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RESEARCH ARTICLE

Two new species of *Dulcicalothrix* (Nostocales, Cyanobacteria) from India and erection of *Brunnivagina* gen. nov., with observations on the problem of using multiple ribosomal operons in cyanobacterial taxonomy

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Abstract

Two new species of *Dulcicalothrix, D. adhikaryi* sp. nov. and *D. iyengarii* sp. nov., were discovered in India and are characterized and described in accordance with the rules of the International Code of Nomenclature for algae, fungi, and plants (ICN). As a result of phylogenetic analysis, *Calothrix elsteri* is reassigned to *Brunnivagina* gen. nov. During comparison with all *Dulcicalothrix* for which sequence data were available, we observed that the genus has six ribosomal operons in three orthologous types. Each of the three orthologs could be identified based upon indels occurring in the D1–D1′ helix sequence in the ITS rRNA region between the 16S and 23S rRNA genes, and in these three types, there were operons containing ITS rRNA regions with and without tRNA genes. Examination of complete genomes in *Dulcicalothrix* revealed that, at least in the three strains for which complete genomes are available, there are five ribosomal operons, two with tRNA genes and three with no tRNA genes in the ITS rRNA region. Internal transcribed spacer rRNA regions have been consistently used to differentiate species, both on the basis of secondary structure and percent dissimilarity. Our findings call into question the use of ITS rRNA regions to differentiate species in the absence of efforts to obtain multiple operons of the ITS rRNA region through cloning or targeted PCR amplicons. The ITS rRNA region data for *Dulcicalothrix* is woefully incomplete, but we provide herein a means for dealing with incomplete data using the polyphasic approach to analyze diverse molecular character sets. Caution is urged in using ITS rRNA data, but a way forward through the complexity is also proposed.

KEYWORDS
16S-23S ITS rRNA region, *Brunnivagina* gen. nov., Calotrichaceae, Cyanobacteria, *Dulcicalothrix*, multiple ribosomal operons, ITS rRNA region percent dissimilarity

Abbreviations: BI, Bayesian inference; CHAB, Harmful Algae Biology Herbarium of the Institute of Hydrobiology, Wuhan, China; ESS, estimated sample size; GCC, Global Collection of Cyanobacteria; ITS, internal transcribed spacer; ML, maximum likelihood; PCR, polymerase chain reaction; PD, percent dissimilarity among ITS sequences; PI, percent identity among 16S rRNA sequences; PSRF, potential scale reduction factor.

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INTRODUCTION

Cyanobacterial taxonomy has received a great deal of interest in the last 20 years, with descriptions of new species and new genera in the phylum being made at an ever-increasing pace (Komárek et al., 2014; Kaštovský et al., 2023). This has been a result of the increasing ease of obtaining high-quality sequences of the 16S rRNA gene and the associated ITS rRNA region between the 16S and 23S rRNA genes (ITS rRNA region) and the acceptance and application of several pragmatic criteria for species and genus recognition using these data. Specifically, percent identity thresholds for the 16S rRNA gene (≤98.7% indicates different species, and ≤94.5% indicates different genera; see Yarza et al., 2014) and percent dissimilarity thresholds for the ITS rRNA region (≥7.0% indicates different species, and ≤3.0% indicates same species; see Osorio-Santos et al., 2014; Becerra-Absalón et al., 2018; Pietrasiak et al., 2014, 2019, 2021) have been used regularly as evidence for lineage separation that allows application of evolutionary species concepts, particularly the monophyletic species concept (Johansen & Casamatta, 2005). Phylogenies based on both 16S rRNA gene sequences and ITS rRNA regions have also provided critical evidence for species and genus recognition (Heidari et al., 2018; Saraf, Dawda, & Singh, 2019; Kaštovský et al., 2023; Luz, Cordiero, Kaštovský, Johansen, Dias, Fonseca, Urbatzka, & Vasconcelos, 2023; Luz, Cordiero, Kaštovský, Johansen, Dias, Fonseca, Urbatzka, et al., 2023). Furthermore, the secondary structures of the conserved helices in the ITS rRNA region have been used to provide evidence of lineage separation for use in species-level differentiation (Becerra-Absalón et al., 2018; Johansen et al., 2011, 2014; Jung et al., 2020; Martins et al., 2019; Saraf et al., 2020).

Often 16S rRNA gene sequence data and resultant analysis of those data have resolved genera but have been poor at species-level recognition. This has led to increasing use of ITS rRNA region data for species recognition. However, cyanobacteria usually have multiple ribosomal operons, and targeted polymerase chain reaction (PCR) has demonstrated that the ITS rRNA regions of each operon in a cyanobacterial genome usually have different sequences and secondary structures (Johansen et al., 2008, 2011). The heterocytous taxa are particularly rich in ribosomal operons, having 3–5 operons (Boyer et al., 2001; Johansen et al., 2017; Bohunická et al., 2024). For this reason, it is critical to either clone the PCR amplicons to get clean ITS rRNA region sequences (Hentschke et al., 2016, 2017; Kumar, Saraf, Pal, Mishra, Singh, & Johansen, 2022) or purify the PCR products, check them on a gel, and then sequence the different bands (Kumar, Saraf, Pal, Mishra, & Singh, 2022). Researchers have often separated ITS rRNA sequences based on presence or absence of tRNA genes in the ITS rRNA region, but if more than two operons are present in a genus, then there will be multiple operons that have the same number of tRNA genes but are still paralogous (Bohunická et al., 2024; Jusko & Johansen, 2023). Paralogous operons within a single genome can have similar to very divergent sequences, and the ITS rRNA regions can be as different as those observed between species in that genus. Consequently, researchers could recognize and describe two different strains as different species based on ITS rRNA region data when actually they have only recovered different ribosomal operons in the same species. We suspect that this has already occurred.

We recently isolated and characterized two different strains in the tapering, heterocytous genus *Dulcicalothrix*. These appeared to be different species based on a number of different lines of evidence. However, in examining the sequence data available for *Dulcicalothrix* species, we discovered that there are likely five ribosomal operons with six different orthologs present in the genus. This complicated the use of the ITS rRNA region, as we did not have all ITS rRNA regions for the many strains of *Dulcicalothrix*, nor even a single orthologous operon for all strains. However, even with incomplete data, we were able to utilize the polyphasic approach to resolve species-level clades in this genus. In this manuscript, we describe the two strains as new species and illustrate how taxonomic resolution can be achieved even when molecular data are incomplete. We also continue revision of the Calotrichaceae by providing a new genus name for *Calothrix elsteri*.

MATERIALS AND METHODS

Sample collection and strain isolation

Strain ZW1-PS was isolated from a shallow water periphytic sample collected from a fresh water body located in a small township of Mumbra in the Thane District in the state of Maharashtra. Mumbra is surrounded by hills in the northwestern region, whereas the northeastern region is bordered by the tributary of the Ulhas River. It is near coastal (less than 10 m above sea level). The Thane District is located in a tropical rainforest climate (Af in the Köppen-Geiger classification system). Strain 25C-PS was isolated from the sample collected from the surface of the soil of a wet rice field located in the village of Basantgarh in the district of Udhampur, Jammu and Kashmir. Basantgarh is situated at an altitude of 1860–2060 m above sea level, and the village is surrounded by high mountain ranges. The sampling site was surrounded by the tributary Mandura on one side; the other side was surrounded by shrubs and grasses. The region around Basantgarh has a temperate climate with dry cold winters and warm summers (Cwb in the Köppen-Geiger classification system).

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In the laboratory, the samples were examined microscopically to determine the microflora. Furthermore, the natural samples were subjected to isolation on a solidified BG-11_0 media containing 1.2% agar with pH of the media adjusted to 7.2 (Rippka et al., 1979). After the appearance of colonies, a single colony was picked and transferred to a culture tube containing 5 mL of sterile BG-11_0 media. The purification of the strains was done by alternately culturing on solid and liquid medium until a unicyanobacterial isolate was obtained. After purification, the strains were maintained on agar slants in a culture room at 28 ± 2°C, illuminated with 50–55 μmol photons · m^{-2} · s^{-1} illumination with a photoperiod of 14:10 h light:dark (L:D) cycle. To check formation of terminal hairs, both strains were grown in low phosphorous concentrations (50%, 25%, and no phosphorous) of BG-11_0 media.

Morphological analysis

Morphological evaluations of strains ZW1-PS and 25C-PS were performed on a bright-field Olympus BX53 microscope (in case of strain ZW1-PS) and Olympus BX60 (in case of 25C-PS) microscope with Nomarski DIC optics equipped with an Olympus SC50 digital camera, respectively. Morphological characters such as the appearance of the filaments (coiling, branching, degree of tapering, presence of sheath, nature of tapering, etc.), size and shape of heterocytes and vegetative cells, position of heterocytes, presence of hormogonia, thickness of sheath, constrictions between the vegetative cells, and the occurrence of necridia were determined.

Molecular analyses

Genomic DNA was extracted from 14- to 16-day-old cultures using Qiagen DNeasy® Power Soil® Pro Kit (Qiagen, USA). The 16S rRNA gene and associated ITS rRNA region were subsequently amplified using primers VRF1 (5'-CTC TGT GTG CCT AGG TAT CC-3′; Boyer et al., 2002, after Willmotte et al., 1993) and VRF2 (5′-GGG GAA TTT TCC GCA ATG GG-3′; Boyer et al., 2002 after Nübel et al., 1997). The PCR reaction was set in 25 μL reactions containing 12.5 μL of LongAmp® Taq 2× Master Mix (NEB), 0.5 μM each of the primers, and 2 μL of genomic DNA. The PCR amplification was performed on a Bio–Rad C1000™ Thermal Cycler, and the cycling conditions were set at 94°C for 45 s, 57°C for 45 s, and 72°C for 135 s for 35 cycles. The final extension was performed for 3 min at 72°C, and the reaction was held at 4°C until further use. The amplicons were cloned using StrataClone PCR Cloning Kit (Agilent Technologies, USA), and eight clones for each strain were sequenced at Functional Biosciences Inc. (Madison, WI, USA) on a 3730xl DNA Analyzer. Two primers in the plasmid, M13F and M13R, were used for sequencing, together with three internal primers, VRF5, VRF7, and VRF8 (Flechtner et al., 2002). Sequences were assembled and proofread using Sequencher (version 4.8, Ann Arbor, MI, USA). GenBank accession numbers for the resulting sequences are OK138871–OK138873 and OK257844.

