2022

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J. Phycol. **59,** 204–220 (2023)

DOI: 10.1111/jpy.13297

CIRCUMSCRIPTION OF *FULBRIGHTIELLA* GEN. NOV. AND *SHERWOODIELLA* GEN. NOV., TWO NOVEL GENERA IN THE CALOTRICHACEAE (NOSTOCALES, CYANOBACTERIA)[1]

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Three novel strains in Calotrichaceae from tropical habitats were isolated and characterized with regard to their morphology, phylogenetic placement, and secondary structures of conserved domains in the 16S-23S internal transcribed spacer (ITS). The strains fell into two clades formerly identified as *Calothrix* from freshwater and brackish habitats. Based on both morphology and ecology, they differed from the type species of *Calothrix*, *C. confervicola*, which is marine, has wide trichomes with short cells, and narrows abruptly to a hyaline hair. The first clade grouped species with heteropolar filaments widened at the base and narrowed gradually toward the apex but not ending in a hair, with basal heterocytes that are formed in series as the apically placed heterocytes senesce; this clade is being named *Fulbrightiella* gen. nov., with two named species, *F. bharadwajae* sp. nov. and *F. oahuensis* sp. nov. The second clade was comprised of a single species with isopolar trichomes that are untapering as hormogonia, but which widen midfilament and taper toward both ends following growth. These trichomes develop pairs of heterocyte mid-filament, causing fragmentation into heteropolar trichomes with basal heterocytes and ends that taper, but not to a hair. This clade consists of a single species at present, *Sherwoodiella mauiensis*. With this action, four clades in the Calotrichaceae have been named: *Macrochaete*, *Dulcicalothrix*, *Fulbrightiella*, and *Sherwoodiella*. *Calothrix* sensu stricto is truly marine, morphologically distinct, and unsequenced; finding and sequencing the generitype for *Calothrix* remains as the most important and unfinished task in the revision of the Calotrichaceae.

Key index words: **16S-23S ITS; *Calothrix*; Calotrichaceae; heteropolarity; isopolarity; Nostocales; Rivulariaceae**

Nostocales, among the eight orders of the phylum cyanobacteria (Komárek et al. 2014), consist of thalli containing specialized cells (heterocytes and akinetes) in filaments that may be isopolar or heteropolar, tapering or untapering, or false branched or true branched. With the emergence of the polyphasic approach, the taxonomy of cyanobacteria has been moving at a fast pace, particularly in the Nostocales, but with phylogenetic inconsistencies being reported within the order at both family and genus levels (Hauer et al. 2014, Komárek et al. 2014, Berrendero et al. 2016, Saraf et al. 2018, 2019). New families such as Gloeotrichiaceae (Komárek et al. 2014), Tolypothrichaceae (Hauer et al. 2014), Godleyaceae (Hauer et al. 2014), and Cyanomargaritaceae (Shalygin et al. 2017) have been recently described. Currently, there are 19 families within the order Nostocales (www.cyano.db, accessed September 19, 2022) out of which four families, Rivulariaceae (Bornet and Flahault 1886), Gloeotrichiaceae (Komárek et al. 2014), Cyanomargaritaceae (Shalygin et al. 2017), and Calotrichaceae (Bennet and Murray 1889, Saraf et al. 2019), mainly consist of heteropolar, tapering filamentous forms.

The family Calotrichaceae currently contains the type genus *Calothrix* (Agardh ex Bornet and Flahault 1886), as well as *Macrochaete* (Berrendero et al. 2016) and *Dulcicalothrix* (Saraf et al. 2019). The genus *Calothrix* is probably the most difficult

[1]Received 11 June 2022. Accepted 21 October 2022.
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Editorial Responsibility: M.L. Vis (Associate Editor)

genus among tapering taxa based on both morphological and phylogenetic complexities (Whitton 1987, Berrendero et al. 2016, Saraf et al. 2019). *Calothrix* exhibits heteropolar filaments having a basal heterocyte with the apical ends terminating slightly into narrow but chlorophyllous apical cells or into achlorophyllous, hyaline hairs. While many taxa appear to not have the genetic ability to produce hyaline hairs, those that do often do not form hairs, particularly in cultured strains. Variations in media, typically lowered phosphorus, have been found to be required to produce terminal hairs in some of these strains (Whitton 1987, 1992, 2002, Whitton and Potts 2000, Berrendero et al. 2008, Whitton and Mateo 2012, Berrendero et al. 2016, Saraf et al. 2019). Some species of *Calothrix* can facultatively produce basal akinetes, although most species appear to lack this ability (Komárek 2013), and currently no akinete-producing species of *Calothrix* have been reported in published phylogenetic analyses. It has also been noted that strains belonging to the genus *Calothrix* appear as solitary filaments or small bundles of filaments attached to various substrates. *Nunduva* also is comprised of solitary tapering filaments in *Calothrix*-like clusters, but it clearly is phylogenetically allied with the Rivulariaceae (González-Resendiz et al. 2018a, Johansen et al. 2021). In summary, *Calothrix* is a species-rich genus with variable ability to produce hairs and/or akinetes, high levels of plasticity, and broad habitat diversity. It should come as no surprise that this genus is in need of extensive study, revision, and description of new species and genera to arrive at monophyletic genera within the Calotrichaceae and other family-level clades of tapering Nostocales.

In addition to these difficulties (and in part because of them), previous studies using phylogeny based on several markers, including 16S rRNA gene, *cpc*BA-IGS, and *nif*D gene, have consistently found *Calothrix* to be a polyphyletic genus (Henson et al. 2004, Sihvonen et al. 2007, Berrendero et al. 2011, Dominguez-Escobar et al. 2011, Whitton and Mateo 2012). Also part of the problem of apparent polyphyly is the fact that despite the existence of over a hundred species of *Calothrix* (Komárek 2013), a vast number of sequences available in public databases have no species epithets, and these sequences belong to numerous diverse clades. Another difficulty that continues to plague the taxonomy of *Calothrix* is the absence of sequences corresponding to the type species of the genus, *C. confervicola* (Bornet and Flahault 1886, Gardner 1932, Geitler 1942). As a result of this, the exact placement of *Calothrix* sensu stricto is unclear, and thus, in spite of having at least five well-supported genetic clusters (Sihvonen et al. 2007), polyphasic studies on *Calothrix*-like groups have been very few, with only three *Calothrix*-like genera having been described (Berrendero et al. 2016, González-Resendiz et al. 2018a, Saraf et al. 2019). Also, based on robust phylogenetic evidence, Saraf et al. (2019) split the family Rivulariaceae and re-erected the family Calotrichaceae to accommodate *Calothrix*, *Dulcicalothrix*, and *Macrochaete*. At present, the family Rivulariaceae consists of *Rivularia*, *Dichothrix*, *Sacconema*, *Isactis*, *Gardnerula*, *Phyllonema*, *Kyrtuthrix*, and *Nunduva* (Komárek 2013, Komárek et al. 2014, González-Resendiz et al. 2018a,b, Saraf et al. 2019). Thus, from all the above-mentioned studies, it is clearly evident that there are multiple issues complicating the taxonomy of *Calothrix*-like clades. The creation of *Macrochaete* and *Dulcicalothrix* has resolved some issues definitively, but more revisions based on a polyphasic approach need to be completed in order to fully decode the taxonomy of this complicated genus, its morphotypes, and its doppelgangers.

In the present communication, as a part of a first investigation from the freshwater ecosystems of the Jammu and Kashmir region of India, we describe two new *Calothrix*-like genera from tropical freshwater systems in the Jammu and Kashmir region of India as well as the Hawaiian Archipelago, in accordance with the International Code of Nomenclature for algae, fungi, and plants (Turland et al. 2018). We will refer to these genera as *Fulbrightiella* gen. nov. and *Sherwoodiella* gen. nov. in the remainder of this article.

MATERIALS AND METHODS

Sample collection, isolation, and culturing. The sample from which the *Fulbrightiella bharadwajae* gen. nov., sp. nov. strain 18G-PS was isolated was collected from a small puddle in a wetland situated in the village Basantgarh of district Udhampur, Jammu and Kashmir, India (32°48′23″ N 75°32′36″ E; Fig. 1). The sample was collected in a 50 mL falcon tube containing sterile $BG11_0$ liquid medium (Rippka et al. 1979). In the laboratory, the sample was spread on a Petri dish containing $BG11_0$ medium having pH adjusted to 7.2 and solidified with 1.2% agar. After 14–16 days, a single colony was taken, washed thrice with sterile double-distilled water, and transferred to a sterile culture tube containing 5 mL of sterile $BG11_0$ medium. The strain was purified and isolated by culturing alternatively on solid and liquid medium three to four times. The culture was thereafter maintained on agar slants at approximately 22°C with ±2°C temperature, under cool white light of approximately 50–55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a photoperiod of 14:10 h light:dark cycle. In order to check the formation of terminal hairs, the strain was also grown on low phosphorus (50%, 25%, and no phosphorous) containing $BG11_0$ medium.

The sample from which *Fulbrightiella oahuensis* gen. nov., sp. nov. HA4248-MV3 was isolated was collected on July 24, 2009, by Alison Sherwood, Melissa Vaccarino and Rex Lowe from a wet wall on the Kalawahine Trail, Round Top, Honolulu, Oahu, Hawai'i, USA (21°20′6″ N, 157°48′58″ W). The sample from which *Sherwoodiella mauiensis* gen. nov. sp, nov. HA4860-CV1 was isolated was collected on January 7, 2010, by J. Patrick Kociolek, Jennifer Neumann, and Melissa Vaccarino as a composite rock scraping in a stream near Olowalu Beach, Mauai, Hawai'i, USA (20°48′34″ N, 156°36′18″ W). Both samples were transported to the Johansen Laboratory at John Carroll University in a 2 mL Eppendorf vial and plated

FIG. 1. Features of the habitat (a, b) and the naturally occurring sample of *Fulbrightiella bharadwajae* 18G-PS (c) at the time of sampling. [Color figure can be viewed at wileyonlinelibrary.com]

on 1.5% agar-solidified Z8 media (Carmichael 1986). The strains were isolated into liquid Z8 media by Melissa Vaccarino (HA4248-MV3) and Christina Vaccarino (HA4860-CV1), respectively, and transferred to solidified Z8 media for long-term maintenance in a growth room at 16°C under cool white light of approximately 40–50 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a photoperiod of 16:8 h light:dark cycle. In order to check the formation of terminal hairs, both strains were also grown on low phosphorus (10%) containing Z8 medium as well as nitrogen-free Z8 medium.

