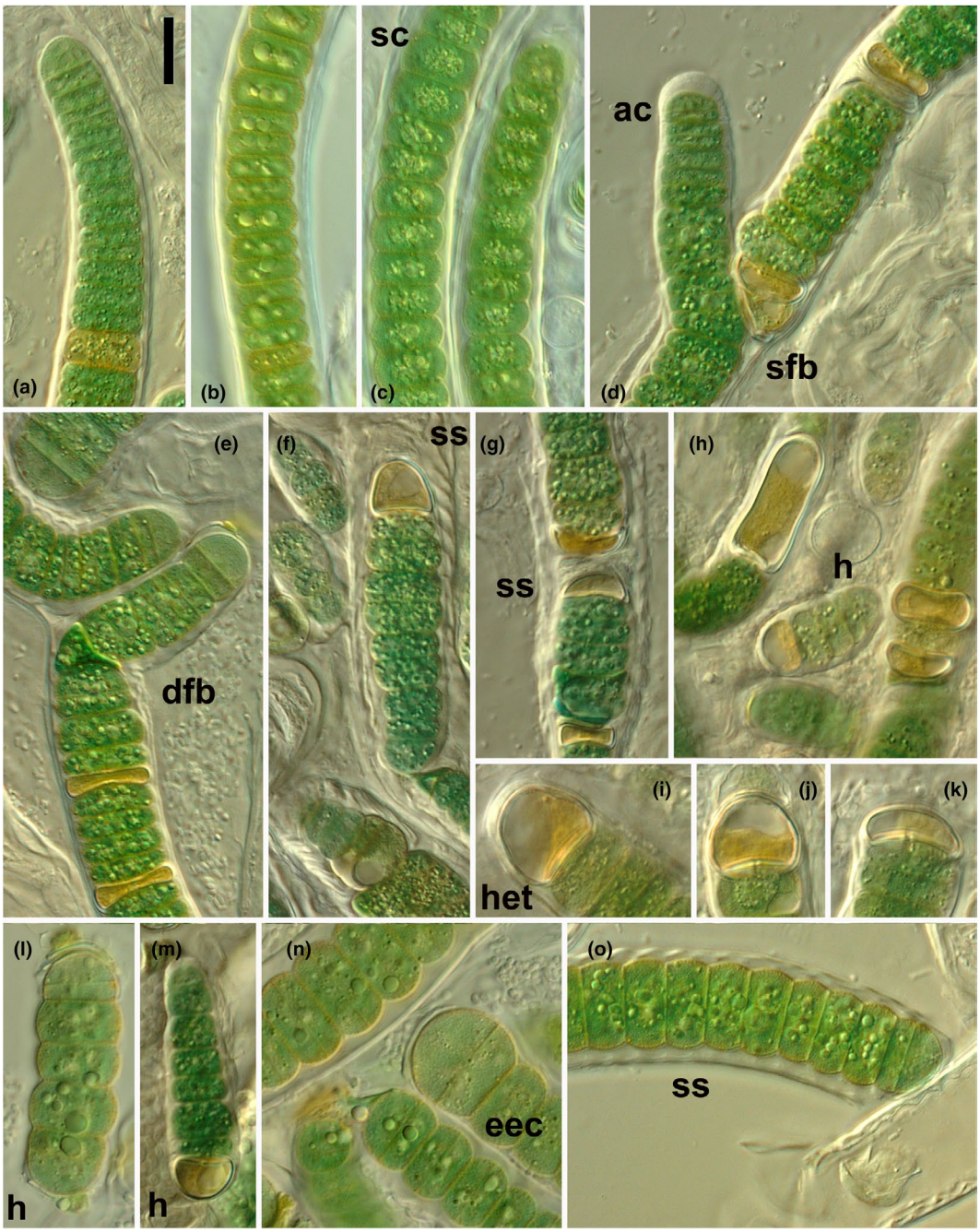

FIGURE 1 *Spirirestis rafaelensis*. Letter codes: Ac, apical cap; dfb, double false branch; eec, enlarged end cell; h, hormogonia; het, heterocyte; sc, strongly constricted at crosswalls; sfb, single false branch; ss, structured sheath. Scale is 10 μm in all panels.

epitype because the holotype for *Hassallia californica* is a figure, and the material used to create the figure had no sequence data attached to the holotype and thus was ambiguous for determination of the species.

Reference strain: SNI-TA17-ML2

Additional strains: Mon65, SNI-TA1-JJ1, SNI-TA17-BJ34, SNI-TA31-BJ5, and SNI-TA31-BJ1

Taxonomic notes: In the initial description, this taxon was characterized by short, straight trichomes with very short cells, a diagnostic feature that allowed us to identify the species in our recent isolations (Figure 2a–c). However, in stationary phase, longer trichomes with longer cells were observed, indicating this character is not always present (Figure 2d–h). Even in this growth phase, cells are shorter than wide and mostly under 5 μm long. This species occurs in dry biological soil crusts on San Nicolas Island, California, United States, and in the Monegros region in Spain. San Nicolas Island has a maritime climate that is considerably milder and cooler than mainland California, whereas the Monegros region frequently experiences drought.

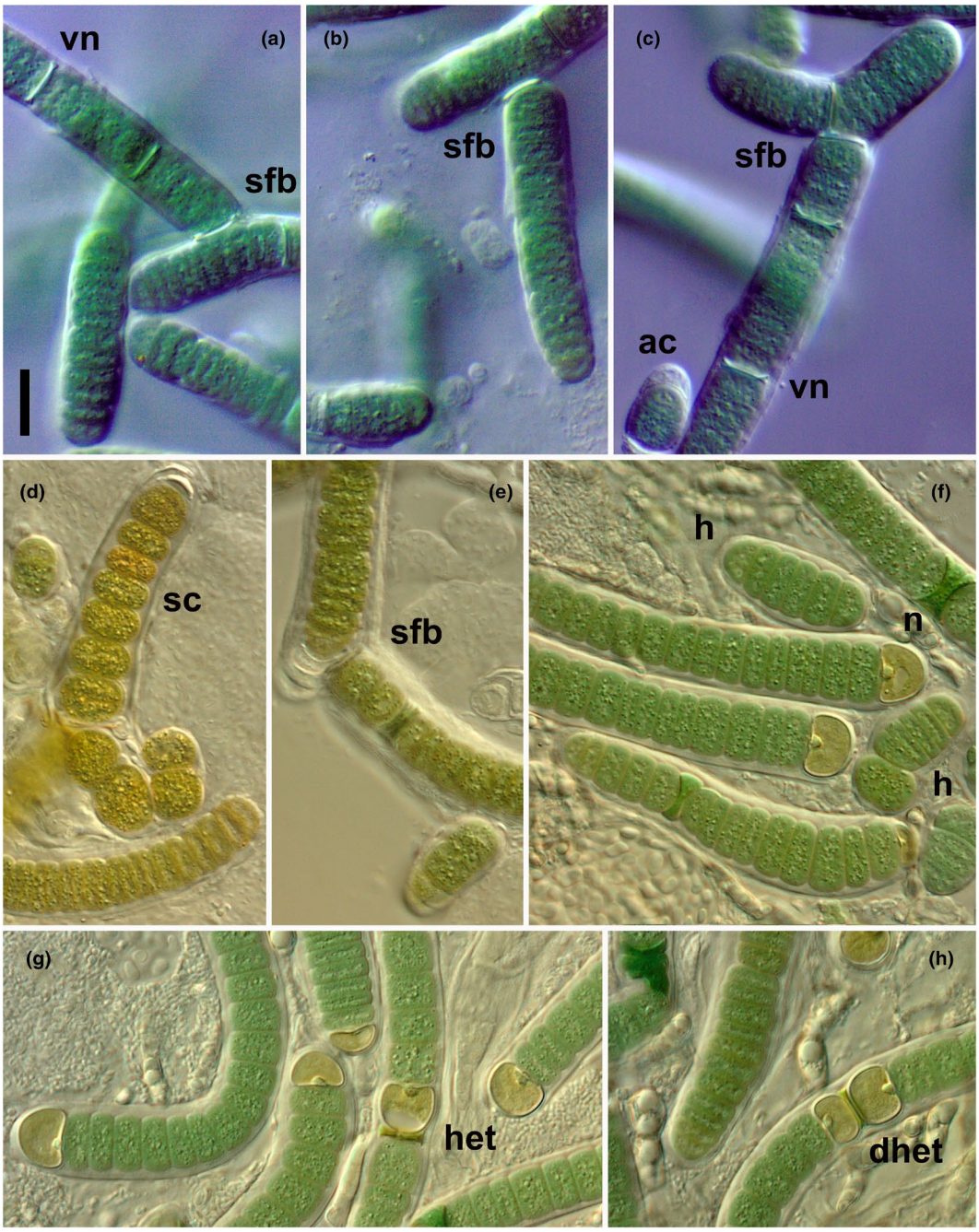

FIGURE 2 *Spirirestis californica*. Letter codes: Ac, apical cap; dhet, double heterocyte; h, hormogonia; het, heterocyte; sc, strongly constricted at crosswalls; sfb, single false branch; vn, vestigial necridia. Scale is 10 μm in all panels.

Spirirestis pseudoramosissima (J.R.Johans. *et* Flechtner) J.R.Johans. *et* B.Jusko comb. nov. Figures 3, 13b.

