FEMS Microbiology Letters, 366, 2019, fnz219

doi: 10.1093/femsle/fnz219
Advance Access Publication Date: 21 October 2019
Research Letter

RESEARCH LETTER – Taxonomy & Systematics

Phylogenetic complexities of the members of Rivulariaceae with the re-creation of the family Calotrichaceae and description of *Dulcicalothrix necridiiformans* gen nov., sp nov., and reclassification of *Calothrix desertica*

Aniket Saraf[1,2], Archana Suradkar[2], Himanshu G Dawda[1], Lira A Gaysina[3], Yunir Gabidullin[4], Ankita Kumat[2], Isha Behere[2], Manasi Kotulkar[2], Priyanka Batule[2] and Prashant Singh[2,5,*]

[1]Department of Biological Science, Ramniranjan Jhunjhunwala College, Station Road, Ghatkopar, Mumbai 400086, India, [2]National Centre for Microbial Resource (NCMR), National Centre for Cell Science (NCCS), Pashan-Sus Road, Pune 411021, India, [3]Department of Bioecology and Biological Education, M. Akmullah Bashkir State Pedagogical University, Oktyabr'skoy revolyutsii, 3A, Ufa 450000, Russia, [4]Department of Information Systems and Technologies, M. Akmullah Bashkir State Pedagogical University, Oktyabr'skoy revolyutsii, 3A, Ufa 450000, Russia and [5]Department of Botany, Institute of Science, Banaras Hindu University, BHU Road, Varanasi 221005, India

*Corresponding author: Department of Botany, Institute of Science, Banaras Hindu University, Varanasi 221005, India. Tel: +91-7755830009; E-mail: sps.bhu@gmail.com

One sentence summary: This paper attempts to solve taxonomic problems in the family Rivulariaceae.

Editor: Aharon Oren
Accession Numbers generated through this study: KY863521-KY863523

ABSTRACT

A freshwater dwelling, tapering, heterocytous cyanobacterium (strain V13) was isolated from an oligotrophic pond in the Shrirampur taluka, Ahmednagar district of Maharashtra in India. Initial morphological examination indicated that strain V13 belonged to the genus *Calothrix*. Subsequent molecular and phylogenetic assessment based on 16S rRNA gene, led us to describe the freshwater/terrestrial clade of *Calothrix* strains without terminal hairs as a new genus *Dulcicalothrix* gen. nov., with the type species *Dulcicalothrix necridiiformans* sp. nov. (Strain V13) on the basis of the necridia forming ability of the strain. Also, the 16S-23S ITS secondary structure analysis clearly differentiated strain V13 from the other members of the clade. Past studies and the current state of knowledge makes it imperative to separate the groups *Calothrix* (marine/freshwater *Calothrix*), *Macrochaete* and *Dulcicalothrix* (freshwater/terrestrial *Calothrix*) into separate genera in accordance with the International Code of Nomenclature for Algae, Fungi and Plants. Robust phylogenetic evidence and

Received: 11 June 2019; **Accepted:** 18 October 2019

previous reports strongly support the re-erection of the family Calotrichaceae distinct from the existing family Rivulariaceae.

Keywords: cyanobacteria; 16S rRNA; 16S-23S ITS; *Calothrix*; Rivulariaceae; necridia