Morphological analysis. Morphological evaluation of the three strains was performed throughout different phases of growth on Olympus BX60 and BH2 photomicroscopes with high-resolution Nomarski DIC optics, equipped with Olympus SC50 digital cameras. Particular attention was paid to the filament characteristics, appearance of sheath, tapering tendencies, shape and size of basal heterocytes, vegetative cells, necridia, and presence/absence of terminal hairs.

Molecular analysis. Genomic DNA was extracted from actively growing unicyanobacterial cultures using the Qiagen DNeasy® Power Soil® Pro Kit (Qiagen, USA). PCR amplification of the 16S rRNA gene and the associated 16S-23S ITS region was performed using the primer pairs VRF1 (5′-CTCTGTGTGCCTAGGTATCC-3′; Wilmotte et al. 1993, Flechtner et al. 2002) and VRF2 (5′-GGGGAATTTTCCG CAATGGG-3′; Nübel et al. 1997, Flechtner et al. 2002). PCR was performed in 25 µL reactions on a Bio-Rad C1000™ Thermal Cycler. A 12.5 µL of LongAmp® Taq 2X Master Mix (NEB), 0.5 µM each of the primers, and 2 µL each of DNA were added to set up the PCR reaction. Cycling conditions were 35 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 135 s. A 3-minute extension at 72°C was performed, and the reaction was held at 4°C indefinitely. After cross check on 1.2% agarose gel, the amplicons were cloned using StrataClone PCR Cloning Kit (Agilent Technologies, USA) followed by restriction digestion (Bohunická et al. 2011). Selected clones of interest were sent to Functional Biosciences Inc. (Madison, WI, USA) for sequencing. The sequences obtained were assembled into contigs, and proofreading was done using Sequencher software (version 4.8, Ann Arbor, MI, USA). The sequences of 16S rRNA gene and 16S-23S ITS which were generated after sequencing were submitted to NCBI Nucleotide (GenBank).

16S rRNA gene analysis. A BLAST search (https://blast.ncbi.nlm.nih.gov/) was used to download all the related sequences with our own sequences. The entire set of sequences was eventually aligned manually to ensure that alignments truly supported the preserved secondary structures. Attention was paid to regions with gaps or missing data. Neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) analyses were used to generate phylogenetic trees. The NJ and ML trees were constructed by MEGA 5.2.2 software (Tamura et al. 2011). The bootstrap method with 1000 replications was utilized to test node support for NJ, ML, and MP trees. The BI tree was constructed using Mr Bayes 3.2.2 (Ronquist et al. 2012). The best fit model for BI was GTR + G + I as determined in jModelTest (Darriba et al. 2012). Fifty million generations were utilized to achieve a 0.01 standard deviation of split frequency. Node support is reported as posterior probabilities.

Pairwise percentage similarities of the 16S rRNA gene between close relatives of strains 18G-PS, HA4248-MV3, and HA4860-CV1 were calculated using either MEGA version 5 (Tamura et al. 2011) or PAUP on XCEDE in the CIPRES Science Gateway.

16S-23S ITS analyses. The different 16S-23S ITS operons (without tRNA/with tRNA) were identified by coding the basal clamps of each semi-conserved helix of our strains of interest along with all phylogenetically related strains, followed by folding of the sequences corresponding to the D1-D1', V2, Box-B, and V3 helices using the Mfold application (ZUKER 2003) on the UNAFold Web Server (https://unafold.org). Apart from the folding of the above four regions, lengthwise comparative evaluation of the leader, D1-D1', Spacer + D2, Spacer + D3 + Spacer, $tRNA^{ile}$, Spacer + V2 + Spacer, $tRNA^{ala}$, Spacer + boxB + Spacer, boxA, D4 + Spacer, and the V3 + end of ITS was also performed for all the strains under investigation. Percent dissimilarity of ITS regions based on p-distance (1 − (100 × p)) was calculated within those taxa belonging to the same genus or to closely related genera in which ITS regions could be aligned.

Line drawings and herbaria preparation. A line drawing of the appropriate characteristic morphological attributes for the strain 18G-PS was drawn using the stippling technique. Herbaria preparations on 25-mm glass fiber filters were prepared in duplicate for holotype and isotype submissions using liquid cultures with standard media and filtration flasks. The filters were allowed to air-dry for at least 48 h and then packed into acid-free envelopes for submission to herbaria.

RESULTS

Taxonomic descriptions. ***Fulbrightiella*** N. Kumar, P. Singh, and J.R. Johansen., **gen. nov.**

Diagnosis: Similar in morphology to *Calothrix*, *Dulcicalothrix*, and *Macrochaete*, but phylogenetically separate from those genera and with percent similarity of the 16S rRNA genes <94.5% in comparison to other tapering genera.

Description: Filaments heteropolar, wider in the basal region, gradually tapering to the distal end, but not forming a terminal hyaline hair. Akinetes absent. Basal heterocytes present, with a senescent heterocyte often seen attached terminally to living heterocytes.

Etymology: Named in honor of U.S. Senator J. William Fulbright, a noted multilateralist in the mid-20th century who founded the Fulbright Fellowship Program, which supported the collaboration leading to this article.

Type species: *Fulbrightiella bharadwajae* N. Kumar, P. Singh, and J.R. Johansen.

Fulbrightiella bharadwajae N. Kumar, P. Singh, and J.R. Johansen., **sp. nov.** (Figs. 2–4).

Diagnosis: Morphologically most similar to *Calothrix clavata*, but differing in its smaller dimensions, particularly width of the basal region of the trichomes, which are 5–7 μm wide in *C. clavata*. Differing from all other *Calothrix* species based on narrower trichome dimensions.

Description: Colony macroscopically visible in small ponds, bluish-green to brownish, mucilaginous and shiny in appearance. Colony in laboratory-grown cultures on agar-solidified media in slants or Petri plates a bluish-green mat with shiny, mucilaginous texture. Colonies grown in low-phosphorous concentrations exhibiting less mucilage, more brittle texture, and less shiny appearance. Cultures grown in liquid media bluish green, with filaments adhering to each other rather than to the walls of the glass tubes. Filaments lacking a bulbous swollen base, gradually tapering from the base to the apex, when mature tapering to a non-hyaline trichome over 2 μm wide, sparsely false branched, less than 20 μm long in young filaments, over 200 μm long

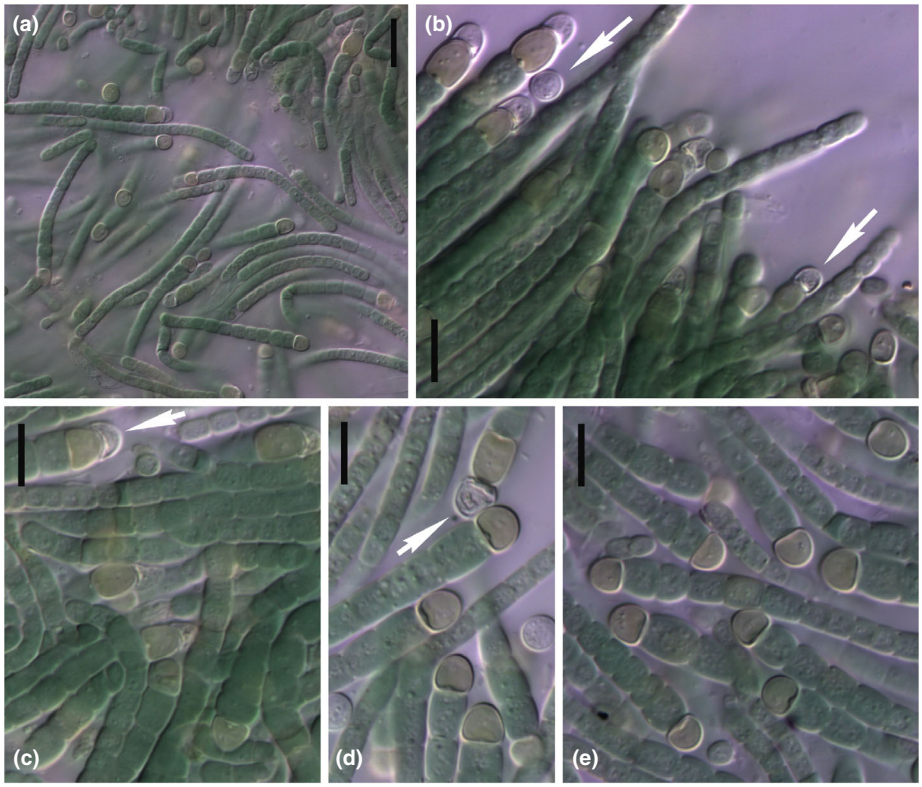

FIG. 2. *Fulbrightiella bharadwajae* 18G-PS grown in $BG11_o$ medium. a. Low magnification view showing consistently short, constricted, and barely tapering trichomes; scale bar 20 μm. b–e. High magnification views showing variability of heterocyte shape and formation; scale bars 10 μm. Arrows: double heterocyte with senescing heterocyte adjacent to a viable basal heterocyte. [Color figure can be viewed at wileyonlinelibrary.com]