Basionym: *Hassallia pseudoramosissima* J.R.Johans. *et* Flechtner *in* Flechtner et al., 2008, *Western North American Naturalist* 68: p. 417, figs 57, 58, 74

Emended description: Filaments entangled, sinuous to straight, never spirally coiled, infrequently singly or doubly false branched, 8–20 μm wide. Sheath colorless, often thick, unlamellated, or irregularly to regularly lamellated, sometimes fibrous on outside layer, not expanded at trichome apex, rarely encasing the apex. Trichomes not constricted in the thinnest trichomes, becoming increasingly constricted with increase in width, 4.6–14 μm wide. Cells blue-green in actively growing culture, becoming olive green in stationary phase, cylindrical in thinner trichomes, barrel-shaped in wider trichomes, with coarsely granular centroplasm, 1.5–9 μm long. Heterocytes commonly occurring in both replete and nitrogen-free media, basal, when intercalary forming at trichome breakage and unipolar, hemispherical,

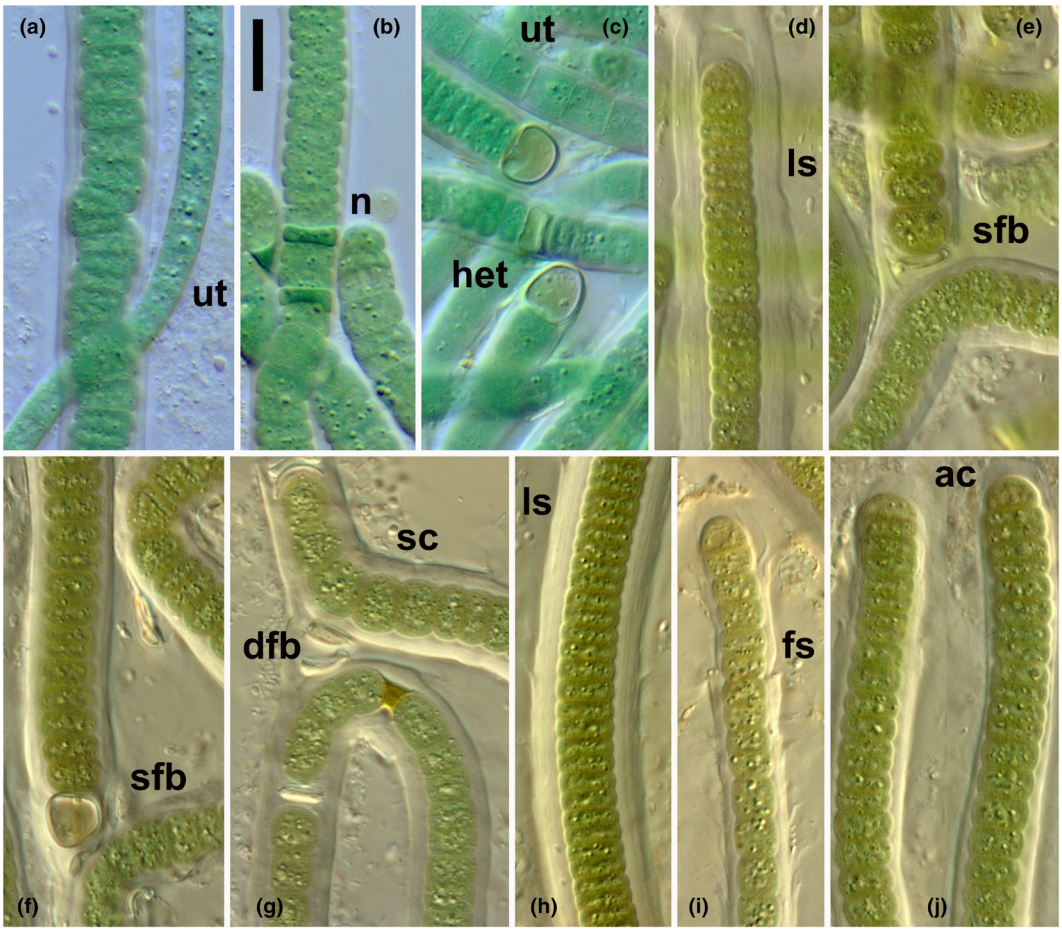

FIGURE 3 *Spirirestis pseudoramosissima*. Letter codes: Ac, apical cap; dfb, double false branch; fs, fibrous sheath; h, hormogonia; het, heterocyte; ls, lamellated sheath; sc, strongly constricted at crosswalls; sfb, single false branch; ut, unconstricted trichome. Scale is 10 μm in all panels.

globose, flattened or compressed, evidently larger in nitrogen-free medium, 5.6–11 μm wide, 3–10 μm long. Necridia present, but vestigial and not bow-shaped.

Epitype here designated: SBBG 238553!, voucher prepared from reference strain, SNI-TA23-BJ7, isolated from exposed caliche sampled in a small hollow on southeastern side of San Nicolas Island, upslope near high elevation site. Collected by J.R. Johansen with W.F. Hoyer and K.E. Hasenstab-Lehman. 33.24653°N, 119.54604°W. Sampled 26 May 2021. Designated as an epitype because the holotype for *Hassallia pseudoramosissima* is a figure, and the material used to create the figure had no sequence data attached to the holotype and thus was ambiguous for determination of the species.

Reference strain: SNI-TA23-BJ7

Taxonomic notes: This species was originally described based on its abundant false branching, a trait also observed in our new strain from San Nicolas Island. To date, only a single isolate of this species has been isolated and characterized. It was growing on exposed caliche sampled in a small hollow on the southeastern side of the island.

Spirirestis lignicolor B.Jusko *et* J.R.Johans. sp. nov. Figures 4, 8c.

Filaments entangled, sinuous to straight, becoming tightly twisted within the sheath, but not spirally coiled, singly or doubly false branched, 10.4–35 μm wide. Sheath thin and colorless in exponential phase of growth, becoming widened and brown with age, often fibrous and telescoping, often encasing the apex. Trichomes slightly to strongly constricted at the crosswalls, commonly fragmenting without formation of necridia, sometimes abruptly tapering at the apices, 7–14 μm wide. Cells blue-green, with slightly granular cytoplasm, lacking polyphosphate bodies, mostly shorter than wide, 3–10 μm long. Heterocytes rare, even in nitrogen-free medium, basal in position, hemispherical to compressed globose, 7–10 μm wide, 4.5–7 μm long. Necridia present, bow shaped. Hormogonia short, 10–66 μm long.

Holotype here designated: SBBG 238531!, herbarium voucher (metabolically inactive) prepared from reference strain, SNI-TA12-AZ3, isolated from soil from blackened hummock above stream bed. Collected from San Nicolas Island by J.R. Johansen with W.F. Hoyer and

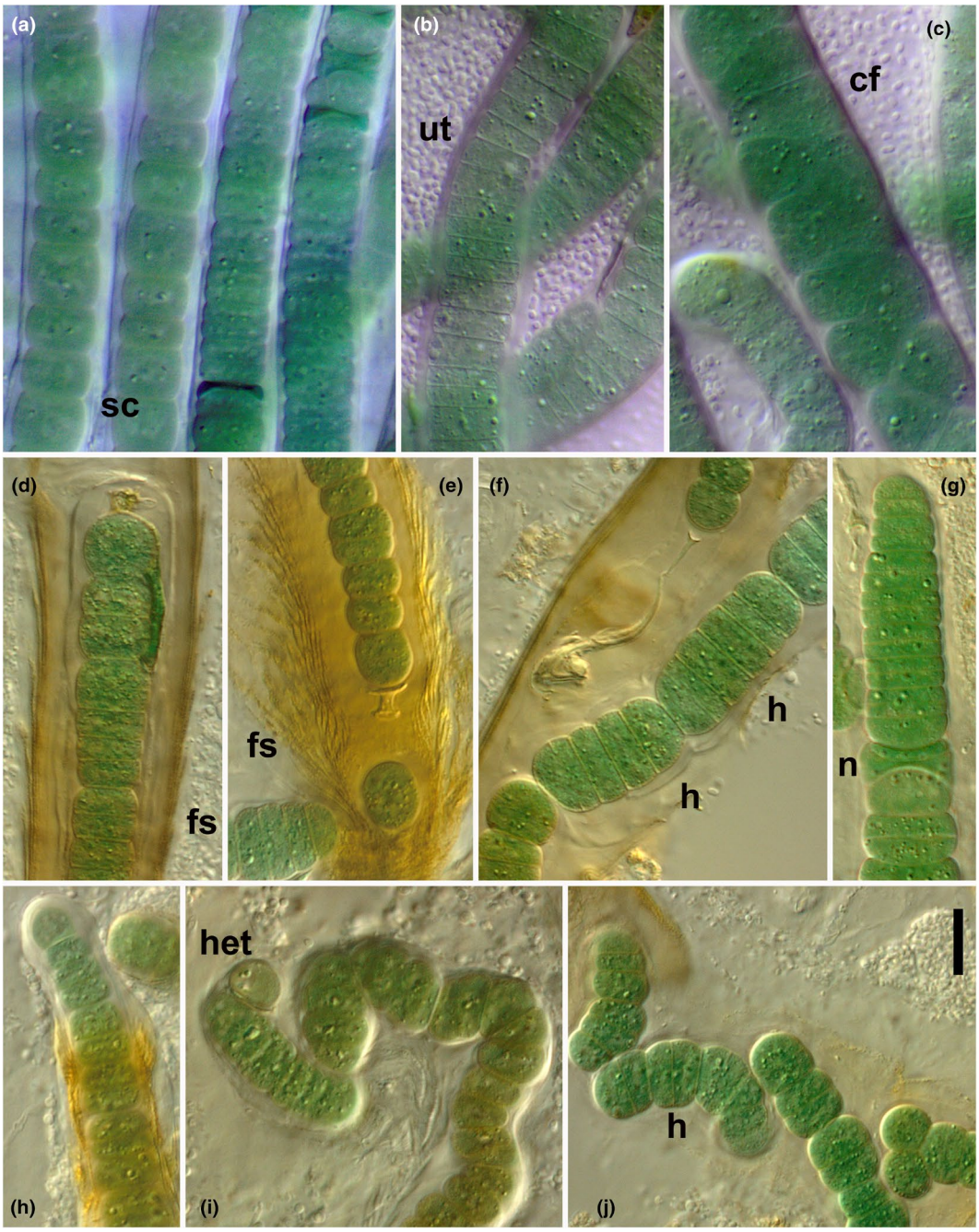

FIGURE 4 *Spirirestis lignicolor*. Letter codes: Ct, coiled trichome; fs, fibrous sheath; h, hormogonia; het, heterocyte; n, necidium; sc, strongly constricted at crosswalls; ut, unconstricted trichome. Scale is 10 μm in all panels.

K.E. Hasenstab-Lehman, 33.230516° N, 119.519103° W. Sampled 25 May 2021. Nearly solid cover by lichen crust.

Reference strain: SNI-TA12-AZ3

Etymology: *lignicolor*, L. color of heartwood before it turns gray, referring to the bright brownish yellow of freshly cut wood evident in the sheath material

Taxonomic notes: This species had very thick sheaths on the filaments which were colored a distinctive light brown color reminiscent of yellow sapwood in conifer trees. It was isolated from a blackened hummock above a stream bend on San Nicolas Island.

Spirirestis williamsae B.Jusko *et* J.R.Johans. sp. nov. Figure 5.