INTRODUCTION

The order Nostocales represent a large monophyletic clade with distinct phenotypic characters that are unique to this order. However, recent studies have indicated considerable phylogenetic inconsistencies existing within the order at the genus and family levels (Hauer *et al.* 2014; Komárek *et al.* 2014; Berrendero *et al.* 2016; Saraf *et al.* 2018). The advent of the polyphasic approach has led to many taxonomic revisions with the description of new families: Gloeotrichiaceae (Komárek *et al.* 2014), Tolypothrichaceae (Hauer *et al.* 2014), Godleyaceae (Hauer *et al.* 2014) and the newly erected Cyanomargaritaceae (Shalygin *et al.* 2017). Moreover, many new genera such as *Mojavia* (Řeháková *et al.* 2007), *Desmonostoc* (Hrouzek *et al.* 2013), *Calochaete* (Hauer, Bohunická and Mühlsteinová 2013), *Roholtiella* (Bohunická *et al.* 2015), *Macrochaete* (Berrendero *et al.* 2016), *Cyanomargarita* (Shalygin *et al.* 2017), *Aliinostoc* (Bagchi, Dubey and Singh 2017), *Komarekiella* (Hentschke *et al.* 2017) and *Desikacharya* (Saraf, Dawda and Singh 2018) etc. have been recently described. The family Rivulariaceae is reported to be morphologically complex and highly polyphyletic in previous studies (Berrendero, Perona and Mateo 2008; Berrendero *et al.* 2016; León–Tejera *et al.* 2016; Shalygin *et al.* 2017; González–Resendiz *et al.* 2018). At present the family consists of 11 genera: *Rivularia*, *Calothrix*, *Dichothrix*, *Gardnerula*, *Isactis*, *Sacconema*, *Microchaete* (Komárek *et al.* 2014), *Phyllonema* (Alvarenga *et al.* 2016), *Macrochaete* (Berrendero *et al.* 2016), *Kyrtuthrix* (León–Tejera *et al.* 2016) and *Nunduva* (González–Resendiz *et al.* 2018). Traditionally, the genera *Calothrix* and *Rivularia* were classified under Mastichotricheae and Rivulariaceae, respectively (Bornet and Flahault 1886). Alternatively, Bennett and Murray (1889) in their work mentioned two different families for tapering cyanobacteria, which placed *Rivularia* and *Calothrix* into the families Rivulariaceae and Calotrichaceae, respectively. However, this scheme of classification was not widely accepted and researchers persisted with Bornet and Flahault's classification for a brief period of time. Later Mastichotricheae along with some non-heterocytous tapering cyanobacteria were merged into Rivulariaceae (Fremy 1929 and Geitler 1932). Subsequently, the non-heterocytous forms were removed from this family (Komárek and Anagnostidis 1989). This plan of classification was followed until the conceptualization of the recent scheme of classification proposed by Komárek *et al.* (2014). The authors created the new family Gloeotrichiaceae from the Rivulariaceae consisting of *Gloeotrichia* as the only genus within the family. In their work, the authors indicated the possibility of more taxonomic revisions taking place in the future at the genus level within Rivulariaceae. Berrendero *et al.* 2016, in their study have shown that *Calothrix* and *Macrochaete* form a phylogenetically distant cluster from the other members of the family Rivulariaceae. Similar phylogenetic clustering was also observed in later studies e.g. Shalygin *et al.* (2017) and González–Resendiz *et al.* (2018). Even in our previous study, we had also observed similar clustering wherein *Calothrix* and *Macrochaete* clustered distantly (Saraf *et al.* 2018). It must be noted that even though the taxon sampling in all the studies was different, similar patterns of clustering in several genera indicated the need for a taxonomic revision of family Rivulariaceae.

The genus *Calothrix* was described by Bornet and Flahault in 1886 with the type species *Calothrix confervicola*. The members of this genus are characterized by the presence of heteropolar filaments with spherical or hemi-spherical heterocytes present usually at the basal end. The trichomes are surrounded by firm sheaths that may be colorless or yellow-brownish in color. The apical cells are usually hyaline, narrow and long, creating a hair-like appearance (Bornet and Flahault 1886). However, there are a few reports of *Calothrix* strains which do not form terminal hairs (Schwabe 1960; Berrendero, Perona and Mateo 2008; Villanueva *et al.* 2019). The *Calothrix* strains without the ability to develop terminal hairs were repeatedly observed to form a separate cluster from the *Calothrix* strains with hairs (Berrendero *et al.* 2016; Shalygin *et al.* 2017; González–Resendiz *et al.* 2018; Villanueva *et al.* 2019). The members within the cluster have been found to have specific ecological preferences and are reported from freshwater or terrestrial habitats (Schwabe 1960; Berrendero, Perona and Mateo 2008; Villanueva *et al.* 2019). Many researchers consider that the *Calothrix* strains without hairs isolated from freshwater/terrestrial habitats belong to a different evolutionary lineage (e.g. Komárek *et al.* 2014; Berrendero *et al.* 2016). This makes *Calothrix* an interesting but challenging genus to study in taxonomic perspectives.

In this study we performed an in-depth phylogenetic analysis of the members of the family Rivulariaceae in order to get insights into the higher level taxonomic status of *Calothrix*. Further, the taxonomic position of the *Calothrix* strains reported from freshwater or terrestrial habitats which do not form terminal hairs were also assessed in detail. Through this study we propose to re-erect the family Calotrichaceae and reclassify the *Calothrix* strains without terminal hairs into a new genus *Dulcicalothrix*. Furthermore, a freshwater strain from India has been characterized by the polyphasic approach and is described as a novel species of *Dulcicalothrix*. The species name *Dulcicalothrix necridiiformans* is proposed in accordance with the International Code of Nomenclature for Algae, Fungi and Plants.

MATERIAL AND METHODS

Sampling, isolation and culturing

The water sample was collected from an oligotrophic stagnant pond located in the Shrirampur taluka, Ahmednagar district of Maharashtra. The samples were immediately put in sterilized 50 ml falcon tubes having $BG11_0$ media. The general evaluation of the landscape of the entire habitat was documented at the time of sample collection. Within a few hours of collection, the sample was analyzed microscopically to estimate cyanobacterial diversity. Purification of the sample and isolation of cyanobacteria was performed by the enrichment spread plate method using 1.2% solidified $BG\text{-}11_0$ medium and the pH was adjusted to 7.2 (Rippka *et al.* 1979). The culture was maintained in a culture room under illumination of approximately 50–55 $\mu Em^{-2}s^{-1}$ with a photoperiod of 14/10 hours light/dark cycle at $28 \pm 2^{\circ}C$. The culture was also grown in low concentrations of phosphorous for testing the ability of hair formation.