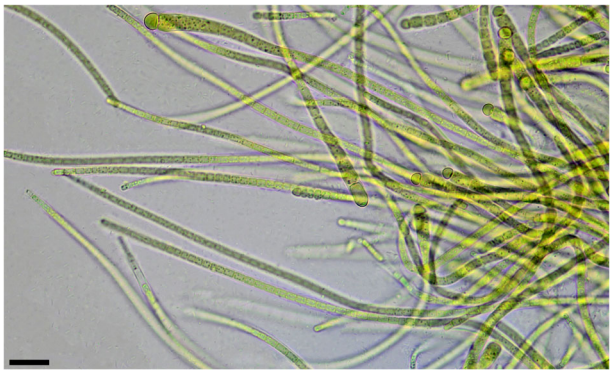

FIG. 3. *Fulbrightiella bharadwajae* 18G-PS grown in low-phosphorus $BG11_0$ medium. Note the thinner, less-constricted trichomes and tapering apices. Scale bar 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

in mature filaments. Sheath thin, firm, colorless, not lamellated and tightly bound to the cells of the trichome. Trichomes not too distinctly constricted at the cross walls, especially in basal regions, appearing less constricted in the long, thin terminal regions of mature filaments, in basal region near heterocyte 2.3–5.1 μm wide, in intercalary region 2.6–3.5 μm wide, and in terminal region 2.2–3.2 μm in wide. Vegetative cells prominently granulated, longer than wide or shorter than wide, 3.3–4.1 μm long near the base, 2.6–6.2 μm long in the main part of the trichome, becoming 2.1–4.7 μm long near the terminus. Heterocytes terminal, basal in position, yellow in color, spherical or with clearly rounded free terminal ends and flattened to the adjacent vegetative cell, sometimes in pairs or series, the newer cell subterminal to the older senescing heterocyte, 3.1–6.7 μm long, 3.1–4.6 μm wide. Necridia present. Akinetes not observed.

Holotype here designated: Portion of a culture of 18G-PS preserved in metabolically inactive form in Global Collection of Cyanobacteria (GCC; Registered Number 1165, Varanasi, India, and available under the accession number GCC20211).

Isotypes here designated: Herbaria deposited in the Global Collection of Cyanobacteria with the number GCC-botanybhu-20,211, and BISH 786237, dried reference strain on glass-fiber filters deposited in the Bishop Museum, Honolulu, Oahu, Hawai'i.

Type locality: Sample was collected by N. Kumar and cultured in November 2019 from a naturally occurring small vernal pool associated with a wet meadow surrounded by evergreen coniferous forests in Village Basantgarh of district Udhampur, Jammu and Kashmir, India (32°48′23″ N, 75°32′36″ E).

Habitat: Naturally occurring macroscopic mats, slightly bluish-green to dark brown in color, with evident mucilage present (Fig. 1) in a small vernal pool associated with a wet meadow surrounded by evergreen coniferous forests. The temperature of the water body was 12.7°C, pH 5.25, electrical conductivity 54.0 $\mu S \cdot m^{-1}$, total dissolved solids 38.5 ppm, and salinity 27.3.

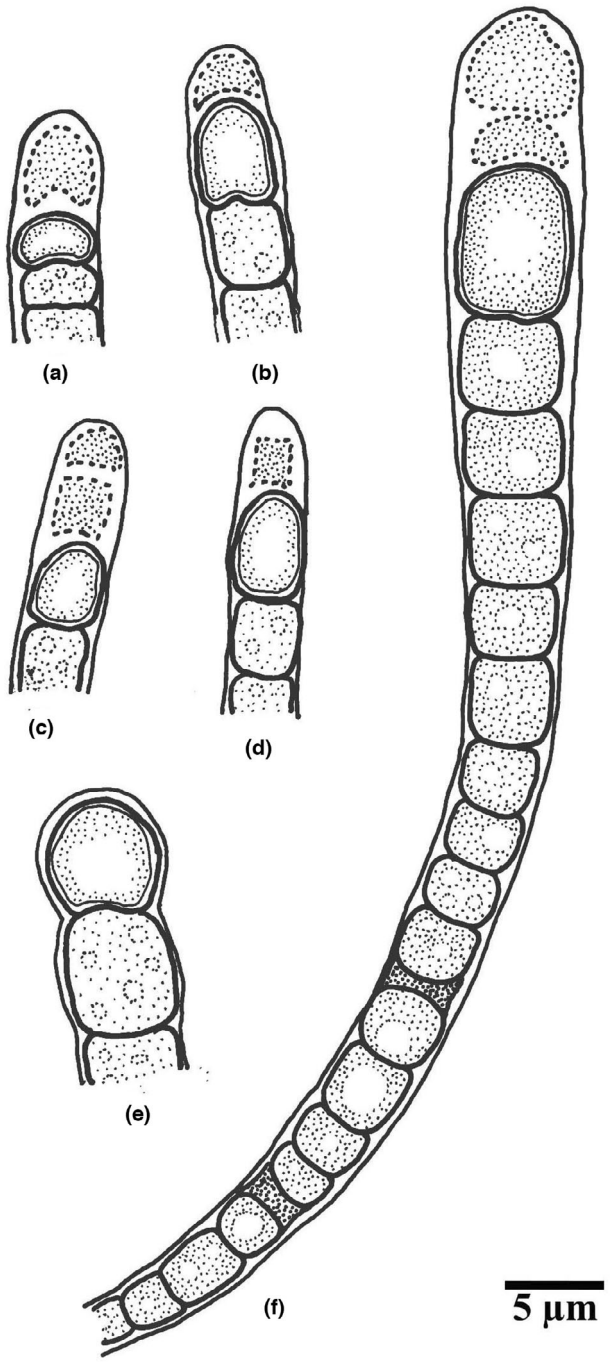

FIG. 4. *Fulbrightiella bharadwajae* 18G-PS line drawing showing key features of heterocyte degeneration, constricted cells, and necridia. Scale bar 5 μm.

Etymology: Named in honor of Professor Yajnavalkya Bharadwaja (1895–1963), who received training with Prof. F. E. Fritsch and established the Banaras School of Phycology at the Banaras Hindu University. Prashant Singh is a fourth-generation

member of this school, and we are pleased to honor this distinguished Indian phycologist.

Reference strain: Fulbrightiella bharadwajae 18G-PS.

GenBank accession number : OK083627, OK161230.

Fulbrightiella oahuensis J.R. Johansen. and P. Singh, **sp. nov**. (Fig. 5).

Diagnosis: Similar to *Fulbrightiella bharadwajae*, but with wider trichomes, less tapering, and purplish cell color. Morphologically similar to *Calothrix elenkinii*, but much more constricted at the cross walls and purplish in color.

Description: Colony macroscopically a blackish-brown mat growing subaerially on a wet rock face, mucilaginous to the touch. Colony in laboratory-grown cultures on agar-solidified media in slants a blackish mat with a purplish-brown cast, not penetrating the agar. Filaments usually lacking a bulbous swollen base, in wider trichomes sometimes with swollen basal region, gradually tapering from the base to the apex, rarely false branched, less than 30 μm long in young filaments, up to 250 μm long in mature filaments. Sheath thin, firm, colorless, not lamellated, usually tightly bound to the cells of the trichome but at times becoming wider with the edge of the sheath clearly visible apart from the cellular portion of the filament, often extending beyond the distal end of the trichome (hormogonia release?), in regions where fragmentation of the trichome has occurred frequently gelatinized and forming a widened sphere or oval around the trichome ends and achieving a diameter of 8–13 μm. Trichomes consistently constricted at the cross walls throughout the length of the trichome, but with constrictions sometimes obscured by the tight sheath, especially strongly constricted in basal and mid-regions regions, appearing less constricted at the terminal regions of mature filaments, in basal region near heterocyte 3.5–8.0 μm wide, in intercalary region 2.5–5.5 μm wide, and in terminal region 2.5–4.8 μm in wide. Vegetative cells purplish in color, granular in the centroplasm, with prominent spherical polyphosphate granules, mostly shorter than wide to isodiametric, sometimes longer than wide, 2.0–11.0 μm long near the base, 1.4–8.0 μm long in the middle part of the trichome, becoming 3.4–6.0 μm long near the terminus. Heterocytes mostly terminal, basal in position, yellow in color, spherical or with clearly rounded free terminal ends and flattened to the adjacent vegetative cell, sometimes in pairs or series, the newer cell