Filaments short, straight, never coiled, frequently single false branched, with double false branching not observed, 12–16 μm wide. Sheath colorless, lamellated, not thickened, encasing the apex. Trichomes distinctly to strongly constricted at the crosswalls, sometimes narrowing toward the apices, 6–12 μm wide. Cells greenish blue-green to olive colored, with conspicuous formation of polyphosphate bodies in stationary phase of growth, shorter than wide, 2.4–9 μm

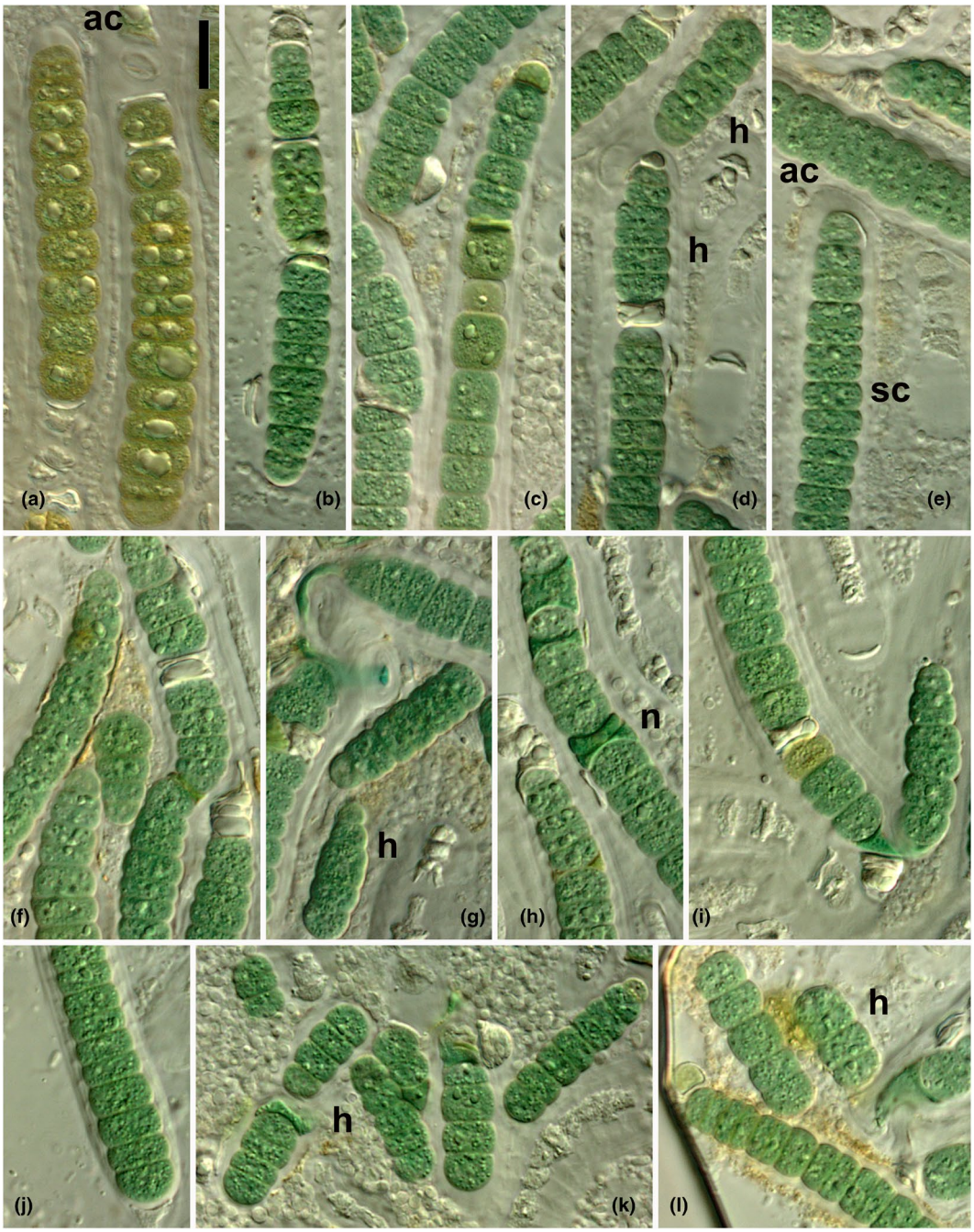

FIGURE 5 *Spirirestis williamsae*. Letter codes: Ac, apical cap; h, hormogonia; n, necridium; sc, strongly constricted at crosswalls. Scale is 10 μm in all panels.

long. Heterocytes rare, forming in intercalary position but causing fragmentation of trichome such that heterocytes appear basal, hemispherical in outline, consistently wider than long, 5.6–9 μm wide, 4–6.6 μm long. Necridia present, bow-like. Hormogonia common, short, 80–150 μm long.

Holotype here designated: SBBG 238542!, herbarium voucher (metabolically inactive) prepared from reference strain, SNI-TA17-BJ30, isolated from poorly developed biological soil crust, upslope from spring origin site, on cliff face with shallow soil pockets. Collected by J. R. Johansen with W. F. Hoyer and K. E. Hasenstab-Lehman. 33.232864° N, 119.517807° W. Sampled 25 May 2021, San Nicolas Island, California, United States.

Reference strain: SNI-TA17-BJ30

Additional strain: LSB87

Etymology: named in honor of Wendy Williams, a prominent soil crust biologist and ecologist in Australia

This is a truly cryptic species identified based upon its phylogenetic isolation (Figures 11, 12) and the percent dissimilarity in the ITS rRNA region when compared to all other strains (Table S3).

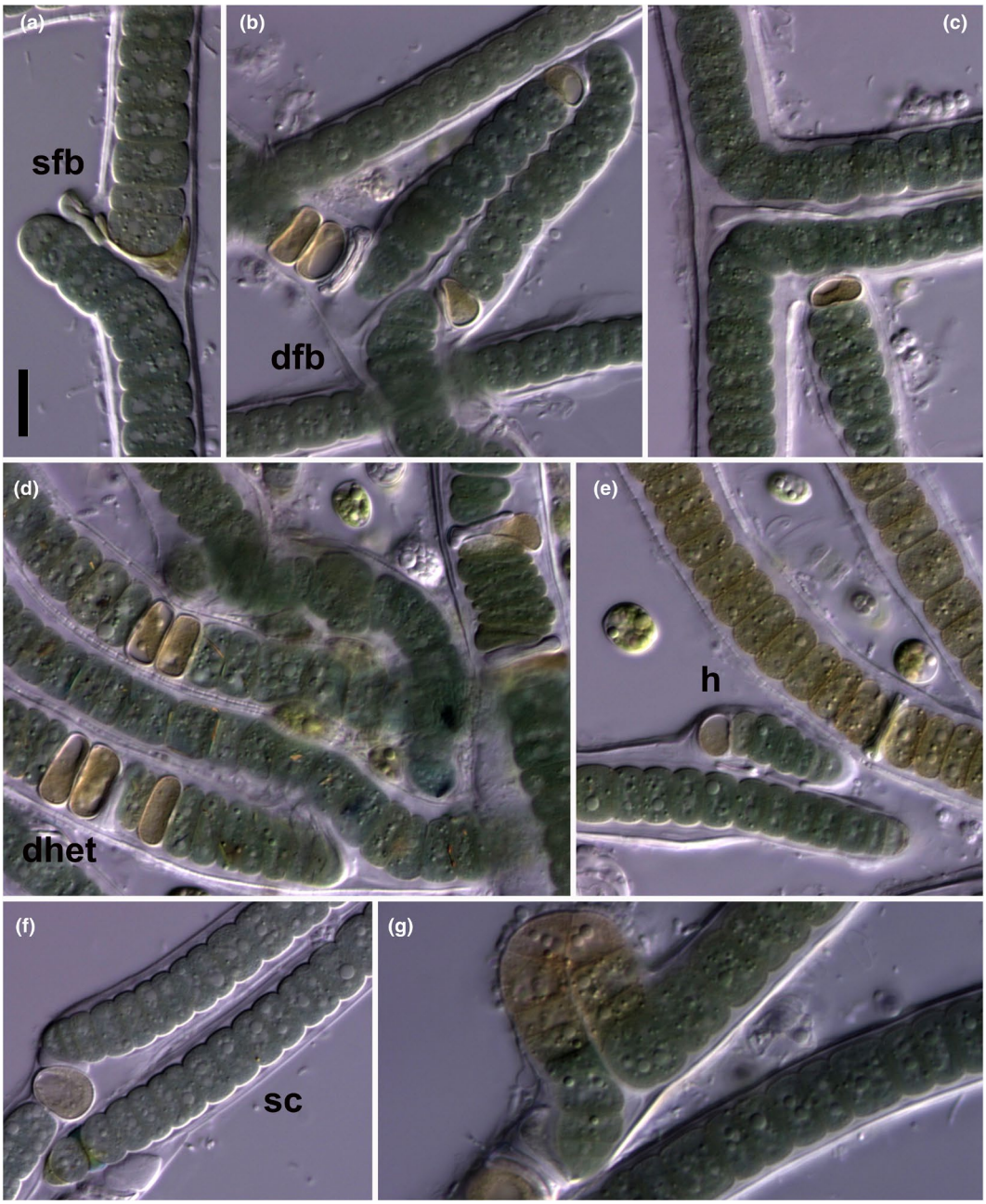

FIGURE 6 *Spirirestis hydroterrestris*. Letter codes: Dfb, double false branch; dhet, double heterocyte; h, hormogonia; sc, strongly constricted at crosswalls; sfb, single false branch. Scale is 10 μm in all panels.

Spirirestis hydroterrestris Prashant Singh sp. nov. Figures 6, 9.

Filaments entangled, sinuous to straight, but never irregularly or spirally coiled, frequently single and double false branched, 10–16 μm wide. Sheath colorless, not lamellated, not thickened, not expanded at the trichome apex, encasing the apex or with trichome extending beyond sheath. Trichomes slightly to strongly constricted at the crosswalls, fragmenting without formation of necridia, sometimes narrowing slightly toward the apices, 7–12 μm wide. Cells slate-blue-green to brownish, with conspicuous formation of polyphosphate bodies, mostly shorter than wide, 3.6–10 μm long. Heterocytes common, forming singly or in pairs, intercalary, compressed and rounded rectangular in outline, 10–11.5 μm wide, 4–8 μm long. Necridia not observed. Hormogonia not observed.