Phenotypic analysis

Intensive morphological characterization of strain V13 was performed, with attention to the shape and size of apical cell,

intermediate vegetative cells, basal cell and heterocytes. Further, the occurrence of sheath, akinetes and necridia were also examined carefully. The microscopic studies were performed using a Nikon YS100 microscope (Nikon, Minato, Tokyo, Japan) and the micrographs were taken using Olympus BX53 (Olympus Corporation, Shinjuku, Tokyo, Japan) fitted with ProgRes C5 camera (Jenoptik, Jena, Thuringia, Germany) under 400X and 1000X magnifications.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from 10 to 14-day-old log phase culture using HiPurA™ Bacterial Genomic DNA Purification Kit (MB505–250PR) with some modifications (Suradkar *et al.* 2017). Amplification of 16S rRNA gene and 16S-23S ITS region was achieved by using primers pA (Edwards *et al.* 1989) and cyanobacteria specific B23S (Gkelis *et al.* 2005). Direct sequencing of the amplified products was performed by Sanger's method using a 3730xl DNA analyzer (Applied BioSystems, USA). The pairwise similarity search for 16S rRNA gene (NCBI accession number KY863521) for the subsequent phylogenetic analyzes was conducted using both the EzBiocloud and NCBI database.

Phylogenetic analysis

The phylogenetic tree was constructed by Bayesian inference (BI), Maximum likelihood (ML) and Maximum parsimony (MP) methods. The BI tree was constructed using MrBayes 3.2.6. (Ronquist *et al.* 2012) and the best fit model was selected using jModelTest (Darriba *et al.* 2012). The analysis was executed using GTR + I + G model. In the analysis, two runs of eight Markov chains were applied for 10 million generations and sampling was done every 1000th generation. The diagnostic was calculated after every 1000 generations and 25% of the samples from the beginning of the chain were discarded. The phylogenetic analysis using ML and MP was performed with MEGA 5.2.2 (Tamura *et al.* 2011). The suitable model of phylogeny for ML was selected using MEGA 5.2.2, which led to the selection of K2 + G + I. The reliability of the ML and MP tree was tested using bootstrap re-sampling method with 1000 replications (Felsenstein 1985). All the phylogenetic trees constructed in this study based on 16S rRNA gene involved 277 nucleotide sequences and the length of the alignment was 1000 nucleotides.

16S-23S ITS secondary structure analysis

The nucleotide sequences corresponding to the D1-D1' helix region and BoxB region of 16S-23S Internal Transcribed Spacer (ITS) were transcribed and folded using Mfold web server (Zuker 2003) and compared with the phylogenetically nearest members. In case of D1-D1', seven structures within the *Dulcicalothrix* clade were available for comparison, however, in case of BoxB only six structures were available for comparisons.

RESULTS

Habitat description

The sampling was done in the month of June from a freshwater stagnant pond located on an isolated agricultural field in Shrirampur, Maharashtra, India. The general ecology of the habitat indicated it to be an oligotrophic system. The climatic conditions were hot and dry and temperature at the time of sample collection was 38.2°C, pH recorded was 7.3 and the humidity was measured at 32%. The area surrounding the water body had deep black soil along with distinctly stratified rocks.

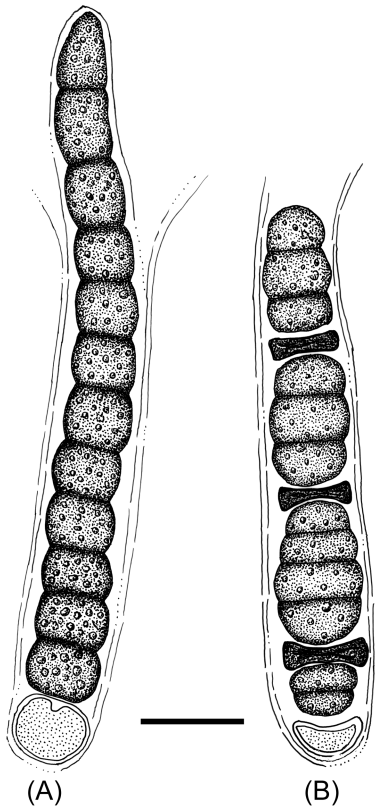

Figure 1. Line drawing of *Dulcicalothrix necridiiformans*. Scale bar 10 μm, **(A)**, Young trichome with basal heterocyte and clear lamellation. **(B)**, Old trichome with bow shaped necridia and formation of potential hormogones

Phenotypic evaluation

In-depth morphological characterization of strain V13 was performed and recorded in minute detail (Figs 1–2; Table S1, Supporting Information). A unique morphological feature of strain V13 was the formation of bow shaped necridia or separation discs that were found to be occurring in almost every filament (Figs 1–2). Heterocytes were present at the basal end and were usually spherical or sometimes elongated. Akinetes were not observed. Strain V13 did not show any terminal hair formation even at very low phosphate concentrations.