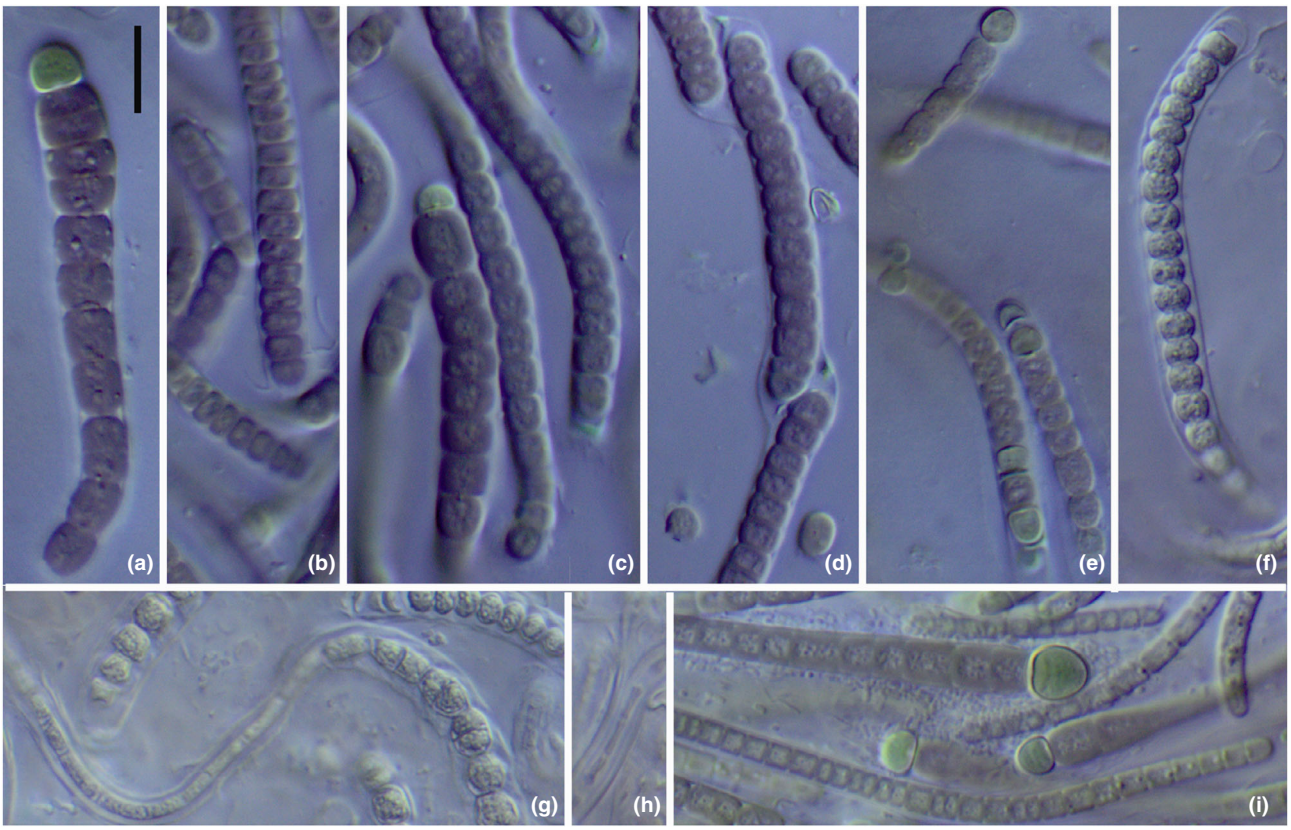

FIG. 5. *Fulbrightiella oahuensis* HA4248-MV3. (a–c) Filaments showing minimal tapering and strongly constricted trichomes. (d) fragmentation of trichome within gelatinizing sheath. (e) Formation of multiple heterocytes, together with degenerating heterocyte at right lower corner. (f–h) Trichomes grown in low-P medium demonstrating intensely constricted trichomes and production of thinner apical trichomes within widened sheaths. (i) Trichomes grown in nitrogen-free Z8 medium. Scale bar 10 μm, all figures at same scale. [Color figure can be viewed at wileyonlinelibrary.com]

subterminal to the older senescing heterocyte, 2.5–5.5 μm long, 3.0–6.4 μm wide, with intercalary heterocytes discoid, 1.6–2.8 μm long, 5.8–6.0 μm wide. Necridia rare. Akinetes not observed.

Holotype here designated: Air-dried reference strain on glass-fiber filters, deposited at the Bishop Museum, Honolulu, Oahu, Hawaii [BISH 786238].

Type locality: Sample collected by A.R. Sherwood, M.A. Vaccarino, and R.L. Lowe on July 24, 2009, from the Kalawahine Trail, Round Top Forest Reserve, near Honolulu, Oahu, Hawai'i, 21°20′6″ N, 157°48′58″ W.

Habitat: Blackish-brown mat growing on a wet rock wall.

Etymology: Named for the island of Oahu, Hawai'i, from which the sample was collected.

Reference strain : Fulbrightiella oahuensis HA4248-MV3.

GenBank accession number: ON706997.

Sherwoodiella J.R. Johansen. and P. Singh, **gen. nov.**

Diagnosis: Similar in morphology to *Calothrix*, *Fulbrightiella*, *Dulcicalothrix*, *Macrochaete*, and *Roholtiella*, but distinctive in the formation of pairs of heterocytes in mid-filament in isopolar filaments, leading to fragmentation of filament to form heteropolar filaments. Also phylogenetically separate from those genera and with percent similarity of the 16S rRNA genes <94.5% in comparison to other tapering genera.

Description: Filaments heteropolar, wider in the basal region, which is bulbous, gradually tapering to the distal end, but not forming a terminal hyaline hair, or isopolar and tapering toward both ends, or isopolar and untapered when present as hormogonia. Akinetes absent. Heterocytes forming in pairs in middle of isopolar filaments, leading to fragmentation of the filament and formation of heteropolar filaments, having the appearance of being basal and solitary.

Etymology: Named in honor of Professor Alison R. Sherwood, noted phylogeneticist, collaborator, and friend who secured NSF support for the sampling of the Hawaiian Islands that led to the discovery of this genus and species.

Type species: *Sherwoodiella mauiensis* J.R. Johansen. and P. Singh.

Sherwoodiella mauiensis J.R. Johansen. and P. Singh, **sp. nov.** (Fig. 6).

Diagnosis: Differentiated from other heterocytous tapering genera by the formation of isopolar filaments that taper toward both ends, which become heteropolar only after fragmentation mid-filament by simple fragmentation or following formation of a pair of heterocytes.

Description: Colony on agar-solidified media a dark olive-green spreading mat. Filaments isopolar and untapered, or isopolar and tapered toward both apices, or heteropolar with a basal heterocyte and tapered distal end, usually lacking a bulbous swollen base, in wider trichomes sometimes with swollen basal region, gradually tapering from the base to the apex, lacking a terminal hair, rarely false branched, less than 30 μm long in hormogonia, up to 80 μm long in mature filaments, heteropolar tapering filaments lacking heterocytes also form through simple fragmentation of isopolar filaments. Sheath thin, colorless, often not evident, not lamellated, usually tightly bound to the cells of the trichome when present, sometimes soft and widened, rarely extending beyond the distal end of the trichome. Trichomes constricted at the cross walls at the basal and mid-trichome regions, becoming unconstructed or only slightly constricted toward the distal ends, with constrictions sometimes obscured by the sheath, in basal region adjacent to heterocyte 6.0–8.4 μm wide, in intercalary region 3.6–5.0 μm wide, and in terminal region 2.0–3.0 μm in wide. Vegetative cells blue-green in color, with prominent spherical polyphosphate granules present in the centroplasm, mostly shorter than wide to isodiametric, longer than wide only in the distal apical regions, 3.0–6.0 μm long near the base, 2.5–4.5 μm long in the middle part of the trichome, becoming 4.0–6.0 μm long at the terminus. Heterocytes rare, forming in pairs in mid-filament, but having the appearance of being terminal after trichome fragmentation, sometimes senescing and forming secondary basal heterocyte adjacent to the degenerating heterocyte, yellow in color, with clearly rounded free terminal ends and flattened to the adjacent vegetative cell, solitary, 2.5–4.0 μm long, 4.0–6.0 μm wide. Necridia and akinetes not observed.

Holotype here designated: Air-dried reference strain on glass-fiber filters, deposited at the Bishop Museum, Honolulu, Oahu, Hawai'i [BISH 786239].

Type locality: Sample collected by J.P. Kociolek, J. Neumann, and M.A. Vaccarino on January 7, 2010, from a unnamed stream above Olowalu Beach near the Honoapiilani Highway, Maui, Hawai'i, 20°48′34″ N 156°36′18″ W.

Habitat: Epilithic in a small stream.

Etymology: Named for the island of Maui, Hawai'i, from which the sample was collected.

Reference strain: Sherwoodiella mauiensis HA4860-CV1.

GenBank accession numbers: KY499906, KT336444, KT336445.

Phylogenetic analysis. The 16S rRNA gene phylogeny was inferred using 527 OTUs belonging to diverse heterocytous cyanobacterial groups (Fig. 7). The family Calotrichaceae itself was comprised of 87 OTUs (Fig. 8). Three separate and strongly supported clades of *Calothrix* sensu lato emerged with the freshwater/terrestrial dwelling genus *Dulcicalothrix* occupying a distinct clade in a sister position to the three clades in *Calothrix* sensu lato (Fig. 8). Clade 1 consisted of strains originating from both brackish (Baltic Sea, salinity 2.5–6.0) and