Holotype here designated: GCC202507, portion of reference strain KH22-PS preserved in a metabolically inactive state in Global Collection of Cyanobacteria, Varanasi, India

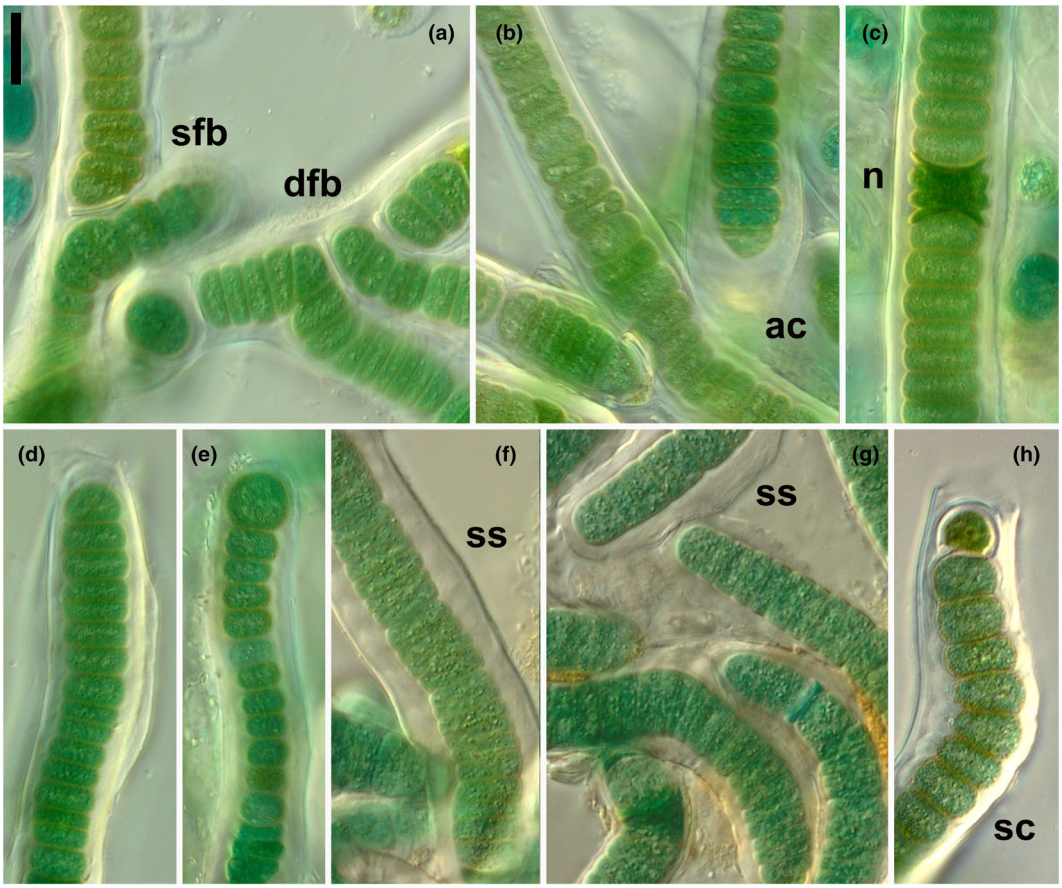

FIGURE 7 *Spirirestis atacamensis*. Letter codes: Ac, apical cap; dfb, double false branch; n, necridia; sc, strongly constricted at crosswalls; sfb, single false branch; ss, structured sheath. Scale is 10 μm in all panels.

Isotype here designated: GCC-botanybhu-202,507, portion of reference strain of KH22-PS on filter paper, archived as a voucher in the herbarium of Banaras Hindu University, Varanasi, India

Reference strain: KH22-PS. Collected by P. Singh, 25.748694° N, 82.698998° E. Sampled 13 June 2019, Khanapatti village, Jaunpur, Uttar Pradesh, India.

Etymology: *hydroterrestris*, L., wet terrestrial, referring to the perennial wet soil from which it was isolated

Taxonomic notes: This species is ecologically separate from all other *Spirirestis* species by its occurrence in perennially wet soil in the tropical climate near Varanasi, India. It is morphologically cryptic but separated from all other species by having 16S rRNA gene PS ≤ 99.1%, ITS rRNA region PD ≥ 6.1% (Table S3), and phylogenetic separation in the ITS rRNA region phylogeny (Figure 12).

Spirirestis atacamensis J.R.Johans. sp. nov. Figures 7, 8d.

Filaments entangled, sinuous, straight to irregularly coiled, but never spirally coiled, singly or doubly false branched, (10)–12–20 μm wide. Sheath colorless, often thick, sometimes irregularly laminated, typically closed, entirely encasing the trichome apex. Trichomes slightly to strongly constricted at the crosswalls, 7–10 μm wide. Cells bright blue-green to olive colored, sometimes with orange coloration in crosswalls, rounded rectangular to barrel-shaped in side view, often with distinct parietal chromoplasm and granular centroplasm evident, 2–6 μm long. Heterocytes very rarely produced, even in nitrogen-free medium, rounded and basal, 9 μm wide, 7–8 μm long. Necridia present, bow-shaped.

Holotype here designated: CBFS-A234-1! Voucher prepared from reference strain, ATA2-1-CV29, isolated from biological soil crust on very fine sandy soil. Collected by J. R. Johansen with K. Osorio-Santos, L. Baldarelli, S. Warren, L. Aguilera, and K. Godoy. 29°18.989′ S, 71°14.919′ W. Sampled 12 May 2009, Atacama Desert near Choros Bajos.

Isotype here designated: CBFS-A235-1! Voucher prepared from strain, ATA2-1-KO21, isolated from biological soil crust on very fine sandy soil. Collected by J. R. Johansen with K. Osorio-Santos, L. Baldarelli, S. Warren, L. Aguilera, and K. Godoy. 29°18.989′ S, 71°14.919′ W. Sampled 12 May 2009, Atacama Desert near Choros Bajos.

Reference strain: ATA2-1-CV29

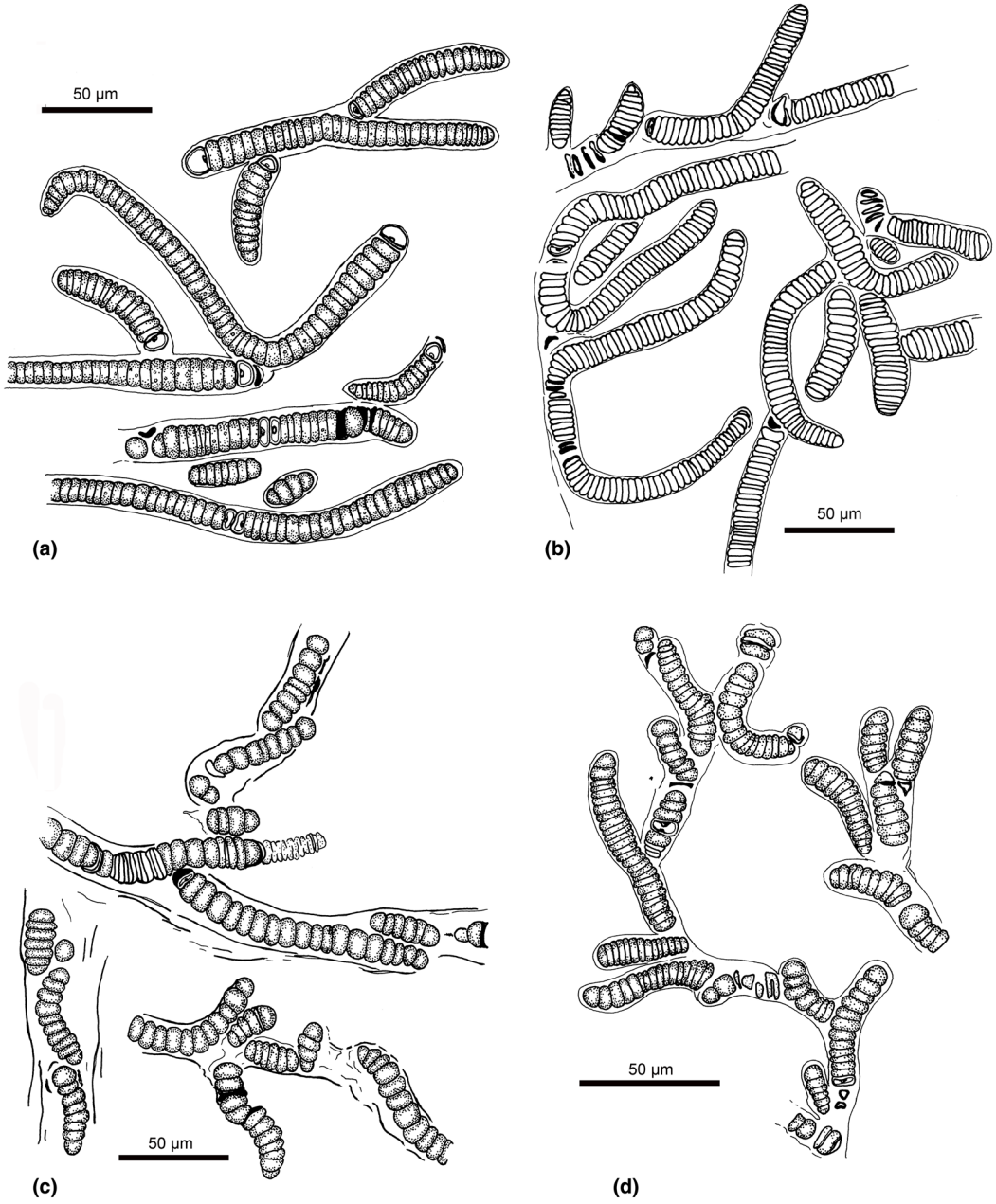

FIGURE 8 Line drawings of select species of *Spirirestis*. (a) *S. californica*, (b) *S. pseudoramosissima*, (c) *S. lignicolor*, (d) *S. atacamensis*.

Additional strains: ATA2-1-KO21, ATA2-3-CV2

Etymology: *atacamensis*, L. living in the Atacama, referring to its collection from soils of the Atacama Desert, Chile

Taxonomic notes: This is the only southern hemisphere species identified so far, occurring in the Atacama Desert in Chile. It is morphologically cryptic, but phylogenetically distinct in the ITS rRNA region BI analysis (Figure 12) and has an ITS rRNA region PD ≥ 2.9% for all interspecies comparisons (Table S3).