Phylogenetic analysis

In the 16S rRNA gene tree, strain V13 clustered within the clade comprising of *Calothrix* strains reported from freshwater and terrestrial habitats with strong probability/bootstrap support (Fig. 3). Strain V13 along with *Calothrix* sp. YK 03 clustered at a completely different node within the freshwater/terrestrial clade (clade C1) and the clustering was supported in all the different trees (BI, ML and MP). This freshwater/terrestrial clade was found to be phylogenetically near to *Macrochaete* and other *Calothrix* strains isolated mainly from marine habitats including few freshwater strains (clade C2) (Table S2, Supporting Information). The robustness of the entire clade comprising of *Calothrix* and *Macrochaete* strains was supported by strong probability/bootstrap values (Fig. 3). The percentage similarity

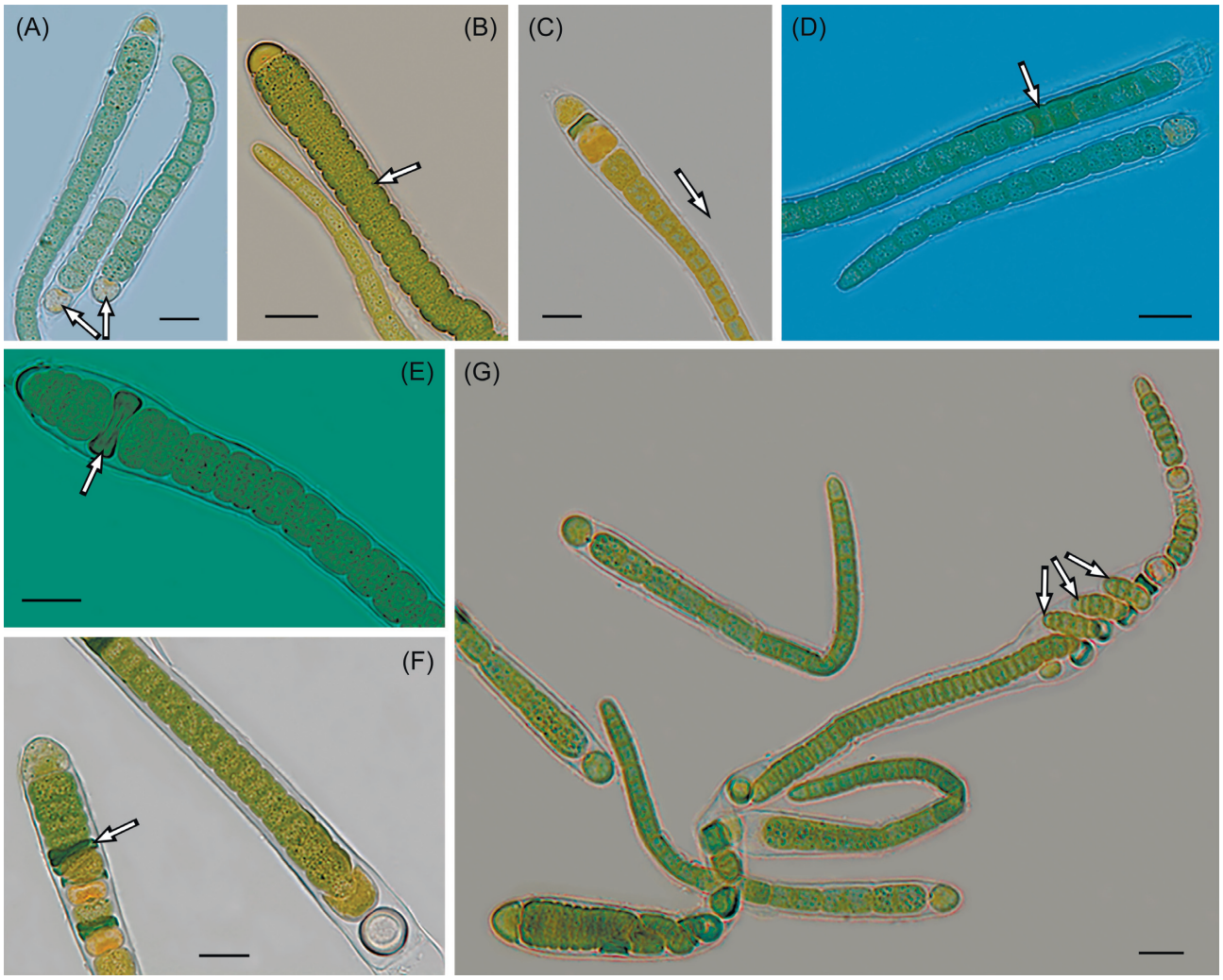

Figure 2. Morphological characteristic of *Dulcicalothrix necridiiformans*. Scale bar 5 μm. Arrows in the Figures 2A-G indicates: **(A)** Young trichome with basal heterocyte, **(B)** Constricted older trichome, **(C)** Narrowing trichome, **(D)** Initiation of necridia formation, **(E)** Trichome with bow shaped necridia, **(F)** Necridia/separation disc, **(G)** Trichome with necridia formation and development of hormogonia.