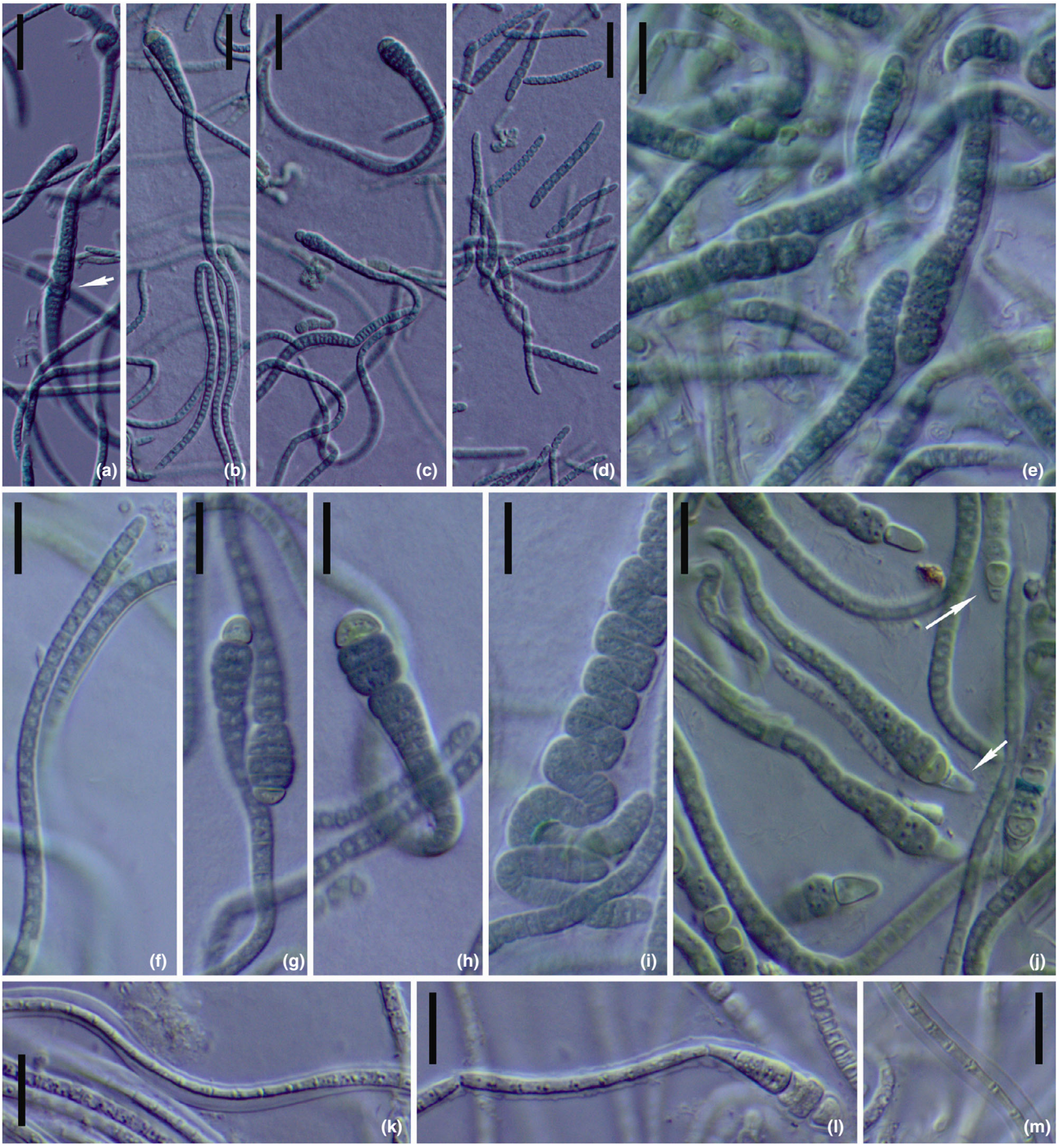

FIG. 6. *Sherwoodiella mauiensis* HA4860-CV1. (a–c) Low magnification views showing isopolar tapering filament (arrow) and heteropolar filaments following fragmentation. (d) Hormogonia. (e) Simple fragmentation of isopolar tapering trichome to produce two heteropolar trichomes in a common sheath. (f) Tapered ends of filaments. (g–h) Filaments developing bulbous bases. (i) Tightly coiled trichome. (j) Filaments grown in nitrogen-free Z8 medium; note the degenerating heterocyte adjacent to the viable heterocyte (arrows) as well as the elongated and/or conical heterocytes. (k–m) Filaments grown in low-P medium, showing production of thin tapered apices with elongated cells. (a–d) Scale bar 20 μm, (e–m) Scale bar 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

freshwater habitats. Our strains 18G-PS and HA4248-MV3 were basal members of this clade, and we consider all "*Calothrix*" strains in this clade to belong to *Fulbrightiella* based on phylogenetic position and high percent identity of the included strains. Clade 2 also consisted of brackish and freshwater forms, and we consider all "*Calothrix*" strains in this clade to belong to *Sherwoodiella*. Clade 3

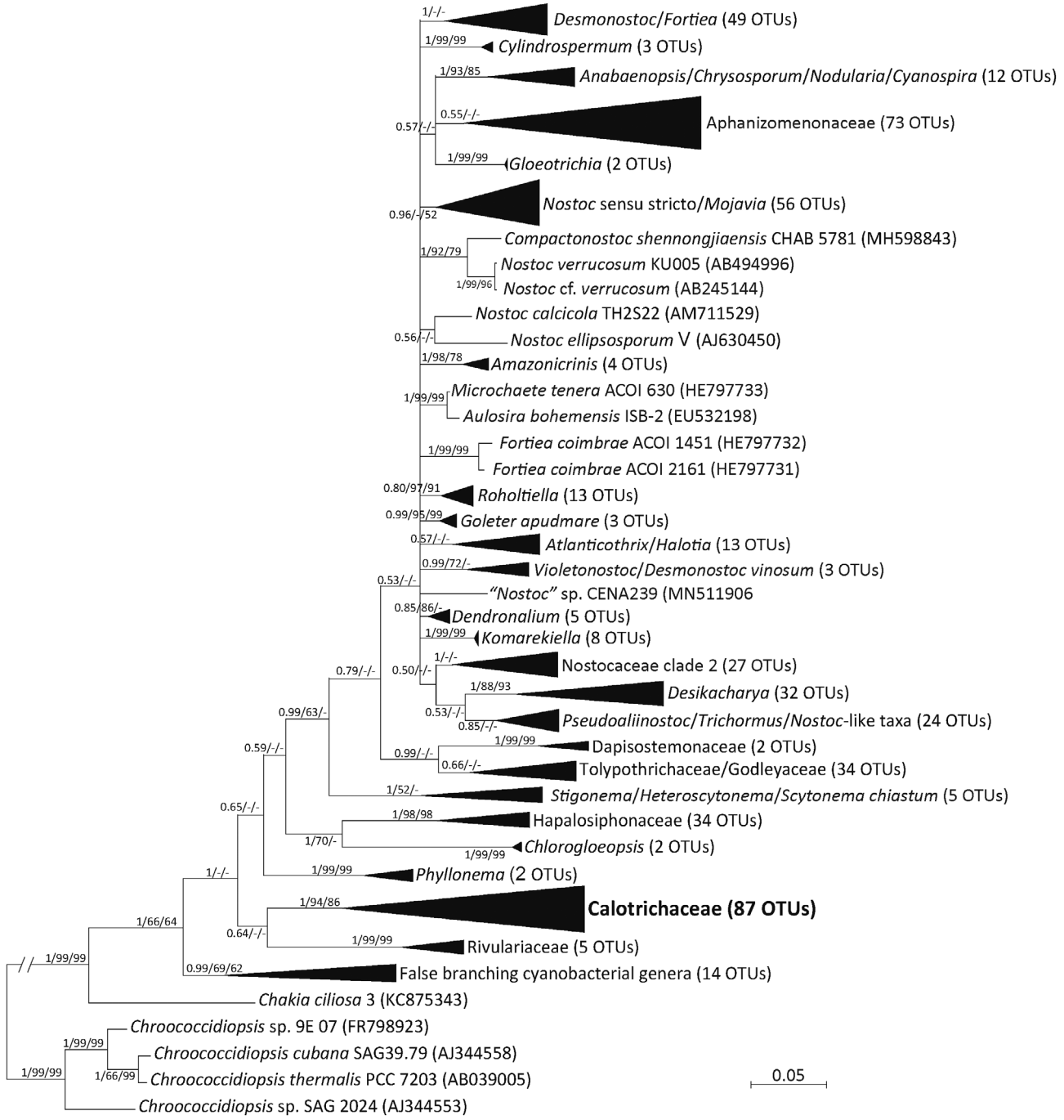

FIG. 7. 16S rRNA gene tree indicating the phylogenetic relationships of members of the heterocytous clade inferred by Bayesian inference with probability/bootstrap values representing BI, ML, and NJ, respectively. Bar represents 0.05 changes per nucleotide position. A total of 527 nucleotide sequences were included in the analysis.

contained only freshwater forms isolated in China, and we consider this to be an unnamed genus that will be described by others. *Dulcicalothrix* formed a separate and distinct node inside the family Calotrichaceae which was sister to the node containing the three Calotrichaceae clades and *Calothrix elsteri*. *Macrochaete* also occupied a position within the family Calotrichaceae. The closely related family Rivulariaceae grouped at the sister node to Calotrichaceae, and both family-level nodes had strong supporting posterior probabilities and bootstrap values, although the node containing both families was not well supported (Fig. 7). Other heterocytous genera (e.g., *Desmonostoc*, *Fortiea*, *Cylindrospermum*,