Molecular analyses

The 16S rRNA gene phylogeny of Tolypothrichaceae plus the Nostocales outgroup showed that habitat preference was strongly aligned with phylogenetic position (Figure 10). All Tolypothrichaceae strains growing on soil, which contained *Spirirestis rafaelensis* (Figure 10, top triangle), were clearly separated from the Tolypothrichaceae from aquatic and subaerial habitats. The single exception was a terrestrial *Tolypothrix* sp. from Canyonlands National Park

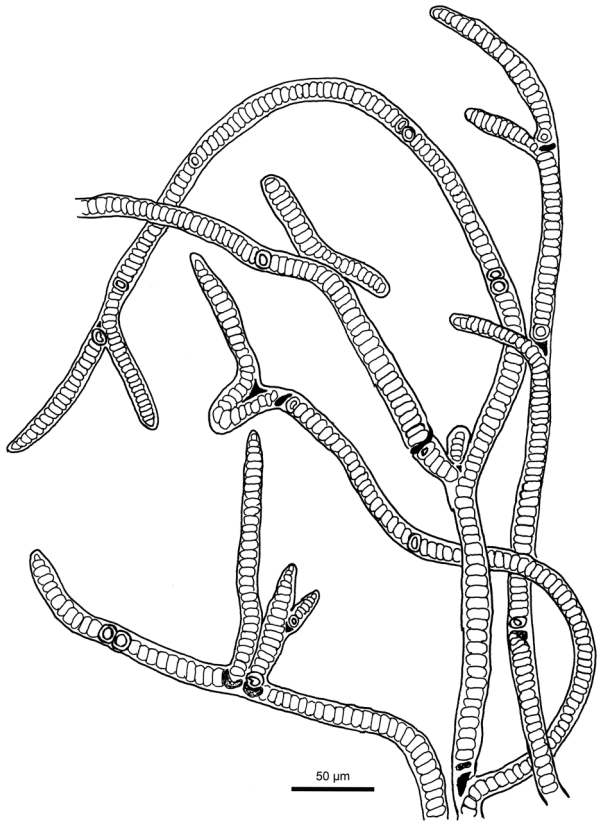

FIGURE 9 Line drawing of *S. hydroterrestris*.

(strain CNP3-B1-c1), which fell outside of the terrestrial *Spirirestis* clade (Figure 10). Wet subaerial strains were intermixed with truly aquatic strains, although trends associating basal Tolypothrichaceae clades with a specific habitat were still evident. The aquatic/subaerial clade contained *Tolypothrix distorta* ACOI 731, *Hassallia byssoidea* CCALA 823, and *Rexia erecta* CAT4-SG4, all considered to be reference strains for those species and genera (Hauer et al., 2014). This clade also contained *Coleodesmium wrangeli* MC-JRJ1, which was collected, characterized, and sequenced by our team and was determined to fit this species based on morphology and ecology. Sequence data of reference strains for *Hassallia antarctica, Hassallia andreassenii, Dactylothamnos antarcticus, Kryptousia macronema, Kryptousia microlepis, Toxopsis calypsus*, and *Godleya alpina* were also present in the phylogeny. The Godleyaceae (*Godleya* and *Toxopsis*) were embedded within the Tolypothrichaceae, but the long branch length suggests this could be an artifact of long-branch attraction. Many genera within the aquatic/subaerial clade were not monophyletic, highlighting the need for further revisionary work in this group.

The soil-inhabiting strains within the collapsed *Spirirestis* triangle in Figure 10 are shown uncollapsed in Figure 11. The clade contains several strains assigned to either *Tolypothrix* or *Hassallia*, all of which we consider to belong to *Spirirestis*. This conclusion is further supported by the high sequence similarity among 16S rRNA gene sequences of these taxa, with all pairwise comparisons showing PS ≥ 97.3% (Table S2). With the exception of KH22-PS, all strains in Figure 11 were isolated from arid to semi-arid climates in soils that remain dry for most of the year (Table S1). KH22-PS was located in perennially wet soils in a tropical climate. The high similarity of *Spirirestis* 16S rRNA gene sequences suggests that this marker alone is insufficient to delineate most species.

The phylogeny based on aligned 16S-23S ITS rRNA region sequences, however, clearly separated the species (Figure 12). The largest clade, *Spirirestis rafaelensis*, contained the two strains known to produce tight coiling (WJT71-NPBG6 and SRS70), and was in a supported clade (1.00 posterior probability from BI analysis, 63% boot strap value from ML analysis) separate from all other *Spirirestis* species. Percent dissimilarity (PD) values for the ITS rRNA regions were low among all *S. rafaelensis* strains (0.0%–3.3%), supporting their classification as a single species (Table S3). *Spirirestis californica* strains (primarily from San Nicolas Island) showed evidence of possessing two ribosomal operons. Both operon types were present in strain SNI-TA31-BJ5, allowing us to distinguish operon types across other sequences from this species. The two operon types had PD = 7.5%, a value that would typically indicate distinct species if they did not occur in the same strain (Table S3).

Spirirestis species named in this study—*S. rafaelensis, S. californica, S. williamsae, S. atacamensis, S. pseudoramosissima, S. lignicolor* and *S. hydroterrestris*—were separated from each other by having PD ≥ 2.9%. The single exception was the species pair *S. williamsae* and *S. californica*, which had PD = 2.1%–2.9% (Table S3), values often observed within a single species. However, both 16S rRNA gene (Figure 11) and ITS rRNA region (Figure 12) phylogenies supported their separation into distinct species.

The ITS rRNA region secondary structures were also informative (Figure 13). The D1–D1′ helix exhibited sequence variability, but its secondary structure was identical across all species. Variable bases were indicated in both *Spirirestis rafaelensis* and *S. californica*, the two species for which multiple strains were analyzed (Figure 13a,b). The Box-B helix was structurally identical across all species (Figure 13h). The V3 helix displayed five different structural variants evident across all species (Figure 13i–m). *Spirirestis rafaelensis* had Types A-D (Figure 13i–l), whereas other species were limited to a single V3 helix type, possible due to undersampling of operons. *Spirirestis hydroterrestris* had type C, *S. atacamensis* and *S. lignicolor* had Type D, and

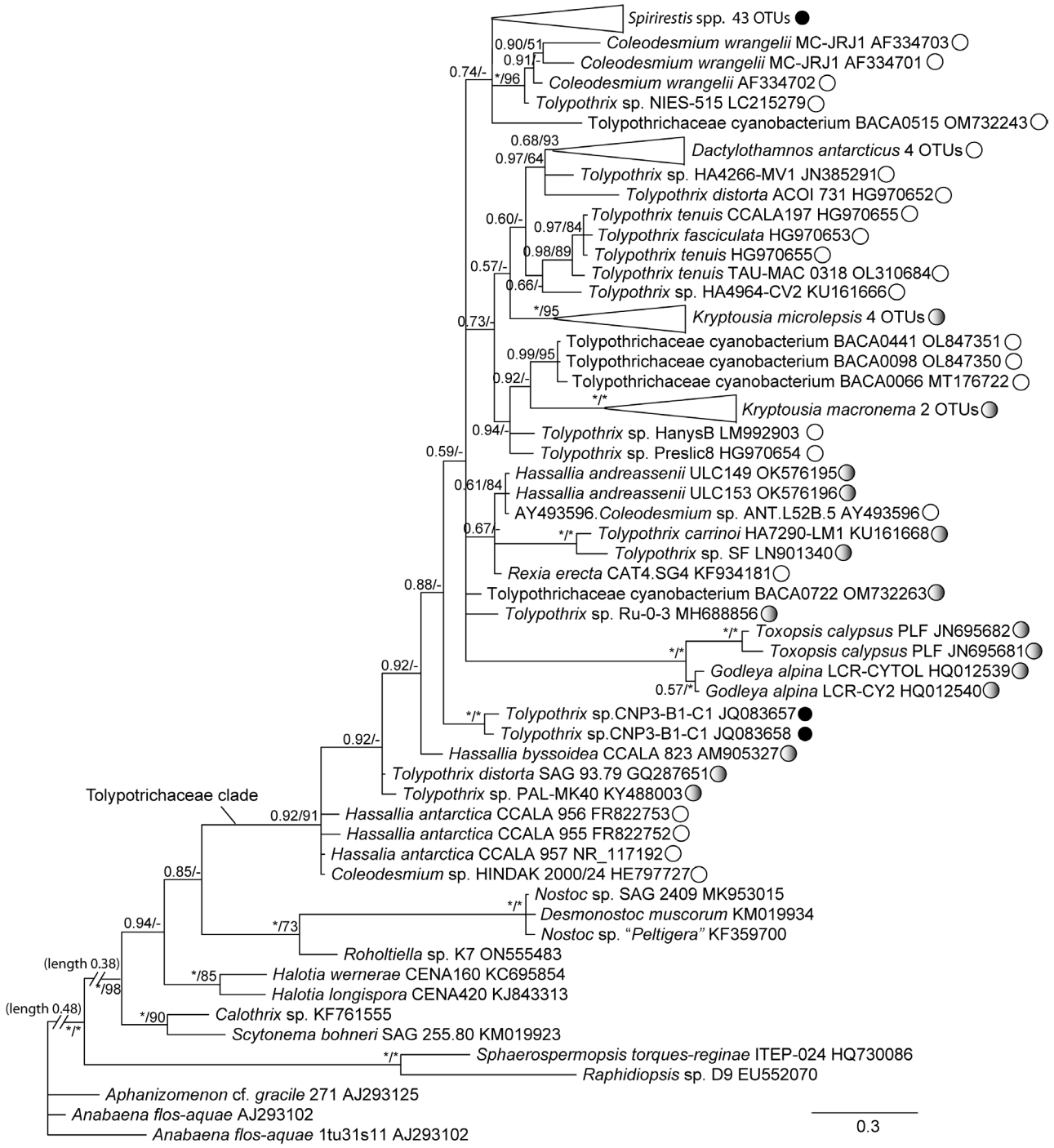

FIGURE 10 Tolypothrichaceae BI 16S rRNA gene analysis with bootstrap support from ML analysis mapped to nodes. The soil clade containing *Spirirestis* species appears as a collapsed triangle at the top of the tree (see Figure 2 for uncollapsed *Spirirestis* clade). Black circles indicate soil strains, clear circles indicate aquatic strains, partially gray and white circles indicate subaerial on hard damp substrate strains.

S. californica had Type E. The V2 helices were highly variable, even within species (Figure 14). *Spirirestis rafaelensis* and *S. californica* exhibited considerable intraspecies variation (Figure 14a–k), with the second operon in *S. californica* differing substantially but remaining consistent within operon type (Figure 14u–w). Species with fewer analyzed strains had distinct V2 structures, including *S. pseudoramosissima*, *S. lignicolor, S. williamsae*, and *S. atacamensis*. Unspeciated strains (the KZ strains and SEV2-5-2) had unique structures (Figure 14q–s), although KZ-2-2-3 closely resembled the V2 structure of *S. lignicolor*.

These molecular analyses confirm that the species of *Spirirestis* are well separated by genetic criteria.