Phylogenetic analysis

The 16S rRNA gene-based phylogenetic trees were inferred using Bayesian inference (BI) and maximum likelihood (ML) methods, and analyses were run on the CIPRES Science Gateway (Miller et al., 2010). First, a total of 521 nucleotide sequences of maximum length of 1517 nucleotides from the Nostocales (including four sequences of *Chroococcidiopsis* as outgroup taxa) were aligned. The alignment was annotated for secondary structure, and this alignment appears in Table S1 (in the Supporting Information). The BI tree was constructed using MrBayes on XSEDE 3.2.6 (Ronquist et al., 2012), and the best-fit model was determined using jModelTest (Darriba et al., 2012), which led to the selection of GTR + G + I model. In the analysis, two runs of eight Markov chains were applied, and the analysis was continued until the value of standard deviation of split frequency was below 0.01. The sampling frequency and the diagnostic frequency were set to 1000, and 25% of the samples from the beginning were discarded. The analysis was run for 95 million generations. This analysis did not achieve convergence of the chains, although an ML analysis did provide a phylogeny that was used to reduce the matrix for subsequent analyses. The alignment was reduced by removing many of the Nostocaceae, which are more distantly related to the family of interest (Calotrichaceae); however, we added a number of Rivulariaceae sequences, as this family has been combined with Calotrichaceae in the past. We also shortened the sequence length to 1162 nucleotides, a length that most of the remaining sequences achieved. The resulting alignment had 228 sequences (Table S2 in the Supporting Information). A BI analysis on this alignment was run using the same protocol as the first analysis. The mean estimated sample size (ESS) exceeded 400 (range: 495–6280), significantly higher than the average of 100 accepted as sufficient (Drummond et al., 2006). The potential scale reduction factor (PSRF) value for all parameters was 1.00, indicating convergence of the Markov chain Monte Carlo chains was achieved (Gelman & Rubin, 1992). It is this analysis that is reported in the manuscript.

Subsequently, an ML analysis was run on the new alignment, using the GTR + G + I model, with analysis run on RAxML-HPC2 on ACCESS. This phylogeny had very similar topology to the BI tree. The bootstrap support values from this analysis were mapped onto the nodes of the BI phylogeny.

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Two ITS rRNA region alignments were constructed. The first had 12 sequences containing both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (Table S3 in the Supporting Information). The second had 20 sequences containing no tRNA genes (Table S4 in the Supporting Information). A BI analysis was run in which data were in two partitions, one for the DNA and the second with standard coding based on the presence of indels (1 for nucleotide present and 0 for indel at that locus). These analyses were set to run for 80 million generations with a stop value of 0.001. Both achieved convergence. The same alignments were run using a heuristic search in a parsimony analysis in PAUP on ACCESS, with gapmode = newstate, swap = TBR, steepest = no, multrees = yes, and nreps = 10,000. All trees were visualized in FigTree (Rambaut, 2009) and post-edited in Adobe Illustrator v. 26.4.1 (Adobe Systems, San Jose, California).

16S-23S ITS rRNA region analysis

The secondary structures corresponding to the conserved domains within the ITS rRNA region (D1–D1′, BoxB, and V2 and V3 helices) were determined using the Mfold program (Zuker, 2003) on the Unifold web server, setting the draw mode at untangle with loop fix and the other parameters at default for all *Dulcicalothrix* strains for which ITS rRNA region sequences were available as of January 1, 2024. Secondary structures were then redrawn in Adobe Illustrator. Lengths of domains within the ITS rRNA regions were determined for all strains based on the ITS rRNA region alignments created for phylogenetic analyses (Tables S3 and S4). These comparative lengths are reported in Table S5 (in the Supporting Information).

Percent identity for 16S rRNA gene data and PD for 16S-23S ITS sequence data

The p-distance values for the 16S rRNA gene and the ITS rRNA region were determined using the SHOWDIST command in PAUP on ACCESS in the CIPRES Science Gateway. For the 16S rRNA gene, percent identity (PI) was calculated as $100*(1-p)$. For the ITS rRNA region, percent dissimilarity (PD) was calculated as $100*p$. Matrices showing all comparisons were then constructed for each set of like sequences.

Line drawing and preparation of herbarium and cryopreserved materials

Line drawings of the characteristic morphological features of the strains ZW1-PS and 25C-PS were made using pen and ink. The herbarium materials for both strains were prepared in duplicate using liquid cultures in standard media immobilized on glass fiber filters. Air-dried filters were then packed in glassine envelopes and curated in packets made from archival acid-free bond paper. Herbarium materials were deposited in the Herbarium at Banaras Hindu University, Varanasi, India. In addition to herbarium materials, both strains were cryopreserved and, subsequently, provided the basis for holotypes. These cryopreserved materials were cataloged in the Global Collection of Cyanobacteria (https://ccinfo.wdcm.org/details?regnum=1165).

RESULTS

Taxonomy

Dulcicalothrix adhikaryi Saraf, J.R.Johans. & Pras. Singh sp. nov. (Figures 1 and 2a–g).

Thallus in the natural environment macroscopic, periphytic but rafting when actively photosynthesizing, bluish green, in laboratory-grown culture caespitose on solid media with slightly raised elevation. Sheath—firm, colorless, tightly attached to the trichome. Filaments—uniseriate, heteropolar, gradually tapering without the development of hyaline terminal hairs, when young short and straight, when mature long, usually straight, occasionally coiled within sheath, with rare single or double false branching. Trichomes—prominently constricted at cross walls in young filaments, becoming less so with age, 3.0–4.4 μm wide at base, narrowing to 1.6–2.3 μm wide at distal end. Vegetative cells—granulated, barrel shaped when adjacent to basal heterocytes, 2.4–3.3 μm long. Intercalary vegetative cells—barrel shaped, 1.6–2.3 μm long, 2.3–3.7 μm wide. Apical cells—conical, longer than wide, 2.6–3.9 μm long, 1.6–2.2 μm wide. Heterocytes—solitary, sometimes yellowish in color, usually basal, rarely intercalary, hemispherical to oblong, when hemispherical 2.4–3.6 μm long, 2.5–3.7 μm wide, when oblong 3.7–4.6 μm long, 2.6–3.2 μm wide. Hormogonia—present, usually heteropolar, rarely isopolar, when isopolar having heterocytes at both ends with cells irregularly arranged. Akinetes—unobserved. Necridia—present and abundantly formed.

Etymology: Named in honor of Professor Siba Prasad Adhikary, prominent Indian phycologist.

Type locality: Mumbra, Thane, Maharashtra, India (19°09′56.1″ N 73°01′43.5″ E).

Habitat: In fresh water pond, growing as periphytic bluish-green macroscopic mat which can raft when actively photosynthesizing and producing oxygen bubbles trapped in mucilage. The pH was recorded at 7.2. Electrical conductivity was measured at 110 $\mu S \cdot cm^{-1}$.

Holotype here designated: A portion of a culture of *Dulcicalothrix adhikaryi* is cryopreserved in

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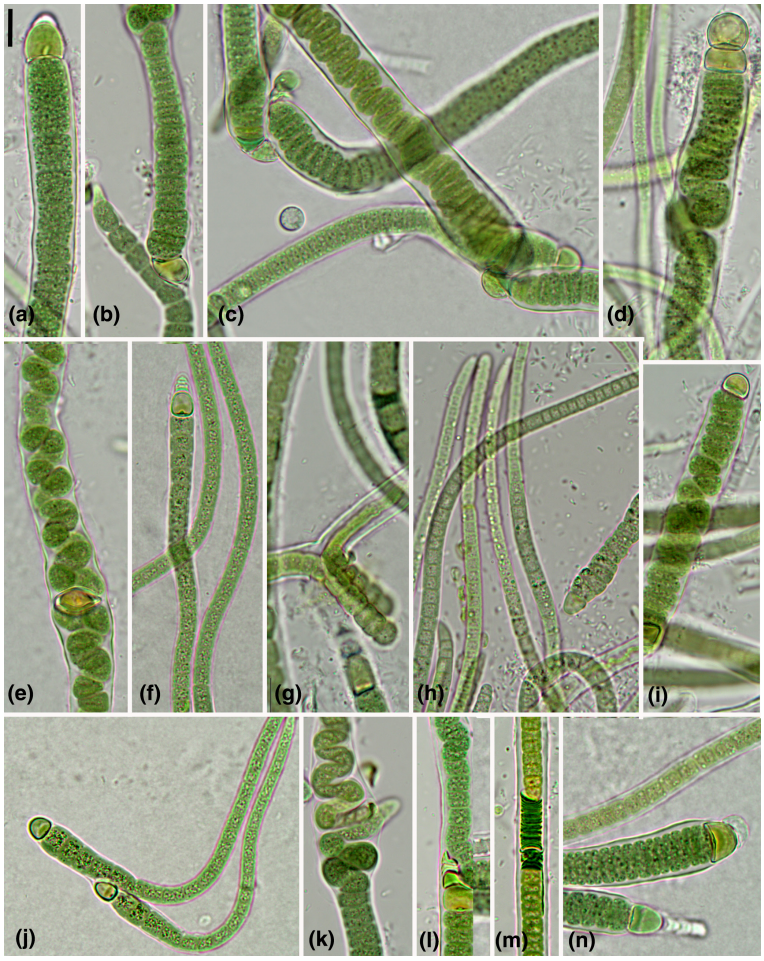

FIGURE 1 Morphological characteristics of *Dulcicalothrix adhikaryi* ZW1-PS. Note both single (b, c, l) and double false branching (g), coiled trichomes (c–e, k), tapered apices (h), basal (a, c, d, f, i, j, n) and intercalary (b, e, l) heterocytes, and necridia (m). Scale bar = 5 μm, applies to all images.

metabolically inactive form cataloged in the GCC located at Banaras Hindu University in Varanasi, India (GCC; Registered Number 1165), and is available under the accession number GCC20247.

Isotype here designated: Herbarium preparation deposited in the GCC with the number GCC-bota nybhu-20,247.

Reference strain: ZW1-PS

GenBank accession numbers: OK138871 and OK138873.

Dulcicalothrix iyengarii N.Kumar, J.R.Johans. & Pras.Singh sp. nov. (Figures 2H–K and 3).

Thallus in the natural environment is a bluish-green macroscopic mat growing luxuriantly on the surface of the soil; in laboratory, bluish green, with discrete initial colonies on solid media, later developing a mat-like appearance with slightly mucilaginous texture and raised elevation. Filaments—uniseriate, unbranched, heteropolar, with gradual tapering toward the apical end, not developing terminal hyaline hairs, when young short and straight, when mature long, usually straight or slightly curved. Sheath—thin, colorless, wide at the basal part, and tightly attached to the trichome in other parts, with multiple trichomes within a single sheath. Vegetative cells in the basal part are shorter than wide, barrel shaped, 4.5–8.1 μm long, 5.8–8.8 μm wide; and in the middle part, isodiametric to longer than wide, 2.9–8.3 μm long, 3.9–6.1 μm wide. Apical cells—conical, 2.5–3.7 μm long, 3.0–4.2 μm wide. Heterocytes basal, solitary, oval to hemispherical or dome shaped, sometimes yellowish in color, 4.9–5.5 μm long, 6.9–7.6 μm wide. Akinetes not observed. Necridia present.

Etymology: Named in honor of Professor M.O.P. Iyengar, prominent Indian phycologist.

Type locality: Basantgarh, Udhampur, Jammu and Kashmir, India (32°48′57″ N 75°32′16″ E).

Habitat: Growing luxuriantly as a bluish-green mat on the surface of rice field soil. The pH of the soil sample was recorded at 5.85 ± 2. The electrical conductivity was 294 $\mu S \cdot cm^{-1}$.