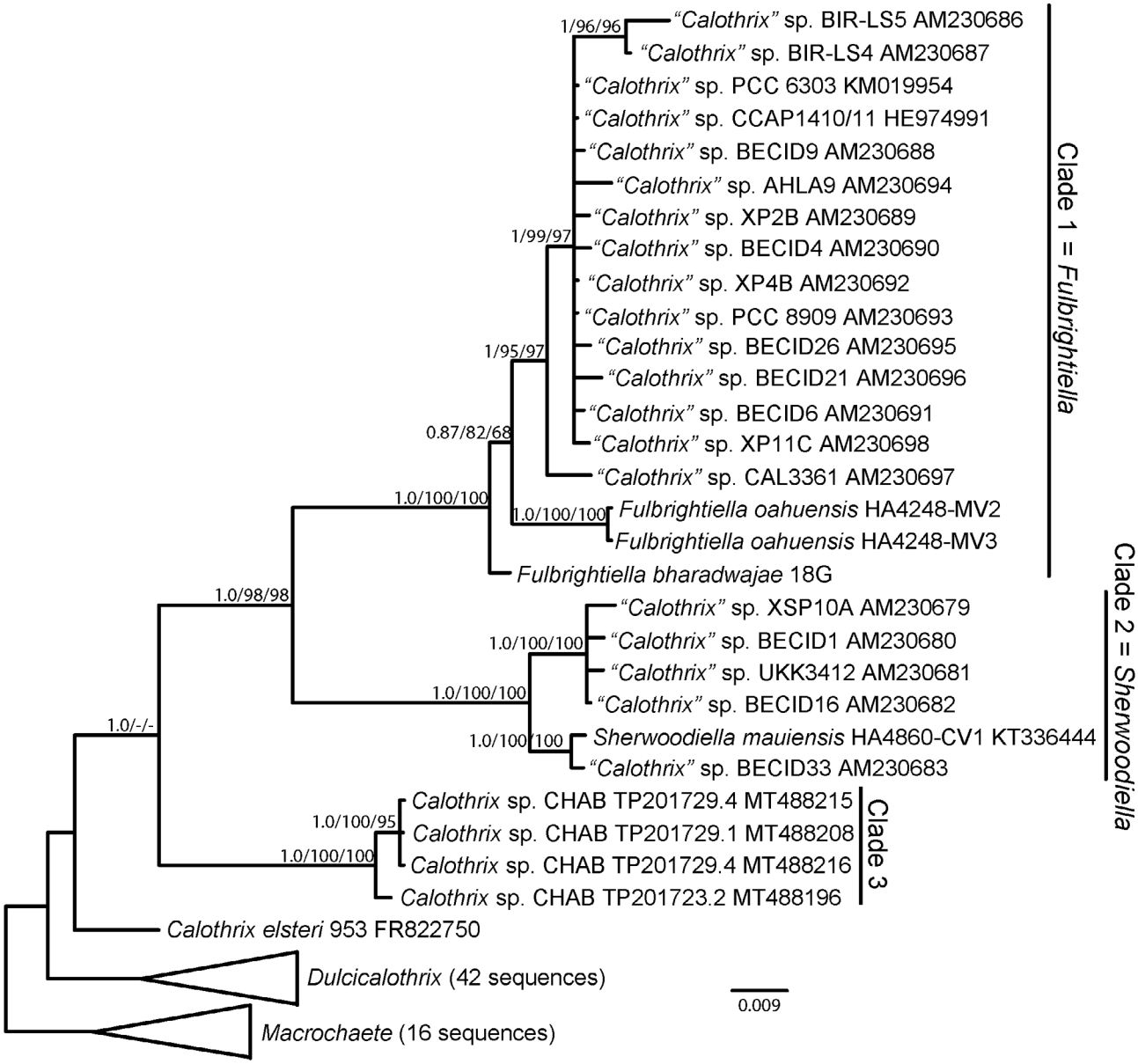

FIG. 8. Expanded node for the Calotrichaceae in analysis shown in Figure 2.

Aliinostoc, *Nostoc*, *Amazonocrinis*, *Roholtiella*, *Atlanticothrix*, *Halotia*, *Dendronalium*, *Komarekiella*, *Camptylonemopsis*, and *Desikacharya*) were clearly outside of the tapering Calotrichaceae and Rivulariaceae, but their relationships to each other were not resolved well, and some genera (especially *Nostoc*) are still polyphyletic based on incomplete taxonomic revision of that genus or incorrectly identified sequences (Fig. 7).

According to the currently accepted genetic standards for prokaryotes based on 16S rRNA gene similarity (<94.5% = different genera), all three clades are distinct genera. *Fulbrightiella* (clade 1) members have internal pairwise similarity values ranging from 97.8% to 100% (Table 1), well above the threshold of 94.5%. Furthermore, all members of *Fulbrightiella* are <94.1% similar to all members of *Sherwoodiella* (clade 2) and the set of strains from China (clade 3), and this combined with the phylogenetic clustering supports recognizing it as a distinct genus separate from the other two clades as well as more distant clades (*Dulcicalothrix*, *Macrochaete*). *Sherwoodiella* species have internal pairwise similarity values ranging from 98.1% to 99.8% (Table 1), also well above the threshold of 94.5%, and it is likewise less than 94.5% in comparison to the other two clades. Clade 3 members are completely freshwater and show the greatest distance from the other two clades (≤91.5%). This is likewise a new genus, and we are aware that the keepers of this set of strains intend

TABLE 1. Percentage similarity matrix of representative members of clades 1, 2, and 3. Member pairs belonging to the same genus clade are shaded. Members of different clades all have percent similarities <94.5% (a commonly used threshold for recognition of members of different prokaryotic genera).

	Fulbrightiella bharadwajae 18G-PS	*Fulbrightiella oahuensis* HA4248-MV2	*Fulbrightiella* sp. PCC6303	*Fulbrightiella* sp. BECID26	*Fulbrightiella* sp. AHLA9	*Sherwoodiella mauiensis* HA4860-CV1	*Sherwoodiella* sp. BECID33	*Sherwoodiella* sp. XSP10A	*Sherwoodiella* sp. BECID1	*Sherwoodiella* sp. UKK3412	*Sherwoodiella* sp. BECID16	"*Calothrix*" sp. CHAB TP201729.4
Fulbrightiella bharadwajae 18G-PS												
Fulbrightiella oahuensis HA4248-MV2	98.4											
Fulbrightiella sp. PCC6303	98.9	98.2										
Fulbrightiella sp. BECID26	98.7	98.1	99.8									
Fulbrightiella sp. AHLA9	98.4	97.6	99.5	99.3								
Sherwoodiella mauiensis HA4860-CV1	94.2	93.7	93.5	93.3	93.4							
Sherwoodiella sp. BECID33	94.1	93.6	93.5	93.3	93.4	99.7						
Sherwoodiella sp. XSP10A	93.7	93.0	93.5	93.4	93.5	98.2	98.2					
Sherwoodiella sp. BECID1	94.0	93.2	93.8	93.6	93.7	98.5	98.5	99.5				
Sherwoodiella sp. UKK3412	94.0	93.2	93.8	93.6	93.7	98.5	98.5	99.5	99.7			
Sherwoodiella sp. BECID16	94.0	93.2	93.9	93.8	93.9	98.6	98.6	99.6	99.8	99.8		
"*Calothrix*" sp. CHAB TP201729.4	93.6	93.3	93.6	93.4	93.7	92.6	92.6	92.0	92.3	92.4	92.4	
Calothrix elsteri CCALA 953	93.9	93.7	93.5	93.3	93.5	93.7	94.0	93.5	93.8	93.8	93.9	95.1

Members of the same genus clade for all strains available have percent similarities ≥97.8%.

to describe this new genus in a subsequent publication.

16S-23S ITS sequence analysis. The 16S-23S ITS region for *Fulbrightiella bharadwajae* 18G-PS was sequenced and out of the selected eight clones, two distinct operons were obtained, one with both $tRNA^{Ile}$ and $tRNA^{Ala}$ and one with no tRNA genes. We also retrieved from GenBank ITS sequences for PCC 6303, and this strain also had two operons, one with both tRNA genes and one with no tRNA genes. Finally, we have one consensus sequence for *Fulbrightiella oahuensis* HA4238-MV3 based on three clones, but only the operon with no tRNA genes was recovered in this strain. The 16S-23S ITS sequence data for both operons were also recovered for both *Sherwoodiella mauiensis* HA4860-CV1 and "*Calothrix*" clade 3 CHAB TP201729.

All ITS regions differed in length, both within taxa and between taxa (Table 2).

The length of the D1-D1' helices within *Fulbrightiella* species was very similar (88–92 nt), as was the D2 region with beginning spacer (31–35 nt), the Box-A (11 nt), and the D4 region (23 nt). However, the spacer+D3 + spacer, Box-B + spacer, V3 helix, and end regions were markedly different between taxa, although the V3 helix was consistent between operons in the same species (Table 2).

With the differences in lengths observed in the ITS, it was not surprising to see that percent dissimilarity between species was high. For example, the percent dissimilarity between *Fulbrightiella bharadwajae* and *Fulbrightiella* PCC 6303 in operons with both tRNA genes was 19.5%. Percent dissimilarity among *Fulbrightiella* species in the operons with no tRNA genes was >22.3% in all three between-species comparisons (Table 3). Percent dissimilarity in ITS regions >7.0% is considered strong evidence of lineage separation between species, and consequently, this analysis alone justifies the recognition of these three species of *Fulbrightiella*. Unfortunately, only a single strain of *Sherwoodiella* has ITS data available, and the freshwater clade 3 strains are all from a single locality and represent a single species.

Finally, the secondary structures of the conserved domains of the ITS region also provide evidence of species separation in *Fulbrightiella* (Figs. 9, 10). The D1-D1' helices in *Fulbrightiella* are consistent in their basal regions up to the 5′-GUGA–UCAC-3′ sequence in mid-helix (Fig. 9, a–d). There is also a high level of consistency between operons in the same species (Fig. 9, a–c). The D1-D1' helix in *Sherwoodiella* is similar to *Fulbrightiella* only in the basal region up to the 5′-CCA–UGG-3′ helix above the 3′ unilateral bulge (Fig. 9, a–f), and the D1-D1' helix in "*Calothrix*" CHAB TP201729 is highly divergent from both *Fulbrightiella* and *Sherwoodiella* (Fig. 9g).

The Box-B helices are markedly different among species of *Fulbrightiella*, and in the case of *Fulbrightiella* sp. PCC 6303, they are markedly different

between operons (Fig. 10, a–e). *Sherwoodiella* also had markedly different structures between operon types (Fig. 10, f and g). However, D1-D1' structures were consistent between operons in both *F. bharadwajae* and "*Calothrix*" CHAB TP201729 (Fig. 10, a–b, h–i). It has been customary to report the Box-B helix only with the basal clamp 5′-AGCA–UGCU-3′ as this sequence is present in all Box-B structures across the phylum Cyanobacteria. However, in many instances, additional pairings more basal to this clamp can form, typically 2–3 extra bp, but up to as many as extra 6 bp. In the Calotrichaceae, 2–3 extra bp consistently form, and so we show the extended possible Box-B for all of our studied taxa (Fig. 10, a–i).