among the strains of the Clade 1 ranged from 96.8% to 100% (Table S3, Supporting Information), above the identity threshold of 94.5% recommended for separation of different genera (Yarza *et al.* 2014). However, strain V13 was < 98.7% similar in 16S rRNA gene sequence to all other members of Clade 1, evidence that it was a species unique from the other members of the clade (Yarza *et al.* 2014). Further, the other members of the family Rivulariaceae including *Rivularia*, *Phyllonema*, *Kyrtuthrix* and *Nunduva* along with one sequence of *Microchaete* were grouped distantly from *Calothrix* and *Macrochaete* (Fig. 4). Surprisingly, some *Calothrix* and *Microchaete* sequences were phylogenetically close to the members of Nostocaceae rather than Rivulariaceae (Fig. 4). Similar clustering of the members of Rivulariaceae was observed in all the phylogenetic trees constructed in this study. Further, the sequences representing other genera like *Nostoc sensu stricto*, *Aliinostoc*, *Desikacharya*, *Scytonema sensu stricto*, *Brasilonema*, etc. clustered with good probability/bootstrap support.

16S-23S ITS secondary structure analysis

The secondary structures corresponding to D1-D1' helix region and BoxB region of 16S-23S ITS region were obtained for strain V13 and compared with selected secondary structures of phylogenetically related strains. Genus-consistent features of the D1-D1' helix include a 5 bp basal clamp with a bilateral bulge 2–3 bp above the basal 3' unilateral bulge, the later consisting of 3–8 nucleotides. Several strains were distinctive in the presence of a second, often large, bilateral bulge below the terminal loop (e.g. V13, PCC 7103, SEV5-4-C5 and MU27-UAM315). The D1-D1' helices were quite variable between strains, ranging from 65 to 100 nucleotides in length and differing in both sequence and structure. The D1-D1' helix region of strain V13 was most similar to that of PCC 7103 (Fig. 5) but clearly differed in sequence and structure. The BoxB helices had a common basal clamp (5'-AGCA—TGCT-3') but differed significantly in the remainder of the helix, with strain V13 being especially set apart by a large sub-basal bilateral bulge (Fig. 6).

DISCUSSION

The aim of the present study was identification and taxonomic characterization of the freshwater strain V13 using the polyphasic approach. The morphological characterization indicated that the strain belonged to the family Rivulariaceae and probably