Holotype here designated: A portion of a culture of *Dulcicalothrix iyengarii* is cryopreserved in metabolically inactive form in GCC located at Banaras Hindu University in Varanasi, India (GCC; Registered Number 1165), and is available under the accession number GCC20248.

Isotype here designated: Herbarium preparation deposited in the GCC with the number GCC-bota nybhu-20,248.

Reference strain: 25C-PS

GenBank accession numbers: OK138872 and OK257844.

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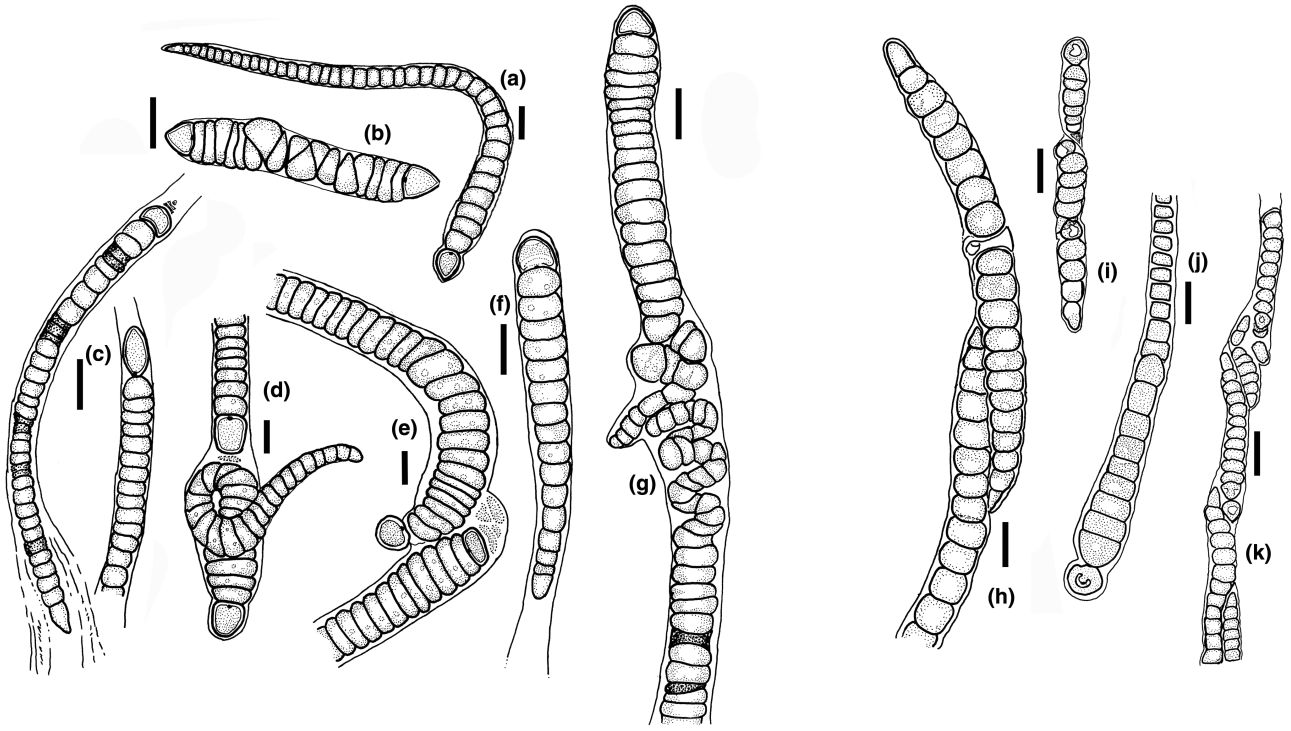

FIGURE 2 Line drawing of both species. (a–g) *Dulcicalothrix adhikaryi* ZW1-PS. (h–k) *D. iyengarii* 25C-PS. Bars, 5 μm.

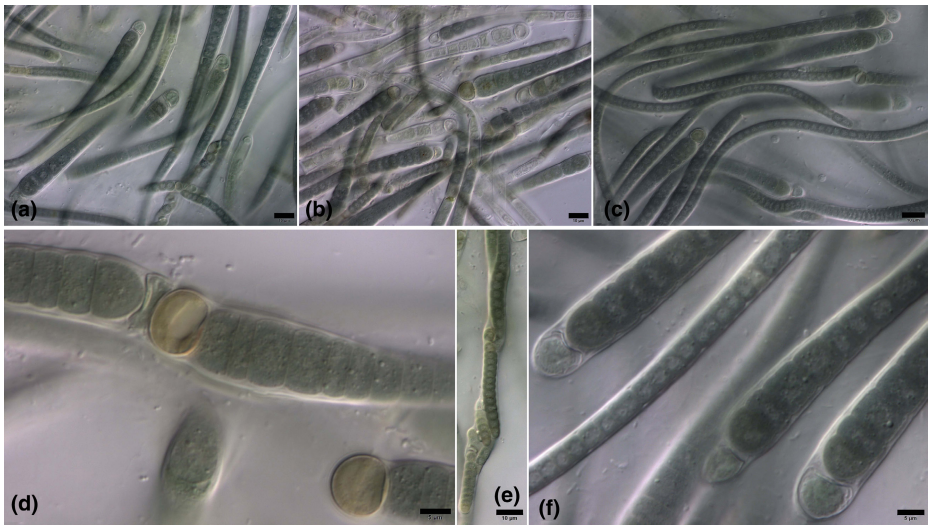

FIGURE 3 Morphological characteristics of *Dulcicalothrix iyengarii* 25C-PS. Note tapering trichomes (a–c, f), multiple trichomes in a common sheath (e), and an intercalary heterocyte (d). Scale bars in (a–d, f = 5 μm, scale bar in e = 10 μm.

Tapering heterocytous strains have been repeatedly identified as *Calothrix parietina*, and most of those sequenced fell within the *Dulcicalothrix* clade. The name has been applied to fresh water, subaerial, and soil populations. However, this species has been reported to taper to a hair, and this is not a feature consistent with the *Dulcicalothix* protolog. Consequently, we are not reclassifying *Calothrix parietina* to *Dulcicalothrix* at this time.

The strain *Calothrix scopulorum* SAG 36.79 belongs to *Dulcicalothrix* and was isolated from a moist stone in the Botanical Garden at the University of Göttingen, whereas *C. scopulorum* was described in Bornet and Flahault (1886) as occurring in marine habitats throughout Europe. Also, *Calothrix scopulorum* has been reported to form terminal hairs and is likely more closely related to the type species for *Calothrix*, *C. confervicola*, which is also marine. Consequently, we are not moving this species into *Dulcicalothrix* based on the sequence data for SAG 36.79

Two other genera were represented in our Calotrichaceae tree, *Calothrix elsteri*, and an unnamed

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Calothrix represented by 10 sequences isolated in China and deposited in GenBank by Y. Wang (GenBank accessions MT488196, MT488209–MT488217). *Calothrix elsteri* is genetically similar not only to *Macrochaete* species (percent identity 94.75%–95.10%) but also to the Chinese *Calothrix* strains by a similar amount. Phylogenetically it is separate from both *Macrochaete* and the CHAB strains (Figure 4). *Calothrix elsteri* has PD ≤94.5 to all other genera in the Calotrichaceae so cannot belong to any of these genera if this threshold is accepted. The genus description is based on the original description for *C. elsteri* (Komárek et al., 2012), which was described from ice-free alkaline lakes in Antarctica. As a fresh water species, we consider it very unlikely to belong to *Calothrix*, as the type of that genus is temperate marine. We here have given it a new name. The description is based on the original published description of *C. elsteri* (Komárek et al., 2012).

Brunnivagina Saraf, J.R.Johans. & Pras.Singh gen. nov.

Filaments—heteropolar with basal heterocytes at the attached base and tapering to a thin hair. Sheath—deep, dark brown in color, firm, sometimes telescoping or fraying near the tapered end. Trichomes not constricted at the cross walls, up to 360 μm long, up to 20 μm wide at base, and narrowing to a hyaline hair less than 1 μm wide at the apex. Cells—blue green when growing in absence of sheath (in culture), shorter than wide to isodiametric in main part of filament, and becoming longer than wide in the hyaline hair. Heterocytes—mostly solitary and basal, occasionally in pairs, rarely intercalary, hemispherical to rounded. Akinetes—not observed.

Etymology: *L. brunneus* = deep brown and *L. vagina* = sheath. Deep brown sheath, named for its appearance in nature.

Type species: *Brunnivagina elsteri* (Komárek, Nedbalová & Hauer) Saraf, J.R.Johans. & Pras.Singh comb. nov.

Basionym: *Calothrix elsteri* Komárek, Nedbalová & Hauer, phylogenetic position and taxonomy of three heterocytous cyanobacteria dominating the littoral of deglaciated lakes, James Ross Island, Antarctica, *Polar Biology* 35: 764. 2012.

Comparative morphology

Both of the new *Dulcicalothrix* species were heteropolar in nature and gradually tapered toward the apical ends (Figures 1–3; Table S6 in the Supporting Information). Hair formation was not observed in either strain even

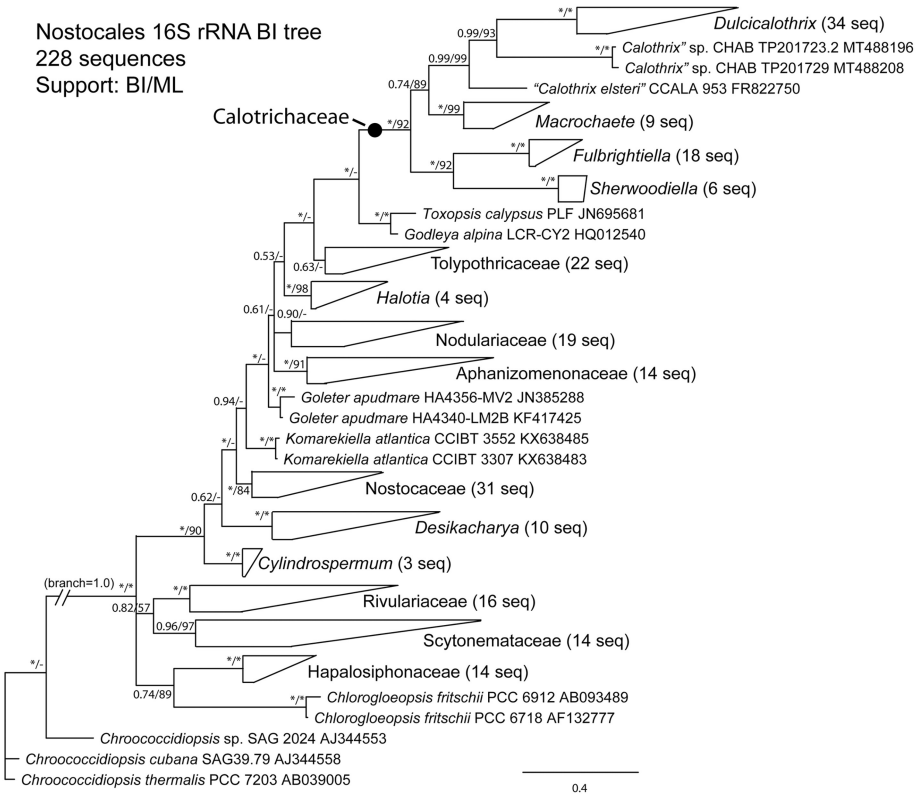

FIGURE 4 Bayesian inference analysis based on 228 sequences of the 16S rRNA gene, with ML bootstrap values mapped to nodes; *Means full support, –means less than 0.50% or 50% support. Polygons representing multiple sequences show the minimum branch length in the cluster at the bottom of the polygon and the maximum branch length in the cluster at the top of the polygon. The uncollapsed tree on which this is based is Figure S1 (in the Supporting Information).