The V2 helix only forms in operons with both tRNA genes. All V2 helices were markedly different between genera and species, not even sharing the same sequence in the basal clamp (Fig. 10, j–m). The V3 helices did not vary between operons in the same species, but were all different between species and genera (Fig. 10, n–r). There was some consistency in structure in the most basal portion (6 bp) of the V3 helix in the three *Fulbrightiella* species (Fig. 10, n–p), but these helices in general differed significantly among species.

Morphological and ecological differentiation. *Fulbrightiella* species share several features. They never produce long hyaline terminal hairs, although their terminal portions can be notably thinner than the main body of the trichome, particularly in low-P media. They consistently are distinctly constricted at the cross walls except for the most terminal parts, which are still slightly constricted if seen without sheaths. They can form heterocytes in series up to three in a row, with only the heterocyte adjacent to the vegetative cells of the basal region remaining viable, while the more terminal heterocytes are senescing even while remaining attached to the filament. *Fulbrightiella* are thinner than almost all other described *Calothrix* (basal diameter ≤8 μm), although *Sherwoodiella* is also thin (basal diameter 6.0–8.4 μm). Although the *Fulbrightiella* clade has Baltic Sea representatives that are brackish water inhabitants, the two species we describe are freshwater.

Sherwoodiella had considerably longer trichomes than *Fulbrightiella*, and the bulbous basal region was very characteristic. It also had more regions in the trichome that were not constricted at the cross walls. Like *Fulbrightiella*, it never produces hyaline hairs, although thinner trichome apices were observed in low-P medium. Its most diagnostic feature is the production of isopolar trichomes which taper toward both ends that fragment in mid-filament either at the site of double heterocyte formation or at the site of simple fragmentation. At least with the species described in this article, *Fulbrightiella* spp. and *Sherwoodiella mauiensis* can be delineated morphologically.

We do not know the morphology of the strains CHAB TP201729 and CHAB TP201723 isolated by Yilang Wang. However, we do know that the set of strains belonging to this clade were all collected from a wetland puddle near Lake Cuo'e on the Qinghai-Tibet Plateau in Tibet. Given the elevation of this region (>4500 m), we conclude that the ecology of this interesting set of strains is different from both *Fulbrightiella* and *Sherwoodiella*, and given its low percent similarity in the 16S rRNA gene, it almost certainly represents a genus new to science.

Fulbrightiella and *Sherwoodiella* are both morphologically and ecologically separated from the type species of *Calothrix*, *C. confervicola*, that was described from the Atlantic coast of Denmark by Agardh (prestarting point), and validated by Bornet et Flahault. It has been reported from both the Atlantic coast of Europe and the Americas. It is clearly a marine taxon. It differs morphologically from both *Fulbrightiella* and *Sherwoodiella* in that it has wide, short cells in basal and middle regions of the trichomes, and unconstricted trichomes which narrow suddenly to form elongated hyaline hairs. No sequence data have been reported for this species, although it has been well illustrated by diverse authors (Geitler 1932, Komárek 2013). Even with the absence of molecular data for *C. confervicola*, we are confident that *Fulbrightiella* and *Sherwoodiella* belong to different genera based on morphology and ecology (see Table S1 in the Supporting Information for morphological comparison of all genera assigned to the Family Calotrichaceae).

DISCUSSION

The central question remaining from this work is "What is *Calothrix*?" And further, why are *Fulbrightiella*, *Sherwoodiella*, *Dulcicalothrix*, and *Macrochaete* excluded from *Calothrix* sensu stricto when *Calothrix confervicola*, the generitype, is not present in our phylogeny (Figs. 7, 8) or phylogenies of others (Sihvonen et al. 2007, Berrendero et al. 2008, 2011, 2016, Saraf et al. 2019)? The two clusters formerly called *Calothrix* (*Fulbrightiella* and *Sherwoodiella*) appear in Sihvonen et al. (2007) as clusters 2 and 3, respectively (Sihvonen et al. 2007, see Fig. 2). Those authors suggested that clusters 2 and 3 were related to *Calothrix*, but likely did not belong to the genus. Their cluster 4 contained freshwater strains from the Pasteur Culture Collection that were subsequently reassigned to *Dulcicalothrix* (Saraf et al. 2019). They gave measurements of their strains, and based on their measurements, none of their strains would be placed in *C. confervicola* based on morphology, and furthermore, the Baltic Sea is only brackish (salinity ~5), not marine, which also excludes placement in *C. confervicola*. Berrendero et al. (2011, see Fig. 4) also resolved three clusters (C, D, E) corresponding to *Dulcicalothrix*, *Fulbrightiella*, and *Sherwoodiella*, respectively. They did not

TABLE 2. Lengths of ITS conserved domains for selected Calotrichaceae.

Strains	Leader	D1-D1' Helix	Spacer + D2	Spacer + D3 + Spacer	tRNA^{Ile} gene	Spacers + V2 Helix	tRNA^{Ala} gene	Post-tRNA Spacer	Box-B helix + Spacer	Box-A	D4	V3 helix	End Region (D5)	Total length of ITS
Fulbrightiella bharadwajae 18G-PS	7	89	32	29	74	91	73	93	80	11	23	68	42	**712**
Fulbrightiella bharadwajae 18G-PS	7	89	35	38	–	–	–	–	80	11	23	68	42	**393**
Fulbrightiella oahuensis HA4248-MV3	7	92	35	45	–	–	–	–	69	11	23	59	0	**341**
Fulbrightiella sp. PCC 6303	7	88	31	29	74	96	73	43	56	11	23	60	4	**595**
Fulbrightiella sp. PCC 6303	7	89	35	36	–	–	–	–	67	11	23	60	4	**332**
Sherwoodiella mauiensis HA4860-CV1	7	70	35	28	74	88	73	109	61	11	24	83	20	**683**
Sherwoodiella mauiensis HA4860-CV1	7	70	40	20	–	–	–	–	65	11	24	83	20	**340**
"*Calothrix*" sp. CHAB TP201729	8	84	30	21	74	80	73	19	63	11	23	32	72	**590**
"*Calothrix*" sp. CHAB TP201729	8	84	30	37	–	–	–	–	63	11	23	32	72	**360**

make specific taxonomic recommendations, but did affirm Sihvonen et al. (2007) in concluding that cluster C (*Dulcicalothrix*) was a new genus separate from *Calothrix*. When Berrendero et al. (2016) described *Macrochaete*, these three clusters appeared again, and they referred the clades containing *Fulbrightiella* and *Sherwoodiella* to *Calothrix* Marine Clade I and *Calothrix* Marine Clade II, not realizing that neither clade contained truly marine representatives, only freshwater and brackish taxa. Saraf et al. (2019) also considered these clades to be marine/freshwater when they separated out the freshwater/terrestrial *Calothrix* that do not taper to a hair into *Dulcicalothrix*.

The four tapering sequenced members of the Calotrichaceae (*Macrochaete*, *Dulcicalothrix*, *Fulbrightiella*, and *Sherwoodiella*) form stable clades in a number of phylogenies with varying taxonomic sampling. All four genera do not form mucilaginous thalli like those produced in the Rivulariaceae, and hence their current placement in the Calotrichaceae. *Calothrix confervicola* is morphologically and ecologically very distinct from all species within these genera, and we are confident that it is not synonymous with any of these recently described genera. But the Holy Grail of all researchers interested in the tapering heterocytous clades is finding an exact morphological match to *C. confervicola* from the Atlantic Coast of Europe, isolating it into culture, and sequencing it. We solicit our fellow phycologists to engage in this quest.

This work has further demonstrated the value of utilizing cultures in taxonomic efforts. The discovery that *Sherwoodiella* produces isopolar trichomes that lead to heteropolarity through midfilament fragmentation, typically associated with formation of paired heterocytes, would not have likely been made if only field material had been examined. This is a very distinct autapomorphy that separates this genus from other Calotrichaceae. Similar but certainly analogous formation of heteropolar trichomes from isopolar trichomes has been seen in *Tapinothrix clintonii* (Bohunická et al. 2011) and *Leptolyngbya corticola* (Johansen et al. 2011). Like *Sherwoodiella*, *T. clintonii* tapers to both apices, but attaches to the substrate by its swollen midfilament and fragments to produce two attached tapering filaments. In contrast, *L. corticola* develops widened apices on isopolar filaments, which fragment in the narrower midfilament, also giving rise to tapered heteropolar trichomes. It has been assumed that *Calothrix* is heteropolar and reproduces through hormogonia or fragmenting trichomes from false branching. Heterocytes are obligately basal and do not form in series (Komárek 2013), although apical senescing heterocytes have been observed in *Macrochaete*, *Fulbrightiella*, and *Sherwoodiella*. Further detailed study of the life cycles of tapering taxa that have been isolated into pure culture are needed to

TABLE 3. Percent dissimilarity for 16S-23S ITS region for *Fulbrightiella* strains.

a. Operons without tRNA

	Taxa	1	2
1	*Fulbrightiella bharadwajae* 18G operons 2 and 3	–	
2	*Fulbrightiella* sp. PCC 6303 operon 2 (KT336443)	19.5	–

b.Operons with both tRNA

	Taxa	1	2	3
1	*Fulbrightiella bharadwajae* 18G operon 1	–		
2	*Fulbrightiella oahuensis* HA4860-CV1 (KT336444)	23.9	–	
3	*Fulbrightiella* sp. PCC 6303 operon 1 (KT336442)	23.1	22.4	–

definitively understand the origin and consistency of heteropolarity in heterocytous taxa.