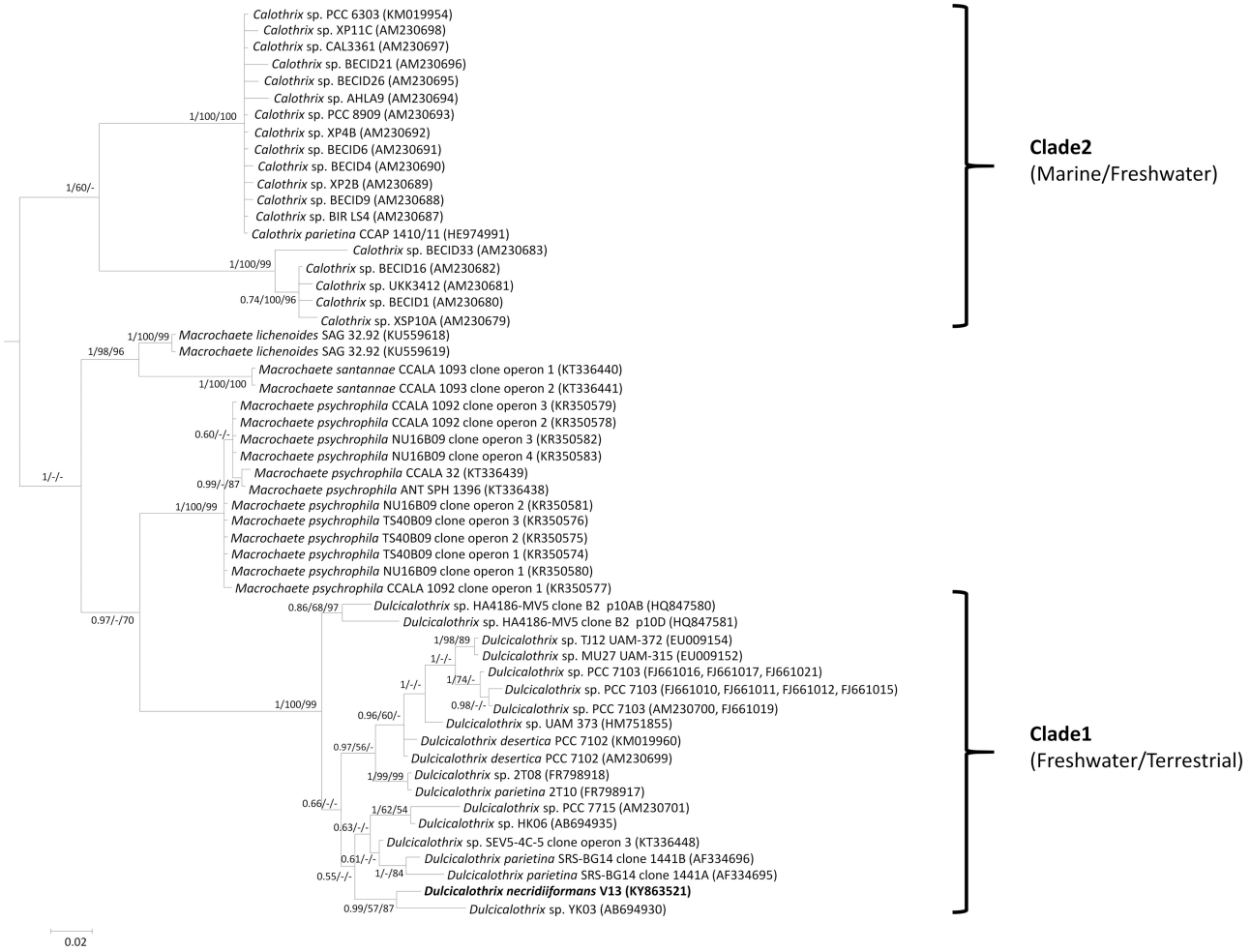

Figure 3. Complete Calotrichaceae clade showing the phylogenetic positioning of the genus *Dulcicalothrix* and *Dulcicalothrix necridiiformans* based on 16S rRNA gene tree inferred by Bayesian inference with probability/bootstrap values representing BI, ML and MP, respectively. Bar 0.02 changes per nucleotide position. The total number of OTUs within the clade are 54 and the length of the 16S rRNA gene of strain V13 is 1480 bp.

to the genus *Calothrix*. Strain V13 exhibited heteropolar filaments without development of terminal hairs even at very low phosphate concentration. One of the characteristic features of the strain is the formation of necridic cells with hormogonia formed adjacent to the necridia. This unique feature indicates the possible role of necridia in vegetative propagation (Figs 1–2; Table S1, Supporting Information). Further, to ascertain the taxonomic status of our strain we reconstructed the phylogeny based on the 16S rRNA gene and certain important observations were made that are discussed hereafter. In the 16S rRNA gene tree, it was found that strain V13 clustered within the *Calothrix* clade consisting of strains isolated from freshwater/terrestrial habitat (clade Cl) (Fig. 3; Table S2 , Supporting Information). It must be noted that according to the available morphological descriptions for the members of the clade C1, no strain has been observed to form terminal hairs (Schwabe 1960; Berrendero *et al.* 2016; Villanueva *et al.* 2019). Further, clade C1 formed a phylogenetically distinct cluster separating it from the other *Calothrix* strains (clade C2). Most of the strains in clade C2 are reported to form terminal hairs and are isolated from marine habitat except *Calothrix* sp. PCC 6303 and *Calothrix parietina* CCAP 1410/11 (Fig. 3; Table S2, Supporting Information). This phylogenetic positioning was consistent in all the trees constructed in this study. Similar observations were also made in previous studies where the possibility of a different evolutionary lineage for the *Calothrix* strains without hairs was also discussed (Komárek *et al.* 2014; Berrendero *et al.* 2016). The two *Calothrix* clades were separated by the *Macrochaete* cluster and a similar result was also observed in the phylogenetic tree represented in Villanueva *et al.* (2019). It was in fact surprising that the authors decided to describe their strain as a new member of *Calothrix* even though there was clear evidence of the polyphyly within *Calothrix*. We believe that characterizing our strain as a new species of *Calothrix* would be incorrect as it would counteract the basic idea on which the current classification system is based. It should be mentioned that *Calothrix dumus* (Villanueva *et al.* 2019) was not included in our analysis even though it falls within the freshwater/terrestrial clade, as it formed a very long branch in the 16S rRNA gene tree. Also the percentage similarity of *Calothrix dumus* with the other members of the clade was found to be very low. This observation is in agreement with Villanueva *et al.* (2019). The phylogenetic positioning of *Calothrix dumus* in our analysis and also in Villanueva *et al.* (2019) may be attributed to the long-branch attraction or possible sequence error, and we excluded it from our study.

Non-existence of *Calothrix sensu stricto* clade is one of the major reasons responsible for the phylogenetic inconsistency and attendant taxonomic confusion surrounding *Calothrix*.