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at lowered phosphate concentration. Heterocytes were observed primarily at the basal ends of the filaments in both species (Figures 1a,c,d,f,i,j,n, 2a–g,j,k, and 3a–d,f), but more rarely developed in an intercalary position (Figures 1E,L and 3D), leading to filament fragmentation and false branching (Figures 1B,L and 3D) where the unipolar heterocyte abutted the filament attached opposite to the polar nodule. A unique feature of *D. iyengarii* is the occurrence of multiple young trichomes within a single sheath during some stages of development (Figures 2h,k and 3e), whereas the formation of isopolar hormogonia with heterocytes at both ends (Figure 2b) and spiraling of the filaments in the older stages of the life cycle are the unique features of *D. adhikaryi* (Figures 1c,e,k and 2g).

Analysis of the 16S rRNA gene

The *Dulcicalothrix* clade consisted of 36 sequences and formed a well-supported distinct cluster with strong posterior probability/bootstrap support within the family Calotrichaceae as defined by Saraf, Suradkar, et al. (2019), together with *Fulbrightiella*, *Sherwoodiella*, *Macrochaete*, and an as-yet-undefined species from China: the CHAB strains (Figure 4). *Brunnivagina elsteri* fell outside of any clade. This analysis with an expanded set of tapering taxa clearly separated the Rivulariaceae from the Calotrichaceae (Figure 4). The uncollapsed tree from this analysis is available in supplemental materials (Figure S1 in the Supporting Information).

Dulcicalothrix iyengarii clustered closely with strains CENA 127 and HA4283-MV5 and was nested in a larger clade containing strains HA4186-MV5, 44-T10 Mareš, 34T8Micr, and HA4395-MV3 (Figure 5). *Dulcicalothrix adhikaryi* was sister to *D. necridiiformans* in a node distant from *D. iyengarii* but still within the genus *Dulcicalothrix*. Other named species included *D. alborzica*, *D. desertica*, *Calothrix parietina*, and *C. scopulorum*. All the *C. parietina* strains within *Dulcicalothrix* were probably misidentified and in need of further taxonomic study (for details, see Saraf, Suradkar, et al., 2019). Based upon the overall morphology and the ecology of *C. scopulorum*, which was described from a number of marine habitats, we consider *C. scopulorum* SAG 36.79 to be misidentified, as it is from a moist rock in the Botanical Garden in Göttingen.

Most of the *Dulcicalothrix* species fell into separate species based upon the 16S rRNA gene sequence percent identity (PI) threshold of 98.7% (Table S7 in the Supporting Information). Although it is incorrect to assume that PI ≥98.7% indicates strains belong to the same species, we consider it likely that when PI is greater than 99.5%, strains are likely the same species. However, any putative congeners require further evidence to be confident in assigning strains to the same species. Based on these percent identity standards, we have around 17–19 species of *Dulcicalothrix* species represented in our analysis, with nine of the "species" represented by multiple strains (Table S7).

16S-23S ITS rRNA region analysis

Two different types of operons (with $tRNA^{Ile}$ and $tRNA^{Ala}$ genes and with no tRNA genes) were observed in the genus *Dulcicalothrix*. However, upon aligning the ITS rRNA regions, we discovered that several patterns of indels were obvious in the D1–D1′ helix region. Furthermore, when we combined all D1–D1′ helices in a single alignment, it became clear that we had three D1–D1′ helix types, with representatives in both the two tRNA operons and the no tRNA operons (Table 1). Two types of D1–D1′ helix, which we have designated types 1 and 2, were shorter than the third type, type 3 (Table S5). We have referred to the operon types as 1N, 2N, and 3N for those operon types with no tRNA genes and as 1B, 2B, and 3B for those operon types with both tRNA genes. Examination of three strains for which complete or near-complete genomes are available (strains PCC 7716, NIES-4071, and NIES-4105) revealed that these *Dulcicalothrix* species each had five ribosomal operons, two with both tRNA genes and three with no tRNA genes. Given that we observed six different operon types, this means that using the ITS rRNA region for species-level taxonomy is extensively complicated in this genus. In particular, we consider that using non-orthologous operons to determine PD should not be used as a separation criterion. Percent dissimilarity between orthologous operons is required for calculating this species-determining metric.

Nevertheless, we aligned all ITS rRNA regions having both tRNA genes in one alignment and all ITS rRNA regions with no tRNA genes in another alignment so that phylogenies could be constructed and PD could be calculated. Percent dissimilarity among operons with both tRNA genes showed that some strain pairs could be compared (Table 2). *Dulcicalothrix alborzica*, PCC 7103, SRS-BG14, and PCC 7716 had type 1B operons, and comparison among them all had PD >7.0%; consequently, they could be considered different species from each other. *Dulcicalothrix iyengarii, D. desertica*, HA4395-MV3, HA4283-MV5, NIES-4017, NIES-4105, and N3-MA1 all had type 3B operons. Of these, the two NIES strains showed no differences, and we conclude easily that they are the same species. *Dulcicalothrix iyengarii* and HA4395-MV3 had PD = 6.6%, a value we consider ambiguous and uninformative on its own. SEV5-4-C5 was the only strain with a 2B operon, so this

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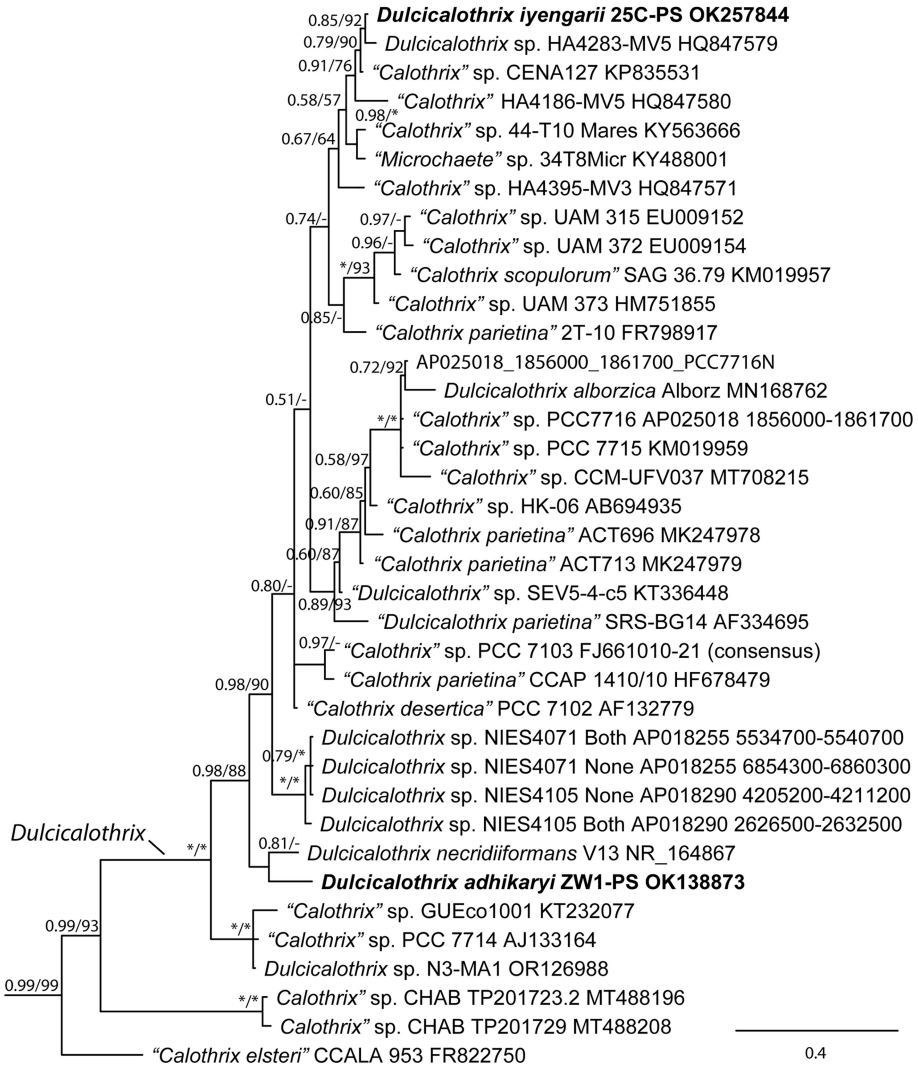

FIGURE 5 The *Dulcicalothrix* phylogeny from the analysis in Figure 4. Unnamed taxa were given species numbers to group same species following all taxonomic analysis, with empty circles denoting individual species. Note, the central clade containing both *D. alborzica* and *D. desertica* had poor species resolution with many species-level clusters being paraphyletic. Nodal support: *Means full support, – means less than 0.50% or 50% support.

sequence cannot be compared with any of the others with two tRNA genes.

We recovered 20 operons with no tRNA genes, so more comparisons are possible. This set was unambiguous. Either strain pairs in the same operon type had PD ≥7.0% (different species) or PD ≤3.0% (same species). Nine strains had a type 1N operon, eight had a type 2N operon, and two had a type 3N operon (Table 3). One strain, SEV5-4-C5, had both a type 1N and type 2N operon, which were only 2.5% dissimilar. It is this operon pair from the same strain that provided convincing evidence that type 1 and type 2 operons were indeed paralogous. Based on this evidence, we have five different species in the type 1N group, four different species in the type 2N group, and two different species in the type 3N group.

The phylogenies based on the two alignments give additional evidence for separation of some species clusters, and in most cases, the evidence was congruent with the 16S rRNA gene phylogeny (Figure 6). For example, *Dulcicalothrix iyengarii*, HA4186-MV5, 44-T10 Mareš, and 34T8Micr have type 2N operons and belonged to the same clades in both the 16S rRNA gene analysis and the ITS rRNA region—no tRNA analysis (Figures 5 and 6). Likewise, SEV5-4-C5 and SRS-BG14 were in the same clades and had ITS rRNA regions only 2.7% dissimilar, even though they were from 1B and 2B operons. Other groups that showed agreement between phylogenies included PCC 7716 and CCM-UFV03, PCC 7103 and CCAP 1410/10, and NIES-4071 and NIES-4105.

Secondary structures of the conserved domains in the ITS rRNA region were also partially informative. Minor differences occurred in the D1–D1′ helices in the type 1 and type 2 operons (Figure 7). Some strains showed that operons of the same major type (1, 2, or 3) were

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TABLE 1 Alignment of the D1–D1′ helix showing all six orthologous operons.