There are now 13 genera of tapering heterocytous genera for which 16S rRNA gene sequence is available: *Macrochaete* (Berrendero et al. 2016), *Dulcicalothrix* (Saraf et al. 2019), *Fulbrightiella*, *Sherwoodiella*, *Rivularia* (Sihvonen et al. 2007, Berrendero et al. 2008, Shalygin et al. 2018), *Kyrtuthrix* (León-Tejera et al. 2016, Johansen et al. 2021), *Nunduva* (González-Resendiz et al. 2018a, Johansen et al. 2021), *Phyllonema* (Alvarenga et al. 2016, González-Resendiz et al. 2018b), *Roholtiella* (Bohunická et al. 2015), *Gloeotrichia* (Sihvonen et al. 2007), *Scytonematopsis* (Vaccarino and Johansen 2011), *Calochaete* (Hauer et al. 2013), and *Cyanomargarita* (Shalygin et al. 2017). These are phylogenetically very diverse and represent seven different family-level lineages. It is evident that the character of tapering has arisen multiple times in evolutionary history, and being in many cases, a homologous character is not as informative as early workers felt it to be. However, within family-level lineages where it occurs, it is very valuable as a character that can be used to define genera as well as families. At one time these 13 genera would all have been placed in the Rivulariaceae, a conclusion that will never get support in the future. Tapering taxa have interesting life

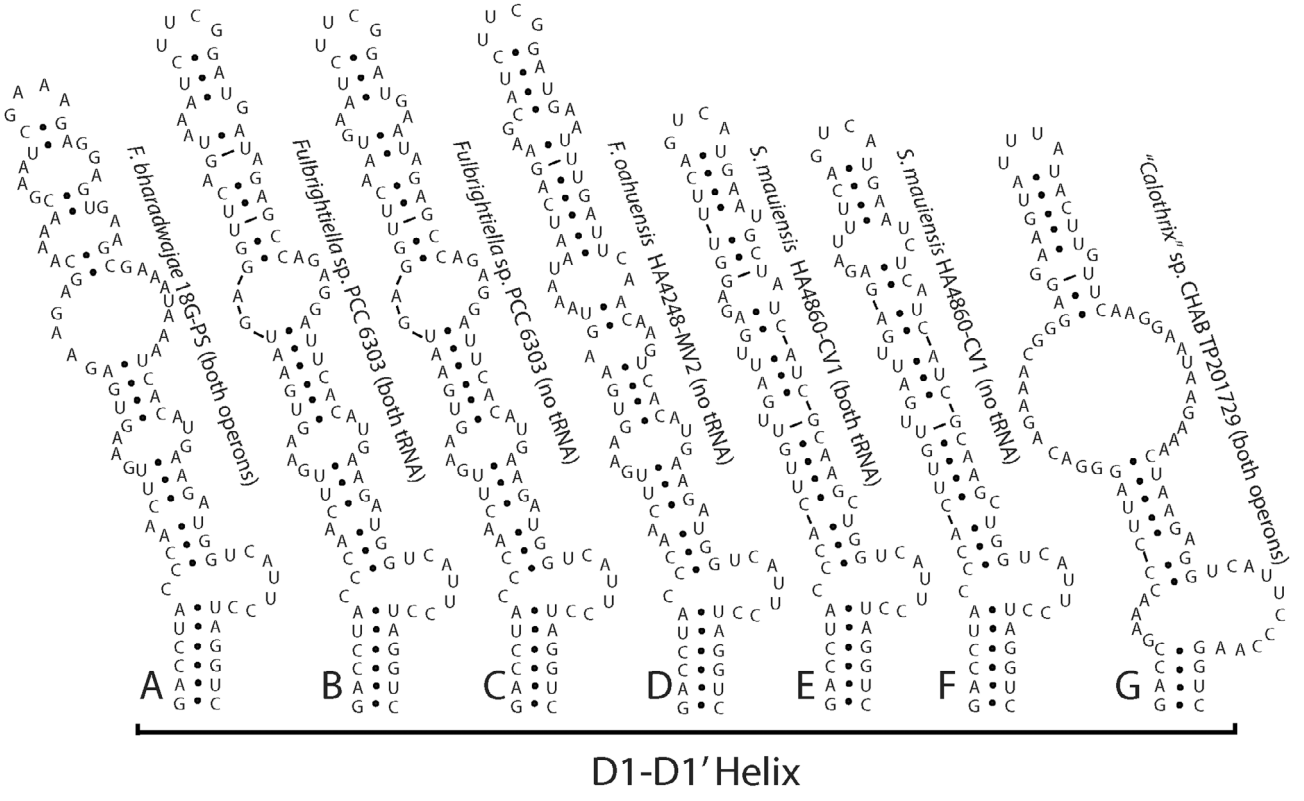

FIG. 9. Secondary structure of the D1-D1' helices for *Fulbrightiella*, *Sherwoodiella*, and a strain of a yet-to-be described *Calothrix*-like taxon from China.

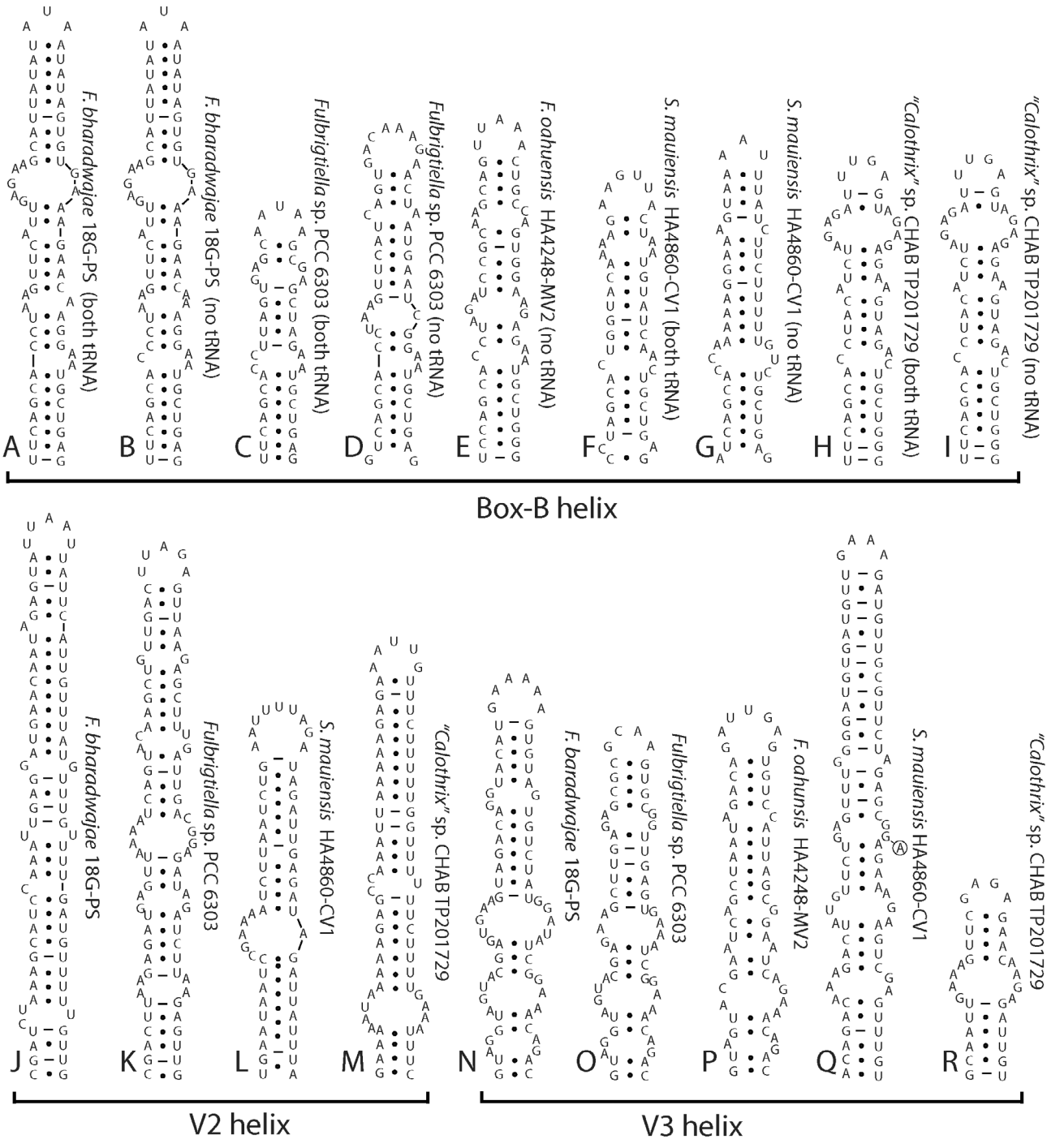

FIG. 10. Secondary structure of the Box-B, V2, and V3 helices for *Fulbrightiella*, *Sherwoodiella*, and a strain of a yet-to-be described *Calothrix*-like taxon from China.

cycles and high morphological variability, making application of species epithets difficult. However, with the definition of these genera, we hope that we are at the dawn of a period when the 300+ species described in *Calothrix* (Komárek 2013) will eventually be placed in a more correct classification that reflects their genetic diversity and evolutionary history.

We thank the Head, Department of Botany, Banaras Hindu University (BHU), for providing the necessary facilities and encouragement. The United States-India Educational Foundation (USIEF) is thanked for the Fulbright Nehru Academic and Professional Excellence Fellowship (FNAPE) to PS. The Department of Biology, John Carroll University, is thanked for all the help, support, and facilities provided to PS during his stay at JCU. Deep gratefulness is extended to all members of the Johansen Laboratory for their help. NK thanks the

University Grants Commission (UGC) for Junior Research Fellowship (JRF). JRJ thanks John Carroll University for research support during the visit of PS.

FUNDING INFORMATION

The work was funded by the Department of Science and Technology (DST-SERB), India, through the Core Research Grant Project CRG/2018/004111. The Banaras Hindu University is also acknowledged for providing Seed Grant under the Institution of Eminence (IoE) Scheme. Support for sequencing was provided by John Carroll University.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Morphological comparison of the genera currently within the family Calotrichaceae.