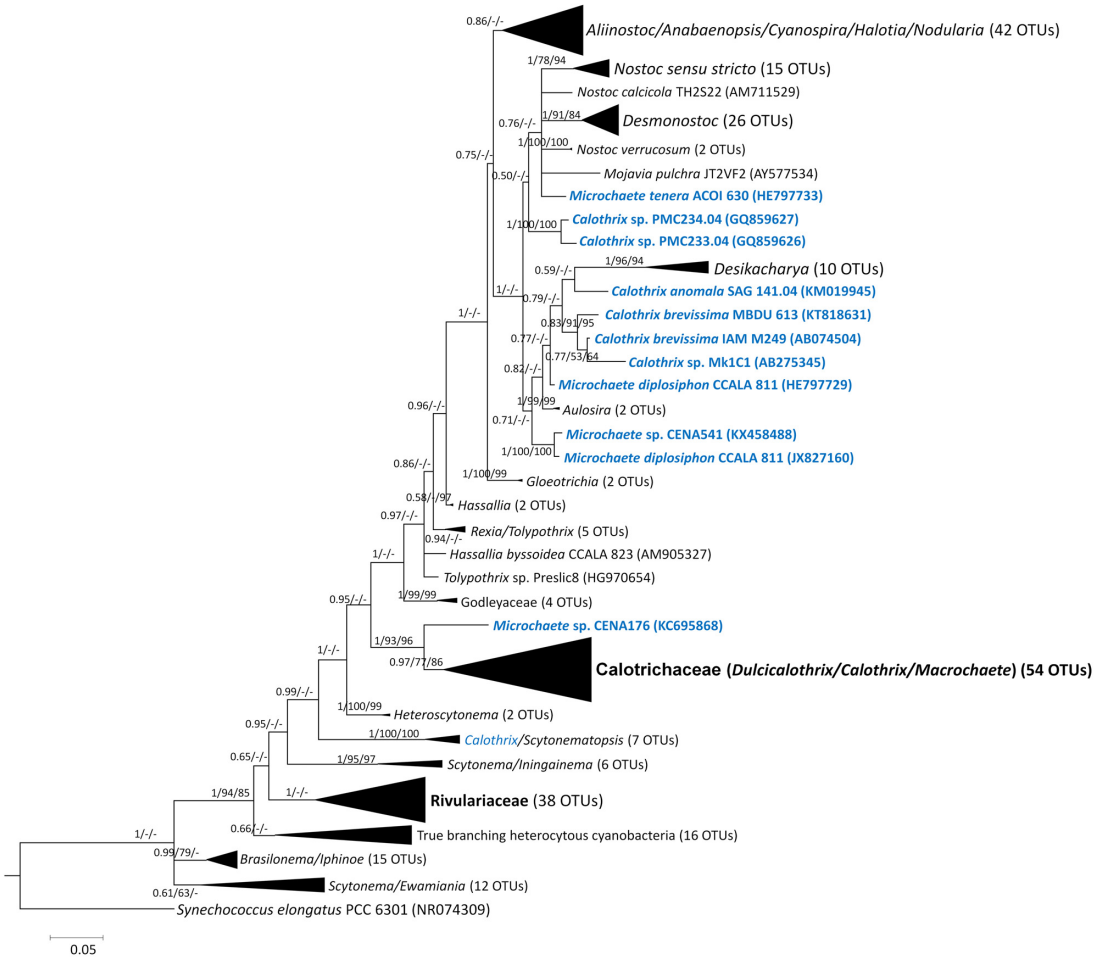

Figure 4. 16S rRNA gene tree indicating the phylogenetic complexity within the members of the family Rivulariaceae inferred by Bayesian inference with probability/bootstrap values representing BI, ML and MP, respectively. Bar 0.05 changes per nucleotide position. The length of the 16S rRNA gene of strain V13 is 1480 bp long and a total of 276 nucleotide sequences were included in the analysis. Blue Color indicates the *Calothrix* and *Microchaete* species whose taxonomic status must be revised in future.