	Type 1B
Dulcicalothrix sp. SRS-BG14 AF236642	GACCTACC-----CAACTTG--TTGTAATACGTACTTCAGTA----------CGT---AAAAGAAGCAACAAGAAGGTCATCACCTAGGTC
"Calothrix" sp. PCC 7103 FJ661020	GACCTACCG----TTCCTTG--TCGTAATACGTACTTTAGTA----------CGT---TAAAAAA-CAACAAGAAGGTCATCACCTAGGTC
D. alborzica CCC-1387 MN699469	GACCTACC-----CAACTTGC-TTGTAATAAGCACTTCAGTG----------C-----TTAAAAACCAACAAGAAGGTCATCACCTAGGTC
"Calothrix" sp. PCC 7716 AP025018	GACCTACC-----CAACTTGC-TTGTAATAAGCACTTCAGTG----------C-----TTAAAAACCAACAAGATGGTCATCACCTAGGTC
	Type 1N
D.adhikaryi Zw1-PS OK138873	GACCTACC-----CAACTTG--TTGTCATGCGTACTTGAGTA----------CGTA--GAAAAAAACAACAAGCAGGTCATCACCTAGGTC
"Calothrix" sp. UAM 315 EU009152	GACCTACC-----CAACTTGC-TCGTAATAACCACTTTAGTG----------G-----TTTCA---TAACAAGATGGTCATCACCTAGGTC
"Calothrix" sp. PCC 7103 FJ661016	GACCTACCG----TTCCTTGT-C-GTAATACGTACTTCAGTA----------CG----TTAAAAAACAACAAGAAGGTCATCACCTAGGTC
"Calothrix" sp. PCC 7716 AP025018	GACCTACC-----CAACTTGC-TTGTAATAAGCACTTCAGTG----------C-----TTAAAAACCAACAAGAAGGTCATCACCTAGGTC
D. parietina CCAP 1410/10 HF678479	GACCTACCG----TTCCTTGT-TTGTAATACGTACTTCAGTA----------CG----TTAAAAAACAACAAGAAGGTCATCACCTAGGTC
"Calothrix" sp. CCM-UFV03 MT708215	GACCTACC-----CAACTTGC-TTGTAATAAGCACTTCAGTG----------C-----TTAAAAACCAACAATAAGGTCATCACCTAGGTC
D. necridiiformans V13 KY863521	GACCTACC-----CAA-TTG--TTGTATTGCGTGCGGAAGTA--------- ----------CGTACTCAAAAAAGCAACACTCAGGTCATGACCTAG GTC
Dulcicalothrix sp. SEV5-4-c5 KT336446	GACCTACC-----CAACTTG--TTGTAATACGTACTTCAGTA----------CGT---AAAAGAAGCAACAAGAAGGTCATCACCTAGGTC
	Type 2B
Dulcicalothrix sp. SEV5-4-c5 KT336448	GACCTACC-----CAACTTG-----TTGATTGTACTTTAGTA----------C-----TCAAAAACCAACAAGAAGGTCATCACCTAGGTC
	Type 2N
Dulcicalothrix iyengarii 25C-PS OK138872	GACCTACC-----CAACTTG-----TAAGTTGTACTTCGGTA----------C-----TGTTAAAGCTGCAAGA-GGTCATTACCTAGGTC
Dulcicalothrix sp. SEV5-4-c5 KT336447	GACCTACC-----CAACTTG-----TTGATTGTACTTTAGTA----------C-----TGAAAAACCAACAAGAAGGTCATCACCTAGGTC
Dulcicalothrix sp. HA4186-MV5 HQ847580	GACCTACC-----CAACTTG-----TAAGTTGTACTTCGGTA----------C-----TGATAAAGCTGCAAGA-GGTCATTACCTAGGTC
Dulcicalothrix sp. 44-T10 Mares KY563666	GACCTACC-----CAACTTG-----TGAGTGGTACTTCGGTA----------C-----TGTTAAAGCTGCAAGA-GGTCATTACCTAGGCC
Dulcicalothrix sp. 34T8Micr KY488001	GACCTACC-----CAACTTG-----TGAGTGGTACTTCGGTA----------C-----TGTTAAAGCTGCAAGA-GGTCATTACCTAGGCC
Dulcicalothrix sp. NIES-4071 AP018255	GACCTACC-----CAACTTG----TCAGACAGTACTTGTGTA----------C-----GATAAATGTCACAAGATGGTCATCACCTAGGTC
Dulcicalothrix sp. NIES-4071 AP018255	GACCTACC-----CAACTTG----TCAGACAGTACTTAGGTA----------C-----GATAAATGTCACAAGATGGTCATCACCTAGGTC
Dulcicalothrix sp. NIES-4105 AP018290	GACCTACC-----CAACTTG----TCAGACAGTACTTGTGTA----------C-----GATAAATGTCACAAGATGGTCATCACCTAGGTC
	Type 3B
D. iyengarii 25C-PS OK138872	GACCTACCCATTTTAGATTGT-GGATTTTGGATTTTGGATTG--AAAGATCG AGAATTTAGAATTTGAGAAAAAAGTCTAAAATAGTGTCATT-CCTAGGTC
Dulcicalothrix sp. HA4395-MV3 HQ847571	GACCTACCCATTTTAGATTTT-GGATTTTAGATTTTGGATTG--AAAGATCG AGAATTTAAAATTTGAGAATGAAGTCTAGAATAGTGTCATT-CCTAGGTC

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TABLE 1 (Continued)

	Type 3B
D. desertica PCC7102 RSCL01000108	GACCTACCCATTTTGGATTGT-CGATTTTGGATTTTAGATTGAATAAAAAT AAAATCCCAAATCGGAAATCAAAAATCCAAAATAGTGTCATT-CCTAGGTC
Dulcicalothrix sp. HA4283-MV5 HQ847573	GACCTACCCATTTTAGATTGT-AAATTTTGGATTTTGGATTT--TTTGAATAG AGTCTAAAATCGGAAATCAAAAATCTAAAATAGTGTCATT-CCTAGGTC
Dulcicalothrix sp. NIES-4071 AP018255	GACCTACCCATTTTAGATTGT-GAATTTTTGAAAATCGATTG-- TGAGAATT-AATCTCAAATCGATTGAAAAAAATCTAAAATTGTGTC ATT-CCTAGGTC
Dulcicalothrix sp. NIES-4105 AP018290	GACCTACCCATTTTAGATTGT-GAATTTTTGAAAATCGATTG-- TGAGAATT-AATCTCAAATCGATTGAAAAAAATCTAAAATTGTGTC ATT-CCTAGGTC
Dulcicalothrix sp. N3-MA1 OR126988	GACCCATCAAGTCAAAACAAGAAAGTGAAGAGTAAAAAGAATTT GAAAAGTAGTTCTTTTGGCTTTAAGCTTTAAATGTTTAACTTGATC TAC-CCGAGGTC
	Type 3N
Dulcicalothrix sp. HA4283-MV5 HQ847579	GACCTACCCATTTTAGATTGT-AAATTTTGGATTTTGGATTT--TTTGAATAG AATCTAAAATCGGAAATAAAAAATCCAAAATAGTGTCATT-CCTAGGTC
Dulcicalothrix sp. HA4395-MV3 HQ847572	GACCCATCAAGTCAAAAGAGCAAAGTAAAAAGTAAAAAGGAT-- GGAAAGTATTTCTTTGAACTTTTGCCTTTTAGCCTTTAACTTGATC TAC-CCTAGGTC

Note: Operon type is indicated above each block of the same type.

identical in structure and sequence for both operons with and without tRNA genes. For example, PCC 7103 types 1N and 1B (Figure 7c), PCC 7716 types 1N and 1B (Figure 7d), SEV5-4-C5 types 2N and 2B (Figure 7j), and HA4283-MV5 type 3N and 3B (Figure 7o) were all identical in structure and differed at most by one nucleotide in the terminal loop. There were also between-strain identities, PCC 7716 and CCC 1387 (type 1B), *Dulcicalothrix iyengarii* and HA4186-MV5 (type 2N), 44-T10 Mareš and 34T8Micr (type 2N), and SEV5-4-C5 and SRS-BG14 (types 1N and 1B, respectively; Figure 7d,i–k). There were also strains in the same numerical designation (type 3) that differed distinctly between operons with and without tRNA genes, for example, HA4395-MV3 (3N and 3B; Figure 7n,q).

The BoxB helices were more consistent in size between types but showed similar patterns of structural diversity (Figure 8). SRS-BG14 (1B) and SEV5-4-C5 (1N), 44-T10 Mareš and 34T8Micr (both type 2N), and NIES-4071 and NIES-4105 (both a 2N pair and a 3B pair) were structurally identical (Figure 8b,m,o,v), whereas SEV5-4-C5 (2N and 2B) and HA4395-MV3 (3N and 3B) were different within strains (Figure 8k,l,q,r). One strain, NIES-4071, even showed evidence of having two different 2N operons (Figure 8n,o).

The V3 helices were the most structurally diverse. Unfortunately, several of the strains did not have complete enough sequences to construct the V3 helix, but what we could see was that although there are some commonalities in the basal regions of each helix, the upper portions of these helices are quite different. There were a few interesting similarities; for example, all three V3 helices for SEV5-4-C5 were identical in structure, although they showed two nucleotide substitutions in the terminal loop. Even more interesting was that SRS-BG14 type 1B had exactly the same structure and matched the sequence of SEV5-4-C5 type 2B (Figure 9b,i). It would appear from ITS rRNA structural analysis that these two North American desert soil crust strains belong to the same species. *Dulcicalothrix iyengarii* (2N and 3B) had nearly identical structures (Figure 9g,l). In contrast, PCC 7716 (1N, 1B) was very different in structure (Figure 9c,d).

DISCUSSION

The complexity in the ribosomal operons of *Dulcicalothrix* makes use of the ITS rRNA region for taxonomic purposes very difficult. If sequence data for all five operons were available for all strains, it would be easy to detect lineage separation through examination of PD and secondary structures in the ITS rRNA regions of orthologous operons. However, the reality is we have a very incomplete data set. About a third of the strains with 16S rRNA gene sequence data lack any ITS rRNA region sequence. Only eight of the 20 strains with ITS rRNA region data have sequences for multiple operons. Only two strains (SEV5-4-C5 and NIES 4071) have data for three operons. Since comparisons between ITS rRNA regions of paralogous operons should not be used for taxonomic purposes, particularly PD thresholds, how to arrive at a decision as to whether a strain pair represents the same or different species is an open question.

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TABLE 2 Percent dissimilarity among *Dulcicalothrix* strains based on ITS in operons containing both tRNA genes.