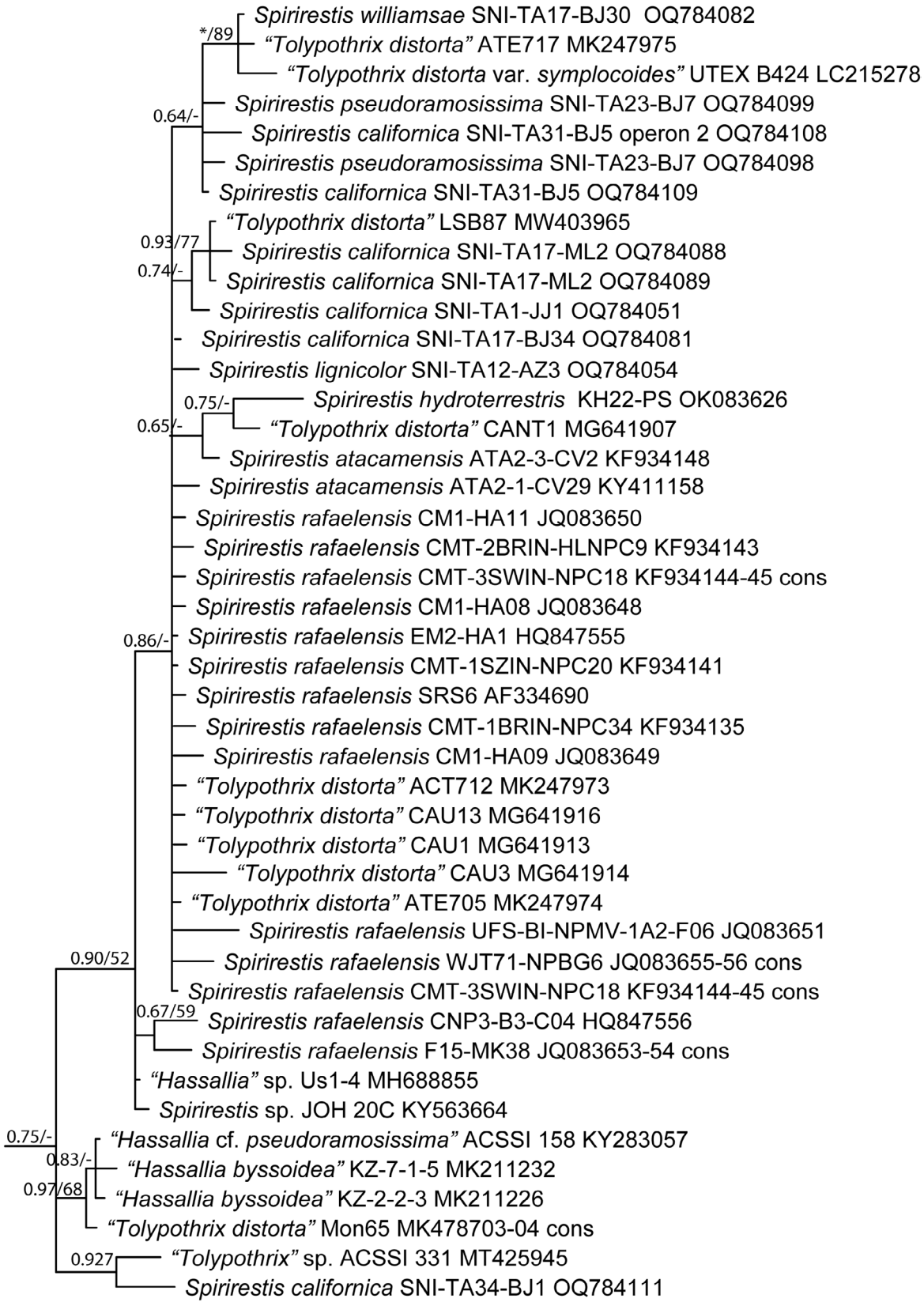

FIGURE 11 *Spirirestis* clade 16S rRNA gene tree uncollapsed from analysis in Figure 1. All strains were isolated from soil.

Additional species may eventually be split from *S. rafaelensis*, given the molecular and geographic diversity among its strains. There are also a number of sequences for strains that have been sequenced by other researchers or lost by us since sequencing (*Spirirestis* species 6–10, see Table S3).

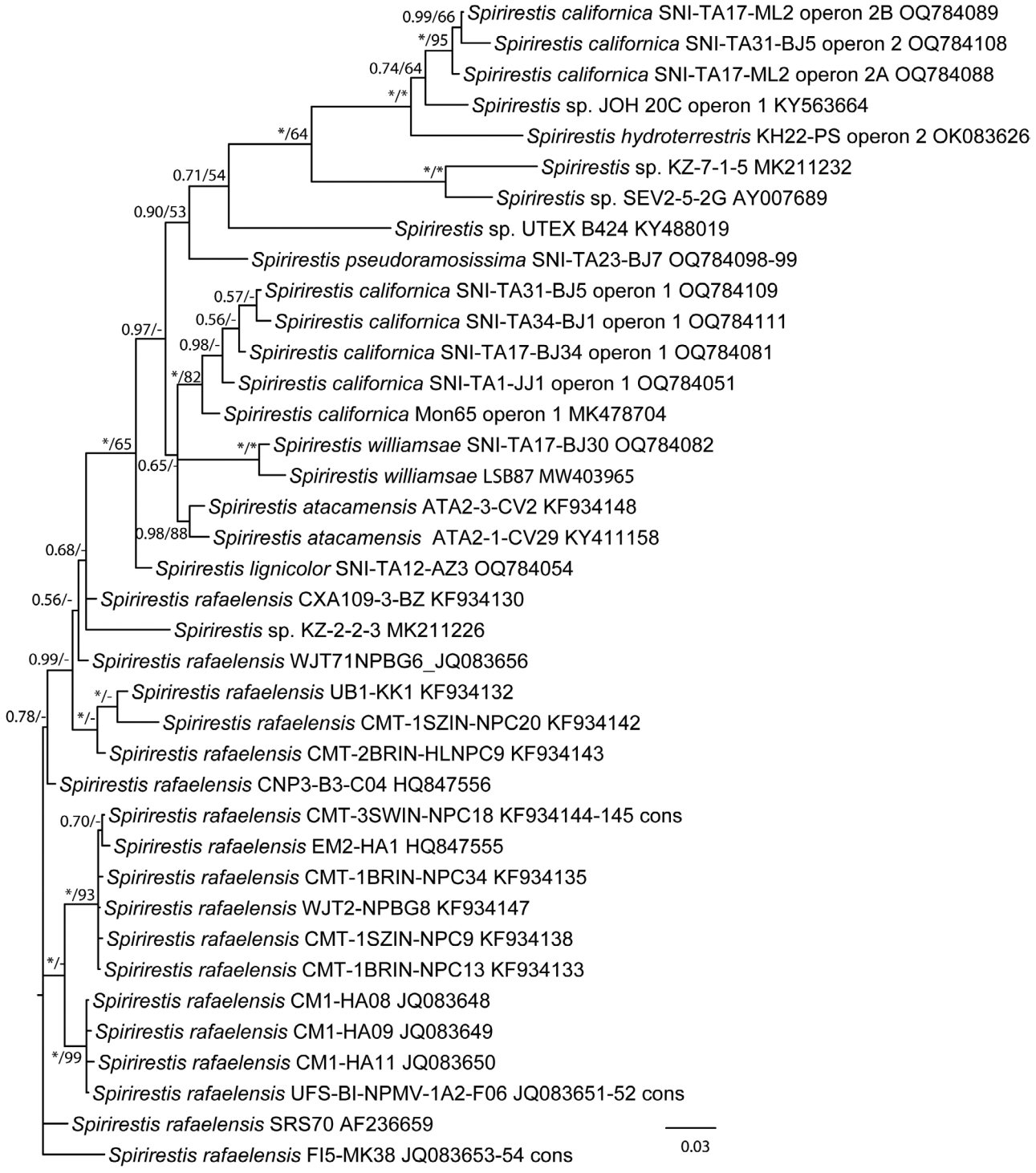

FIGURE 12 *Spirirestis* clade 16S-23S ITS rRNA region BI analysis with bootstrap support from MP analysis mapped to nodes.

Morphological separation

The species of *Spirirestis* are semicryptic. Although some morphological differences exist, species delineation is best achieved by integrating morphological data with biogeography and ecology. Morphometric dimensions often overlap (Table S1), with filaments measuring 10–20 μm wide, trichomes 4.6–16 μm wide, cells 1.5–11 μm long, and heterocytes 5–15 μm wide by 3–12 μm long. Although species have distinct size ranges, they all exhibit some overlap. *Spirirestis lignicolor* is distinctive due to its very wide sheaths (up to 35 μm). *Spirirestis rafaelensis* can form heterocytes up to 20 μm long, whereas all other species have maximum heterocyte lengths of 6.6–12 μm. Only two species, *S. rafaelensis* and *S. lignicolor*, have been observed