Strain designation	*Dulcicalothrix* sp. 9 PCC 7103	*D. Alborzica* CCC-1387	*"Calothrix"* sp. 8 PCC7716	*Dulcicalothrix* sp. 6 SRS-BG14	*Dulcicalothrix* sp. 6 SEV5-4-5c
Dulcicalothrix sp. 9 PCC 7103 FJ661010-17					
D. alborzica CCC-1387 MN699469	21.5				
"Calothrix" sp. 8 PCC7716 AP025018	8.5	19.2			
Dulcicalothrix sp. 6 SRS-BG14 AF236642	7.7	23.0	8.5		
Dulcicalothrix sp. 6 SEV5-4-5c KT336448	7.4	22.6	8.5	2.7	
D. iyengarii 25C-PS OK257844	14.5	28.5	14.8	14.9	12.7
Dulcicalothrix sp. 2 HA4395-MV3 HQ847571	12.9	28.6	12.8	12.7	11.1
D. desertica PCC 7102 RSCL01000108	9.7	23.5	9.9	11.7	10.1
Dulcicalothrix sp. HA4283-MV5 HQ847573	13.7	27.0	13.1	13.4	12.0
Dulcicalothrix sp. 10 NIES-4071 AP018255	17.6	24.8	18.2	17.6	16.0
Dulcicalothrix sp. 10 NIES-4105 AP018290	17.6	24.8	18.2	17.6	16.0
Dulcicalothrix sp. 11 N3-MA1 OR126988	23.7	31.9	23.5	22.7	21.4

Note: Only comparisons between orthologous sequences should be used for species separation (gray highlighted area excluded). Percent dissimilarity ≥7.0% is strong evidence for species separation, and ≤3.0% is evidence strains belong to the same species. Percent similarity <7.0 but >3.0% is ambiguous. Adjacent strains with gray highlights are considered to be same species. Species numbers indicating congeners were added at the conclusion of taxonomic analysis.

We propose using a polyphasic approach to analyze this confusing collection of molecular data. We scored all pairwise comparisons by examining two to five criteria: (1) percent identity of the 16S rRNA gene sequence, (2) phylogenetic placement based on the 16S rRNA gene, (3) phylogenetic placement based on ITS rRNA region sequence alignments, (4) percent dissimilarity of ITS rRNA regions within orthologous operons, and (5) differences in secondary structure among conserved domains in the ITS rRNA regions of orthologous operons. An expanded table summarizing these comparisons is presented (Table S8 in the Supporting Information). Based on each criterion, we assessed whether that criterion was evidence of different species, same species, or uninformative. For example, if PI was ≤98.7%, the pair could be considered different species, whereas if PI was ≥99.5%, the pair was likely in the same species. Between 98.7 and 99.4%, this criterion was ambiguous or uninformative. If the strain pair was in the same clade in the 16S rRNA gene phylogeny, they might be in the same species, but if they were phylogenetically separated, then that could be taken as evidence that they were in different species. If they both had ITS rRNA region sequences and these sequences were from shared orthologous operons, then ITS rRNA region phylogeny and ITS rRNA region PD criteria could be examined. Finally, the number of ITS rRNA region helices that were different could be tabulated. If any criterion indicated lineage separation worthy of species recognition, strain pairs were considered different species. The table containing all this information at a glance was very helpful in visualizing the differences and similarities in these strains (Table S8).

Following this analysis, we numbered the unnamed species so that comparisons were easier to make. This method identified 17 species, 6 of them named and 11 of them unnamed. These 17 species were two species less than our conclusions from the 16S rRNA gene percent identity alone due to the fact we combined two of the species based upon all the evidence. This post-analysis numbering system was employed throughout taxon designations in the phylogenies and percent identity/dissimilarity tables, and empty dots were placed on the nodes in the figures to demarcate the species where possible.

In *Dulcicalothrix*, the 16S rRNA gene data, as represented in both phylogenies and percent identity comparisons, were very robust in differentiating species. If 16S rRNA gene data, morphology, and ecology were our only available character sets, then we would have come to almost the same conclusion as we did with the addition of ITS rRNA region sequence data and analysis. It is encouraging that despite the flaws of incomplete ITS rRNA region data, we came to very similar taxonomic conclusions with the addition of such data. If we had had even more complete ITS rRNA region data, we are confident that our conclusions would have been very similar. However, if this genus did not have as much representation as it did, then we could have made serious errors. If we did not know that there were six different operon types, then we could have concluded that the 2N operon of *Dulcicalothrix iyengarii* 25C-PS and the 3N operon of *Dulcicalothrix* sp. HA4283-MV5 justified describing two species instead of one, as the PD between these two operons was 16.5%. We are concerned that when new species are described without an attempt

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D. Iyengarii 25C-PS	*Dulcicalothrix* sp. 2 HA4395-MV3	*D. Desertica* PCC7102	*Dulcicalothrix* sp. HA4283-MV5	*Dulcicalothrix* sp. 10 NIES-4071	*Dulcicalothrix* sp. 10 NIES-4105	OPERON TYPE
						1B
						1B
						1B
						1B
						2B
						3B
6.6						3B
13.8	12.9					3B
6.8	8.7	11.8				3B
18.5	17.5	15.9	16.2			3B
18.5	17.5	15.9	16.2	0.0		3B
26.3	25.1	24.9	23.0	27.5	27.5	3B

to obtain multiple clones representing multiple operons of the ribosomal genes plus ITS rRNA regions, erroneous taxonomic conclusions could be made, and we are even concerned about some of our own past work in which strains of the same species were described as two different species. Although it is often difficult and economically restrictive to obtain many clones, all cyanobacterial taxonomists should at least be aware of this potential problem and proceed more cautiously in taxonomic description. New genera with a single species are not as problematic as the 16S rRNA gene thresholds and phylogenies are more commonly employed to separate genera. It is when these new genera are expanded to contain more species that caution must be employed.

When multiple operons are recognized, it is important not to use PD of the ITS rRNA region sequences from different operons. Although workers have understood this in comparing operons with or without tRNA genes (Berrendero et al., 2016; Pietrasiak et al., 2019, 2021), when more than two operons are present in the genome, this may have been overlooked. It is interesting to note that not all cyanobacteria appear to have multiple ribosomal operons with differing ITS rRNA region sequences. For example, *Oculatella* (Becerra-Absalón et al., 2020) and, indeed, most Oculatellaceae (Mai et al., 2018), have only been observed to have a single ribosomal operon, and they all contained two tRNA genes in the ITS rRNA region. *Nodosilinea* also has a single operon even after many sequences in many strains have been determined, also with both tRNA genes (Vázquez-Martínez et al., 2018).

Multiple operons within a genus can most confidently be identified when, at least in some strains in the genus of interest, multiple different ITS rRNA region operons are observed in a single strain. In the case of *Dulcicalothrix*, strain SEV5-4-C5 had three very distinct operons, so we knew that this was a potential problem for us. In aligning the ITS rRNA regions, the indels in the D1–D1′ helix region were very evident. When we aligned this region from both tRNA-containing operons and operons lacking tRNA genes, we saw that the three types of D1–D1′ helix clearly were present in both types of ITS rRNA regions. This reflects a pattern we have also observed in *Brasilonema* (Bohunická et al., 2024), in which there are four types of operon based on the D1–D1′ helix sequence and structure, with three of those having representation in both operons with and without tRNA genes. It is possible that orthologous operons could be detected based on differences in other domains in the ITS rRNA region, such as the V2, V3, and BoxB helices, or even the spacer regions. However, these regions do not give a clear signal in *Dulcicalothrix*. In examining the ITS rRNA region alignments for operons with and without tRNA genes (Tables S3 and S4), it can be seen that BoxB sequences are very similar across operons and likely experienced homogenization based upon stabilizing selection forces acting on this helix. However, the V2 and V3 helices are highly variable and likely not under the strict stabilizing selection experienced by the D1–D1′ and BoxB helices. It is likely that chromosomal rearrangements occurred through homologous recombination within strains, leading to the observed complexity in *Dulcicalothrix*

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TABLE 3 Percent distance among aligned 16S-23S ITS sequences in *Dulcicalothrix* species for which ITS operons lacking tRNA genes exist.

Strain designation	*D. Adhikaryi* ZW1-PS	*"Calothrix"* sp. 4 UAM 315	*"Calothrix"* sp. 9 PCC7103	*"Calothrix* sp. 9 CCAP 1410/10	*"Calothrix"* sp. 8 CCM-UFV03	*"Calothrix"* sp. 8 PCC 7716 1,856,000	*"Calothrix"* sp. 8 PCC 7716 9,965,900	*D. Necridiiformans* V13	*Dulcicalothix* sp. 6 SEV5-4-c5
D. adhikaryi ZW1-PS OK138873									
"Calothrix" sp. 4 UAM 315 EU009152	20.0								
"Calothrix" sp. 9 PCC7103 FJ661016	18.2	13.3							
"Calothrix" sp. 9 CCAP 1410/10 HF678479	12.3	13.1	1.5						
"Calothrix" sp. 8 CCM-UFV03 MT708215	19.8	10.8	11.1	11.0					
"Calothrix" sp. 8 PCC 7716 AP02518 1,856,000	18.2	9.3	9.3	8.6	1.2				
"Calothrix" sp. 8 PCC 7716 AP02518 9,965,900	18.2	8.3	9.3	8.7	2.1	0.9			
D. necridiiformans V13 KY863521	23.0	33.6	30.4	26.4	29.0	28.1	28.1		
Dulcicalothix sp. 6 SEV5-4-c5 KT336446	15.2	15.0	13.5	9.1	15.5	14.0	13.6	25.4	
Dulcicalothix sp. 6 SEV5-4-c5 KT336447	15.9	15.4	14.5	12.6	15.4	14.2	13.8	27.2	2.5
D. iyengarii 25C-PS OK138872	19.1	19.2	17.8	20.0	18.5	17.0	17.0	29.6	13.7
Dulcicalothix sp. 3 HA4186-MV5 HQ847580	18.2	18.0	18.9	20.3	17.3	15.7	15.4	25.5	15.1
Dulcicalothix sp. 1 44-T10 Mares KY563666	20.1	16.4	16.8	18.6	18.7	17.1	16.8	31.0	14.2
Dulcicalothix sp. 1 34T8Micr KY488001	20.1	16.4	16.8	18.6	18.7	17.1	16.8	31.0	14.2
Dulcicalothix sp. 10 NIES-4071 AP018255	24.6	27.3	28.0	24.9	27.4	25.9	25.9	29.0	23.5
Dulcicalothix sp. 10 NIES-4071 AP018255	25.9	26.6	27.2	24.8	27.7	26.2	26.2	29.5	23.8
Dulcicalothix sp. 10 NIES-4105 AP018290	25.9	26.7	27.3	24.8	27.5	25.9	25.9	29.5	23.5
Dulcicalothrix sp. HA4283-MV5 HQ847579	18.9	17.8	17.3	27.2	17.0	16.2	16.5	34.1	17.0
Dulcicalothix sp. 2 HA4395-MV3 HQ847572	22.3	18.4	23.2	34.9	20.3	19.4	19.0	40.9	19.9

Note: Strains considered to be the same species are highlighted in matching light gray. Percent dissimilarity thresholds should only be applied within orthologous operons (exclude comparisons highlighted in dark gray). Species numbers indicating congeners were added at the conclusion of taxonomic analysis.

and *Brasilonema*. Certainly, more complete data in many strains in many genera will be needed before the understanding of ITS rRNA region complexity is completely obtained.

The polyphasic approach to cyanobacterial taxonomy should include morphology and ecology in addition to the diverse molecular criteria we employ in this manuscript. However, morphology and ecology alone do not provide clarity among the five species of *Dulcicalothrix* described so far. Currently, the occurrence of multiple young trichomes within a single sheath during some stages of development in

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Dulcicalothix sp. 6 SEV5-4-c5	*Dulcicalothrix iyengarii* 25C-PS	*Dulcicalothix* sp. 3 HA4186-MV5	*Dulcicalothix* sp. 1 44-T10 mares	*Dulcicalothix* sp. 1 34T8Micr	*Dulcicalothix* sp. 10 NIES-4071	*Dulcicalothix* sp. 10 NIES-4071	*Dulcicalothix* sp. 10 NIES-4105	*Dulcicalothrix* sp. HA4283-MV5	OPERON TYPE
									1N
									1N
									1N
									1N
									1N
									1N
									1N
									1N
									1N
									2N
14.0									2N
16.1	9.7								2N
15.2	9.0	9.7							2N
15.2	9.0	9.7	0.0						2N
24.1	24.1	24.2	23.3	23.3					2N
24.4	23.8	24.2	22.9	22.9	4.5				2N
24.2	24.1	24.2	23.9	23.9	3.1	2.5			2N
16.3	16.5	17.0	16.9	16.9	29.3	29.6	29.6		3N
19.0	16.3	17.2	17.9	17.9	29.5	29.6	29.9	21.3	3N

D. iyengarii and the spiraling of the filaments in older stages of the life cycle in *D. adhikaryi* are unique differentiating features for these species. However, other species pairs show overlapping features. For example, *D. necridiiformans* has bow-shaped necridia (for which it was named), but all species have necridia. Similarly, *D. alborzica* and *D. desertica* have swollen basal regions, and the other taxa do not. Furthermore, we compared the heterocytes among the *Dulcicalothrix* species, but we could not differentiate all the species based on this character. *Dulcicalothrix adhikaryi* has heterocytes larger than

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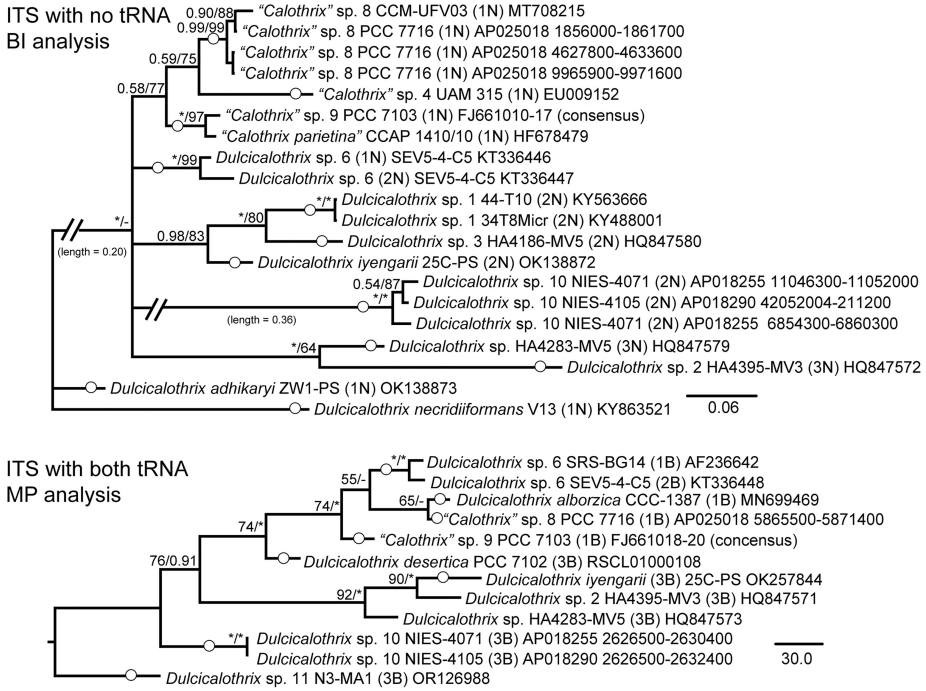

FIGURE 6 Phylogenetic analysis based on alignments of the ITS rRNA regions for operons with no tRNA genes and those with tRNA genes. The top analysis is the Bayesian inference analysis with bootstrap values from the maximum parsimony analysis mapped to the nodes; *Means full support, –means less than 0.50% or 50% support. The bottom analysis is the maximum parsimony analysis with posterior probabilities from the BI analysis mapped to nodes.

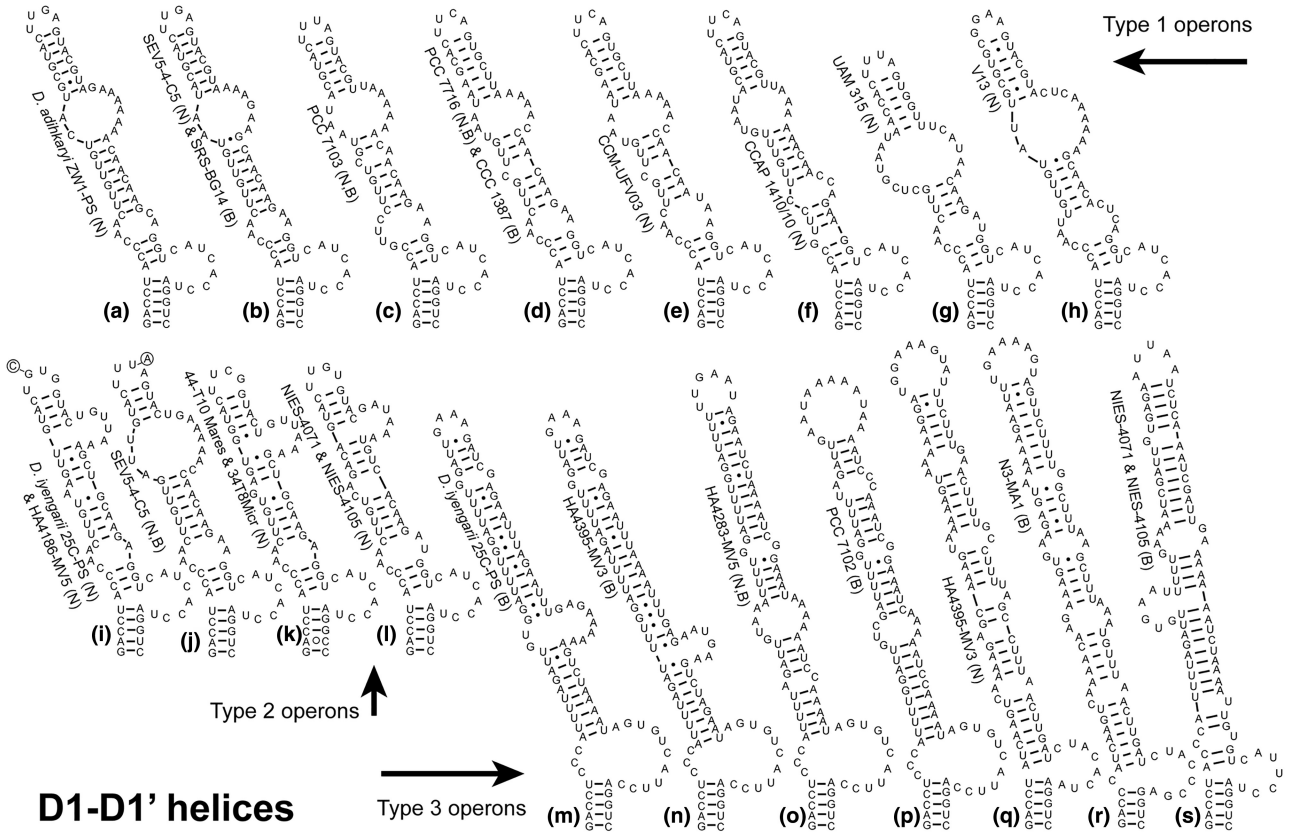

FIGURE 7 The D1–D1′ helices for all *Dulcicalothrix* strains for which ITS rRNA region sequence data are available, organized by numbered type, with indication of no tRNA genes and/or both tRNA genes indicated in parentheses as N or B, respectively.

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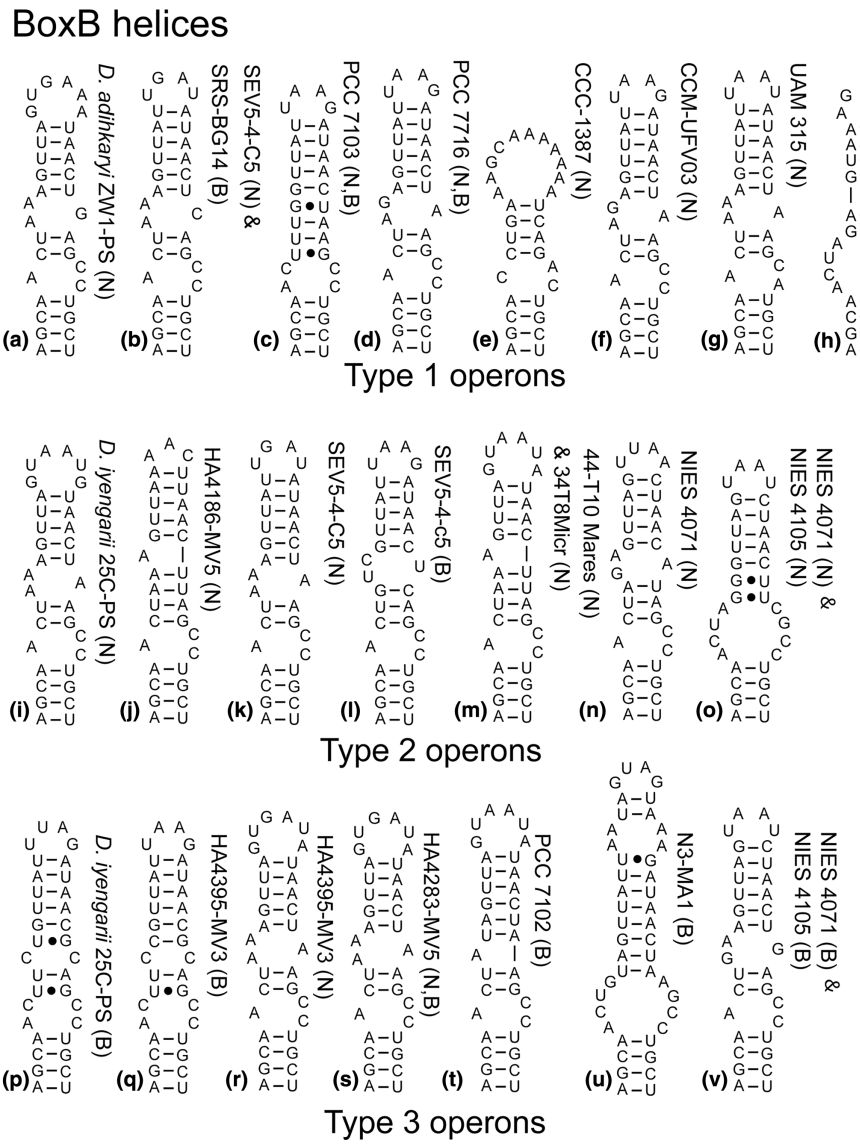

FIGURE 8 The BoxB helices for all *Dulcicalothrix* strains for which ITS rRNA region sequence data are available, organized by numbered type, with indication of no tRNA genes and/or both tRNA genes indicated in parentheses as N or B, respectively.

the heterocytes in other species, and *D. iyengarii* has heterocytes smaller than the heterocytes of other species. However, *D. necridiiformans* and *D. alborzica* have heterocytes that are intermediate and similar in size, and we do not know the size ranges for heterocytes of *D. desertica*. Currently, it is possible that these species could be differentiated based on morphology if morphology is combined with ecology, but we suspect that the 17 species-level clades we identify in this manuscript, if named, would likely be difficult to distinguish based on morphology and ecology alone. We continue to feel these character sets should be combined and considered when conducting cyanobacterial taxonomy, but the molecular data may provide sufficient power to separate taxa even with limited morphological support. Morphology and ecology are critical and required for description of new taxa but often are not sufficient on their own. It is the reason molecular methods of taxonomy have become so widely employed in the modern era.