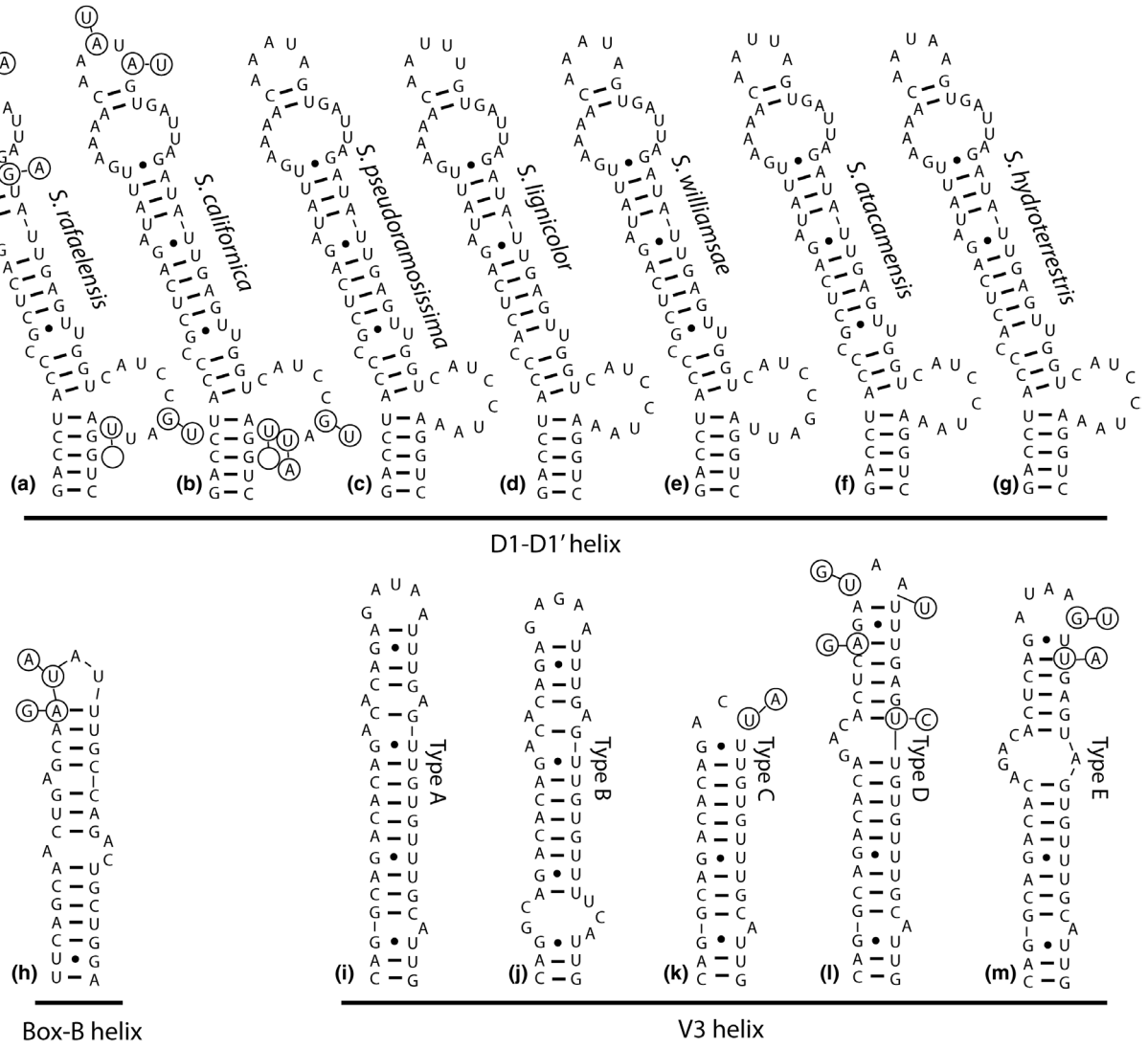

FIGURE 13 Secondary structures of ITS rRNA region conserved domains for D1–D1′, Box-B, and V3 helices. Bases in circles are alternate nucleotides present in some representatives of the species or helix type, with empty circles representing nucleotide loss. For explanation of V3 helix types see text.

producing brownish sheaths, though during exponential growth, their sheaths remain colorless, like those of the other species. *Spirirestis pseudoramosissima* exhibits abundant and repeated false branching, whereas *S. californica* makes short, straight trichomes in exponential growth phase. The degree of trichome constriction at crosswalls and the presence or absence of coiling serve as additional diagnostic features for some species. But unless these characters are considered along with ecology and distribution, it would normally be difficult to identify the species without molecular sequence analysis.

DISCUSSION

With the publication of this manuscript, *Spirirestis* expands from a monospecific genus to a moderately diverse genus with seven described species. Despite this expansion of the genus, we anticipate additional species will soon be described. We have taken a conservative approach in recognizing species. For example, *S. californica* SNI-TA34-BJ1 and *S. californica* Mon65 had 16S rRNA gene PS ≤ 98.6% for all comparisons with other *S. californica* strains, and these two strains were phylogenetically separated from *S. californica* in the 16S rRNA phylogeny (Figure 11), suggesting they could represent one or two separate species. However, the ITS rRNA region phylogeny placed them clearly in the *S. californica* clade with type 1 operons. Given this discrepancy in molecular phylogenetic signals and their morphological indistinguishability from *S. californica*, we have chosen to retain these strains within *S. californica* for now. A similar example can be seen in the block of strains we assigned to *S. rafaelensis*. Although most between-strain comparisons

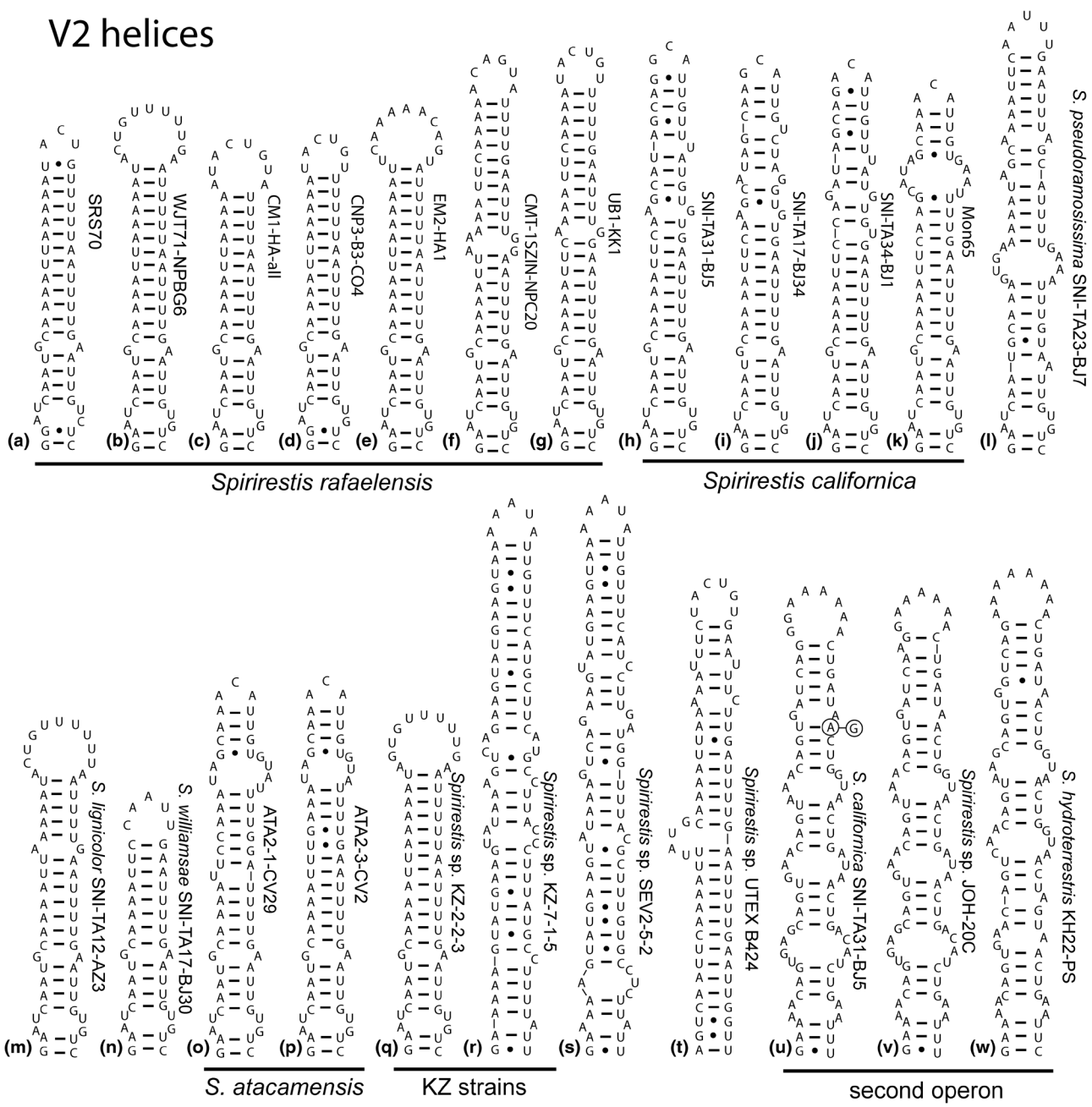

FIGURE 14 Variation in V2 helix structure in all species of *Spirirestis*.

exceeded the 16S rRNA gene 98.7% threshold for same species, some strains fell below this threshold and could instead be considered separate species. However, we lacked ITS rRNA region sequence data for some of these strains, and for many *S. rafaelensis* strains, the ITS rRNA region PD was ≤3.0, which does not support the separation suggested by the 16S rRNA gene percent similarity. Several additional strains that were not in our possession also appear to be new species. In particular, the Ukrainian KZ strains (KZ-7-1-5 and KZ-2-2-3, see Mikhailyuk et al., 2018) appear to be clearly separate from all seven described species. Other strains, such as ATE712 and ATE717 from Mexico (Becerra-Absalón et al., 2019) and the CAU/CANT strains from Spain (Roncero-Ramos et al., 2019) may also represent species new to science. We encourage the researchers in possession of these strains to consider undertaking the description of these potential species. As terrestrial cyanobacteria continue to be isolated and studied, additional species within *Spirirestis* will undoubtedly be discovered.

Some *Spirirestis* species exhibit highly divergent ribosomal operons. For example, *Spirirestis californica* SNI-TA31-BJ5 possessed two operons in which the ITS rRNA regions are quite dissimilar (PD = 7.5%, see Table S3). This allowed us to recognize these

operons in the other *S. californica* strains. The two operons differed extensively in the sequence between the two tRNA genes known as the V2 region (see also Figure 14h,u). Similarly, the Ukrainian strains (KZ-2-2-3 and KZ-7-1-5) also showed major differences in the V2 helix structure (Figure 14q,r) and sequence, with a high PD (7.6%), but these differences could very well be the result of sequencing different operons of the same species. The 16S rRNA gene PS was 99.7%, which suggests these strains are the same species. We applied the DIV metric, developed for detecting paralogous ribosomal operons (Villanueva et al., 2024), but found that the D1–D1′ helices, which commonly serve as a marker to indicate paralogy, were nearly identical across *Spirirestis*. This consistency suggests that these operons were duplicated relatively recently, followed by diversification of the V2 helix, rather than having been acquired by horizontal gene transfer of a ribosomal operon from a closely related genus. Without sufficient sampling and consideration of orthology, the potential for erroneous taxonomic conclusions arising from misinterpretation of operon variation remains a concern (Bohunická et al., 2024; Pietrasiak et al., 2019, 2021; Saraf et al., 2024; Villanueva et al., 2024).

The molecular evolution of *Spirirestis* ITS rRNA region domains provided some insight on the selective pressures acting on cyanobacterial ribosomal operons. The V2 helix exhibited significant sequence and structural divergence among taxa, in contrast to the strong conservation observed in the other helical secondary structures, including the D1–D1′ and the Box-B helices. This disparity suggests that the V2 experienced weaker purifying selection than the other domains, raising the possibility that relaxed constraints on its function facilitate relatively rapid diversification. One plausible explanation for this is that the V2 helix offers adaptive ribosomal structural plasticity that may respond to varying environmental conditions; alternatively, the recent gene duplication event we propose may have led to a neofunctionalization of ancestral helices. However, additional studies on the function of the ITS rRNA region and its individual domains are needed before conclusions can be drawn. Studies involving phylogenetic analyses treating each ITS rRNA region domain as a separate gene may elucidate the differential pressures, if any, acting on these regions.