The tapering heterocytous taxa have now received considerable taxonomic attention, and the Calotrichaceae is particularly well established. Only the strains in the CHAB collection remain to be circumscribed. The family contains *Dulcicalothrix*, *Brunnivagina*, *Fulbrightiella, Sherwoodiella*, and *Macrochaete* (Berrendero et al., 2016; Kumar, Saraf, Pal, Mishra, Singh, & Johansen, 2022; Saraf, Suradkar, et al., 2019). What we lack is the namesake for the family *Calothrix*, which will be established with the sequencing of a strain clearly belonging to the type species, *C. confervicola*. This is the piece of this puzzle that remains. We are hopeful that it will be placed in the puzzle soon.

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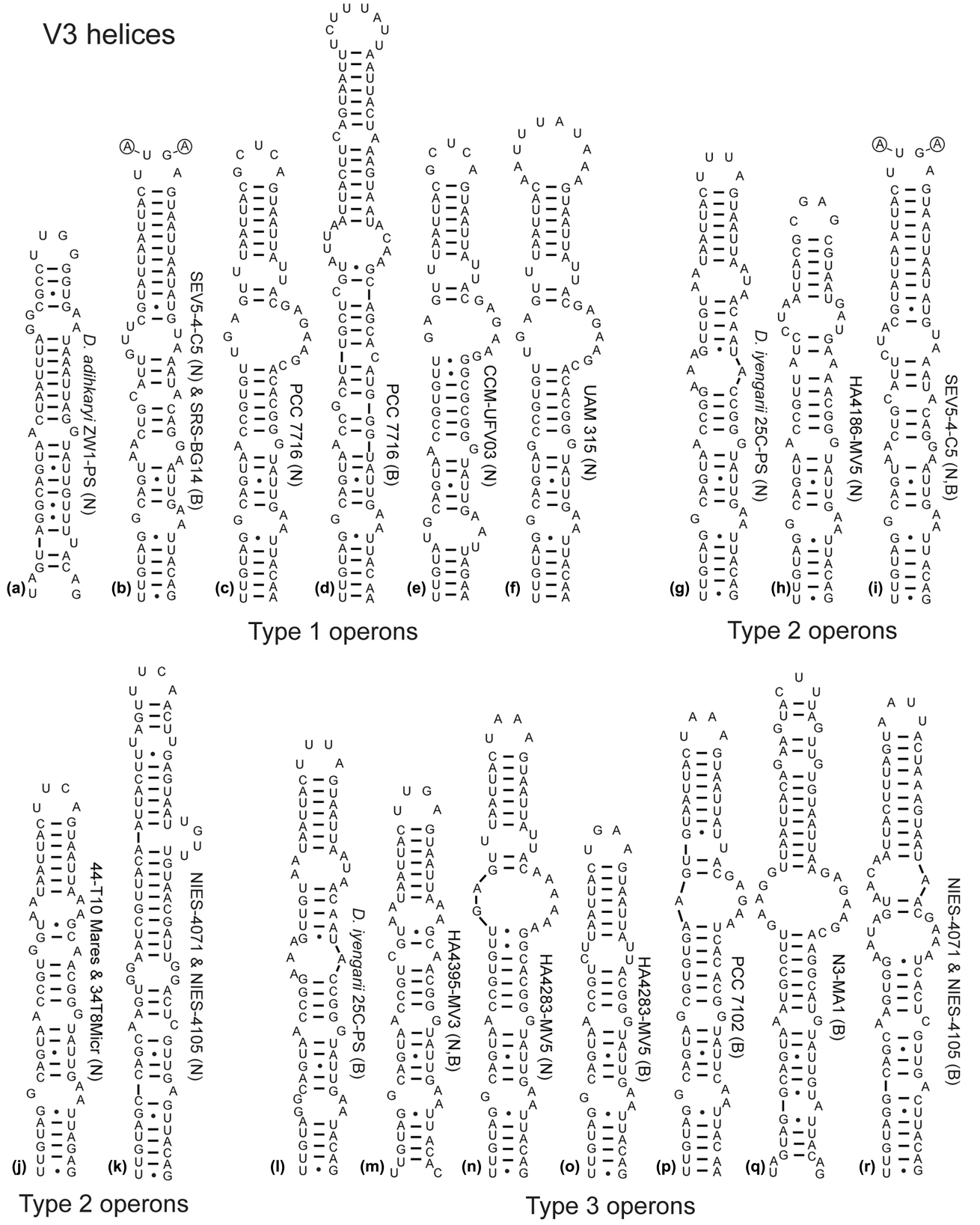

FIGURE 9 The V3 helices for all *Dulcicalothrix* strains for which ITS rRNA region sequence data are available, organized by numbered type, with indication of no tRNA genes and/or both tRNA genes indicated in parentheses as N or B, respectively.

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AUTHOR CONTRIBUTIONS

Aniket Saraf: Conceptualization (lead); investigation (lead); writing – original draft (supporting); writing – review and editing (equal). **Prashant Singh:** Conceptualization (supporting); funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); writing – review and editing (equal). **Naresh Kumar:** Formal analysis (supporting); visualization (equal); writing – review and editing (equal). **Sagarika Pal:** Visualization (equal); writing – review and editing (equal). **Jeffrey R. Johansen:** Conceptualization (supporting); formal analysis (lead); visualization (equal); writing – original draft (lead); writing – review and editing (equal).

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SUPPORTING INFORMATION

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

Figure S1. Bayesian Inference analysis based on 228 sequences (Nostocales plus *Chroococcidiopsis* as an outgroup) of the 16S rRNA gene, with ML bootstrap values mapped to nodes.

Table S1. Master alignment of 521 sequences with a length of 1517 nucleotides color coded and annotated based on secondary structure of the 16S rRNA molecule. Helices labeled H1 to H45 with counter for nucleotide position at top of matrix block. The 5′ and 3′ sides of the helices appear in the same color, and many are interrupted by large sections of other helices, in which case the 3′ side of the helix is indicated with a prime (apostrophe). The reader is referred to diagrams of the 16S molecule (e.g., fig. 2 in Johansen et al. 2017. Highly divergent 16S rRNA sequences in ribosomal operons of *Scytonema hyalinum* (Cyanobacteria). *PLoS ONE* 12(10): e0186393). Researchers are welcome to use this alignment as a starting point for other studies of the Nostocales, but are requested to cite this supplementary table and the manuscript in which it appears.

Table S2. Nexus file for Bayesian Inference Analysis of 16S rRNA gene sequence for 228 sequences containing the ingroup of the Calotrichaceae and outgroup Nostocales + *Chroococcidiopsis* taxa.

Table S3. Alignment of ITS sequences with both $tRNA^{Ile}$ and $tRNA^{Ala}$ gene sequences. This contains the formatting and data blocks for the Bayesian Inference analysis conducted on the CIPRES Science Gateway. Highlighting is coded as follows: (1) first block of turquoise and green—D1–D1′ helix, (2) yellow block ending in AAACT—D2 region, (3) yellow block of 6 nucleotides—D3 region, (4) first red block—$tRNA^{Ile}$, (5) second red block—$tRNA^{Ala}$, (6) second block of turquoise and green—BoxB helix, (7) yellow block of 11 nucleotides—BoxA, (8) maroon—D4 region, (9) third block of turquoise and green—V3 helix, (10) final yellow block—D5 region. Researchers are welcome to use this alignment as a template for other analyses of ITS regions, but should cite this figure and the manuscript in which it appears.

Table S4. Alignment of ITS sequences with no tRNA gene sequences. This contains the formatting and data blocks for the Bayesian Inference analysis conducted on the CIPRES Science Gateway. Highlighting is coded as follows: (1) first block of turquoise and green—D1-D1′ helix, (2) yellow block ending in AAACT—D2 region, (3) yellow block of six nucleotides—atypical D3 region, (4) second block of turquoise and green—BoxB helix, (5) yellow block of 11 nucleotides—BoxA, (6) maroon block—D4 region, (7) third block of turquoise and green—V3 helix, (8) final yellow block—D5 region. Researchers are welcome to use this alignment as a template for other analyses of ITS regions, but should cite this figure and the manuscript in which it appears.

Table S5. Domain lengths for all *Dulcicalothrix* ITS sequences currently available in GenBank. Lengths are organized by operon type, which is indicated at beginning of strain designation. NA means that sequences were incomplete so length could not be determined for that region in the indicated strain.

Table S6. Morphological and ecological comparisons of *Dulcicalothrix iyengarii* 25C-PS and *Dulcicalothrix adhikaryi* ZW1-PS with the other members of the genus *Dulcicalothrix*.

Table S7. Percent identity among *Dulcicalothrix* strains based on 16S rRNA sequence data. Strains with percent identity ≥99.5% are considered to be likely same species (highlighted), those ≤98.7 are considered to be different species (blue font), those between 98.7% and 99.5% may or may not be the same (black font, no highlighting), and require further evidence for a decision. We consider those sharing same color of shading to be likely the same species.

Table S8. Combined molecular comparison of strain pairs using five criteria. D means the criterion indicates that the two strains are in different species, S means the strain pair could be or is likely the same species, A means the evidence is ambiguous using this criterion. The first criterion is percent identity of 16S rRNA gene sequence (<98.7% is D, 98.7%–99.4% is A, and 99.5% is S). The second criterion is phylogeny based on the 16S rRNA gene sequence (D is different clades, S is same clade, A is close clade but long branch), The

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third criterion is ITS phylogeny (D is different clades, S is same clade, A is close clade but long branch). The fourth criterion is percent dissimilarity of between orthologous operons (D is ≥7.0%, A is between 3.0% and 7.0%, S is ≤3.0%). Finally the fifth criterion is the number of ITS helices in orthologous operons that have different structure (0–3 among D1–D1′, Box-B, and V3 helices). If the strain pair does not share an orthologous operon a single question mark follows the first two criteria. If any of the criteria have a "D" the strain pair is considered to represent two different species.

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