Although our molecular evidence for species delineation within *Spirirestis* was primarily based on 16S rRNA gene and ITS rRNA region divergence, these conventional methods have inherent limitations. Recent studies have shown that ribosomal markers fail to capture much of the true genetic diversity, and it is well documented that the 16S rRNA gene is insufficient to resolve species (Fox et al., 1992; Yarza et al., 2014), a problem that is even more pronounced among members of the Nostocales, in which clearly differentiated taxa share high 16S rRNA gene sequence identity, even with high ITS rRNA region dissimilarity (Baldarelli et al., 2022; Bohunická et al., 2015; Casamatta et al., 2006; Jusko & Johansen, 2024). Integrative approaches to taxonomy (i.e., the polyphasic approach) that combine ecological, genetic, morphological, and other types of data offer a more robust framework for resolving species boundaries that would be otherwise ambiguous with only limited genetic information. In the context of *Spirirestis*, multifaceted methods help clarify conflicting signals from the 16S rRNA gene and ITS rRNA region phylogenies. Although genome-level data analyses (e.g., whole-genome sequencing and average nucleotide identity) hold potential to revolutionize our understanding of cyanobacterial evolution, caution is warranted in their current application: Genomic datasets available for cyanobacteria remain relatively limited, and there is a lack of consensus on the best practices for harmonizing these methods with those employing traditional genetic markers. Further studies, for now, should therefore adopt a cautious approach, using established ecological, molecular, morphological, and biogeographical data as a tool to refine genomic insights.

One of the key conclusions from this study is that habitat preference and ecology are critical factors for recognizing genera. We demonstrated unambiguous phylogenetic separation between soil-inhabiting species in the Tolypothrichaceae (*Spirirestis*) from the aquatic and wet subaerial taxa (*Tolypothrix, Hassallia, Coleodesmium, Dactylothamnos*, and *Kryptousia*). This pattern is not a new finding, as several cyanobacterial genera are confined to soil habitats, including *Pseudoacaryochloris* (Johansen et al., 2025), *Myxacorys* (Pietrasiak et al., 2019), *Trichotorquatus* (Pietrasiak et al., 2021), *Symplocastrum* (Pietrasiak et al., 2014), *Mojavia* (Baldarelli et al., 2022; Řeháková et al., 2007), and *Kastovskya* (Jusko et al., 2024; Mühlsteinová et al., 2014). Some genera are restricted to marine environments, such as *Moorena* (Engene et al., 2012; Tronholm & Engene, 2019), *Crocosphaera*, *Rippkaea*, and *Zehria* (Mareš et al., 2019), as well as *Brachytrichia, Kyrtuthrix*, and *Nunduva* (González-Resendiz et al., 2018; Johansen et al., 2021; León-Tejera et al., 2016). Although some genera exhibit broad ecological ranges, habitat of occurrence remains a critical factor in the identification of both genera and species. Within the Tolypothrichaceae, the strong ecological partitioning between terrestrial (*Spirirestis*) and aquatic genera is consistent with studies on other broad microbial groups (Hanson et al., 2012; Martiny et al., 2006) which demonstrate similar trends. However, in those studies, such a strong ecological affinity (i.e., aquatic vs. terrestrial) was typically observed at higher taxonomic levels. Nevertheless, this pronounced partitioning suggests that transitions between these markedly different habitats are intrinsically challenging and rare. Moving from consistently moist aquatic environments with dissolved nutrients, to arid, terrestrial habitats, which are

subject to desiccation and nutrient limitation, presents often-insurmountable evolutionary and physiological challenges. The divergence of *Spirirestis* from related genera may thus have been driven by pre-existing traits that favored survival in these challenging conditions. In contrast, the transition from aquatic to subaerial environments appears to be somewhat easier, which may explain the weaker yet discernable trends in habitat preference among other Tolypothrichaceae genera. This observed correlation between habitat and evolutionary relatedness strongly suggests that environmental selection is a key driver of both functional and genetic divergence. As the taxonomic level at which significant ecological separation can exist seems inconsistent across cyanobacterial lineages, future research integrating genomic analyses with detailed ecological assessments will likely be necessary to determine the relative role of habitat preference in the divergence of each cyanobacterial lineage separately.

In addition to ecological specialization, differences in dispersal potential also appear to play a role in shaping the genetic and biogeographical patterns observed within *Spirirestis*, which is broadly distributed. Although soil-dwelling taxa as a whole are generally more dispersal limited compared to their aquatic counterparts due to lack of vectoring and habitat discontinuity, finer-scale differences in dispersal potential exist among and within terrestrial lineages. Some *Spirirestis* species, such as *S. rafaelensis* and *S. californica*, demonstrate apparently higher dispersal capabilities. Although most *Spirirestis* species and observational records of the genus originate from the United States, representative strains have also been recovered from Mexico, Chile, Spain, Germany, Ukraine, China, and India. Most strains of *S. rafaelensis* are from California and Utah deserts, but a single strain of this species was also observed in soils of the Gurbantünggüt Desert, Xinjiang Province, China. This suggests that *S. rafaelensis* may have fewer limitations on dispersal than other soil taxa, even within its genus. *Spirirestis californica*, conversely, is relatively common on San Nicolas Island in California, but it has also been reported from Spain (strain Mon65, see Table S2). These two examples indicate at least some species have fewer limitations on dispersal than other soil taxa, a hypothesis which is further supported by the single observation of *S. lignicolor*, recorded only once on San Nicolas Island. Because several of these taxa live on San Nicolas Island and theoretically experience similar vectoring potential, it follows that intrinsic variations in dispersal potential underlie the unequal distributional patterns observed. Consequently, both habitat preference and dispersal potential likely interact to drive (especially terrestrial) biogeographical patterns and genetic divergence. In light of our limited understanding of microbial biogeography, more direct studies are needed to refine these hypotheses.

Although *Spirirestis* is monophyletic and ecologically cohesive, the remaining Tolypothrichaceae genera are intermixed, indicating a need for further taxonomic revision. Several genera in the family remain problematic. For example, the recently described *Kryptousia* is already demonstrably polyphyletic (Figure 10). *Dactylothamnos* is sister to the reference strain for *Tolypothrix distorta* (ACOI 731, see Figure 10). *Coleodesmium* and *Hassallia* are polyphyletic as well. Achieving monophyletic genera will require either recognizing additional genera or collapsing some existing ones. Likely, a combination of both approaches will be necessary. Moreover, the long branch on which the Godleyaceae is positioned (Figure 10) raises the possibility that *Toxopsis* and *Godleya* could be separated from the Tolypothrichaceae with additional taxon sampling. However, at present, recognizing additional families within this group is not warranted.

Looking ahead, we anticipate that continued discovery and description of novel cyanobacterial diversity will shift the future of cyanobacterial taxonomy. The Baas-Becking hypothesis that "everything is everywhere *but* the environment selects" (Baas-Becking, 1934; De Wit & Bouvier, 2006) does not appear to hold true for many cyanobacteria, especially terrestrial taxa. Instead, soil and subaerial taxa appear to be more dispersal limited, leading to geographic isolation and subsequent diversification of populations into new, locally-adapted lineages (Johansen et al., 2025; Jusko et al., 2024; Jusko & Johansen, 2024). Thus, while the environment selects, it is clear that everything is not everywhere. Ribosomal genes evolve slowly due to the essential role of rRNA in cellular metabolism, making them useful for phylogenetic analyses given their stability over evolutionary time. However, this slower evolutionary rate necessarily overlooks much of the full spectrum of genetic change occurring among populations. Future genomic studies will likely reveal greater differences in protein-coding genes, particularly in the core genome (house-keeping genes) and the shell genome (genes present in some but not all cyanobacteria, often acquired via horizontal gene transfer). These analyses may help clarify the effects of directional selection on cyanobacterial lineages and further refine species recognition. As genomic data become more widely available, it is anticipated that species delineation will increasingly rely on protein-coding genes, potentially leading to the identification of many new taxa. Going forward, integrating more detailed genomic studies with ecological data will be essential to refine our understanding of how habitat, distributional patterns, and evolutionary processes shape cyanobacterial diversity. By comparing genetic data from various ecological zones, we can gain a deeper insight into the relationship between molecular adaptations, ecology, and phylogeny.

AUTHOR CONTRIBUTIONS

Jeffrey R. Johansen: Formal analysis (lead); investigation (equal); writing – original draft (lead); writing – review and editing (lead). **Brian M. Jusko:** Investigation (equal); writing – review and editing (supporting). **Nicole Pietrasiak:** Investigation (equal); writing – review and editing (supporting). **Hend Alwathnani:** Investigation (supporting). **Natalie Soliman:** Investigation (supporting). **Anastasia Zhydan:** Investigation (supporting). **Salvadore Peron:** Investigation (equal). **Mathew Luknis:** Investigation (supporting). **Karina Osorio-Santos:** Investigation (equal). **Klára Řeháková:** Investigation (supporting). **Bingchang Zhang:** Investigation (supporting). **Kristen E. Hasenstab-Lehman:** Funding acquisition (equal); investigation (supporting). **William F. Hoyer:** Funding acquisition (equal); investigation (supporting). **Sagarika Pal:** Investigation (supporting); writing – original draft (supporting). **Prashant Singh:** Conceptualization (lead); investigation (equal); project administration (equal); visualization (lead); writing – original draft (equal); writing – review and editing (supporting).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Comparison among species. All dimensions in μm, abbreviations: Fila. = filament, enc. = encasing the apex, cons. = constricted at crosswalls, coil. = regularly spirally coiled, mar. = maritime climate.

Table S2. Percent similarity of 16S rRNA gene sequences. For strains not placed in a species by us, we report the taxon name as it appears in GenBank.

Table S3. Percent dissimilarity among aligned 16S–23S ITS rRNA regions for all *Spirirestis* strains for which ITS rRNA region sequence data are available. Strains highlighted in the same color represent the same species according to established ITS rRNA region sequence thresholds (>7.0% strong evidence of different species, >4.0% likely different species, <3.0% likely same species). Note that two different ribosomal operons were observed in *S. californica* and are indicated by different shades of green highlight. The rarer operon in *S. californica* was also recovered in *S. hydroterrestris* and *Spirirestis* sp. 6 JOH-20C. Comparisons between paralogous operons should not be used for making taxonomic decisions.

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