However, determining *Calothrix sensu stricto* is also difficult as the type species of *Calothrix* i.e. *Calothrix confervicola* is not yet sequenced. *Calothrix confervicola* is a marine cyanobacterium characterized by cylindrical trichomes that abruptly narrow into hairs, non-lamellated sheath that may be colorless or yellowish-brown and heterocytes at basal position which are usually solitary or rarely in pairs (Bornet and Flahault 1886). Therefore, absence of terminal hairs and ecological preference for freshwater or terrestrial habitats could be considered as the distinguishing factors for the creation of a new genus. Initially the development of terminal hairs was not perceived to be a diacritical feature for *Calothrix* as it was found to be dependent on environmental conditions (Berrendero *et al.* 2016). However, the ability to produce hairs is genetically determined and its expression is dependent on environmental conditions (Berrendero *et al.* 2016). Berrendero *et al.* (2016) in their study have discussed this issue in detail and the authors have recommended that the development of terminal hairs should be regarded as a distinguishing trait. Thus, unique morphological and ecological features together with the phylogenetic evidence allowed us to reclassify the *Calothrix* strains of clade C1 into a new genus *Dulcicalothrix*. The creation of *Dulcicalothrix* would further contribute in resolving the ongoing taxonomic dispute. At present, we cannot predict the exact clade of *Calothrix sensu stricto*. But we believe that if and when *Calothrix confervicola* is sequenced it will fall into one of the sub-clades of marine/freshwater cluster as also suggested by Berrendero *et al.* 2016. Further, the phylogenetic position and the branch length of strain V13 in the 16S rRNA gene tree indicates that our strain is a new species belonging to the genus *Dulcicalothrix* (Fig. 3). The 16S-23S ITS secondary structure analysis also supported the above result. The folded secondary structure of D1-D1' helix region of strain V13 was similar to the secondary structures of strain PCC 7103 (Fig. 5). However, the number of nucleotides forming the bilateral bulge varied among the structures. In case of the bilateral bulge above the 3' unilateral bulge, strain V13 had 8 nucleotides whereas the structures formed using both the operons of PCC 7103 had 6 nucleotides. The difference in the number of nucleotides was also seen in the second bilateral bulge below the terminal loop where strain V13 had 11 nucleotides whereas PCC 7103 had only 9 nucleotides. The secondary structures of the other members in the analysis could be easily distinguished from strain V13 and PCC 7103 (Fig. 5). As D1-D1' helix region did not provide enough conclusive evidence, we further performed the comparative analysis by obtaining the folded secondary structures of BoxB region. The comparative analysis using BoxB region clearly differentiated strain V13 from PCC 7103 along with the other members of the genus *Dulcicalothrix* (Fig. 6). The shape and the

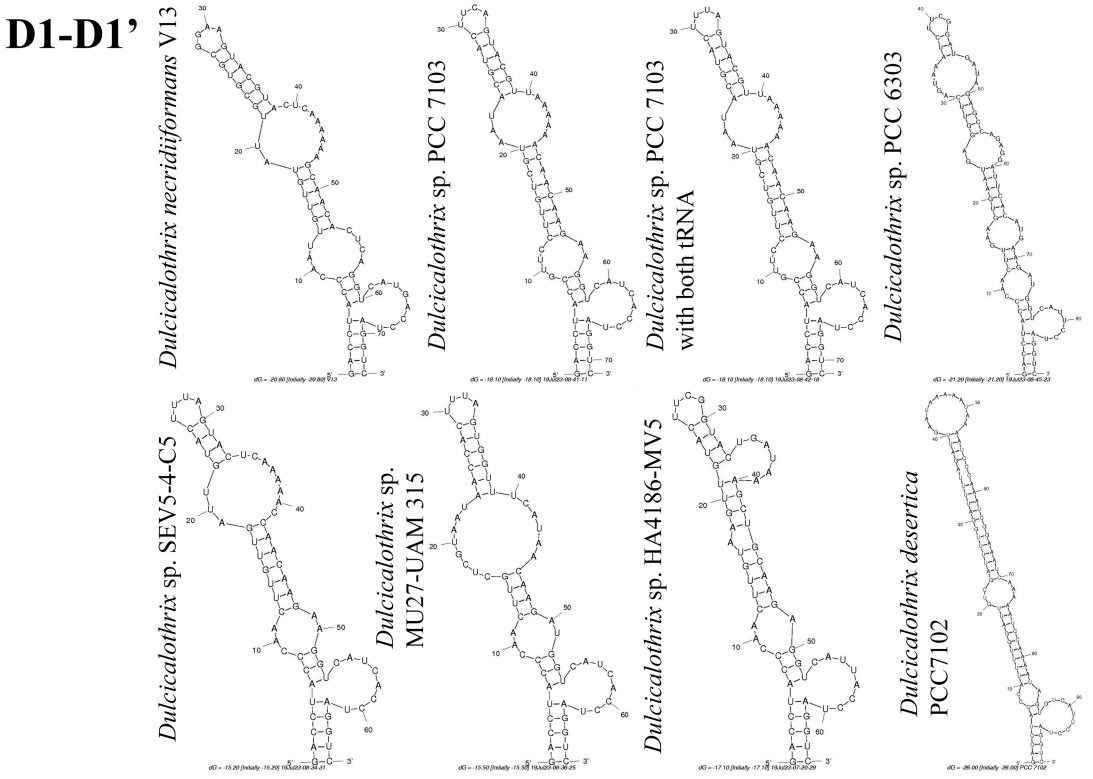

Figure 5. Comparative secondary structure analysis of D1-D1' helix region of *Dulcicalothrix necridiiformans* with the phylogenetically related strains based on 16S rRNA gene phylogenetic analysis.

number of nucleotides in the sub-basal bilateral bulge of strain V13 was found to be different from the other strains involved in the analysis (Fig. 6). The V3 region could not be sequenced as the direct sequencing strategy employed here did not amplify those regions. Still, the results obtained and the critical differences observed in the BoxB region and also the D1-D1' region are sufficient to support our interpretations.

From the 16S rRNA gene tree constructed in this study, it can be clearly observed that the family Rivulariaceae as recently defined (Komárek *et al.* 2014) is polyphyletic (Fig. 4). *Rivularia* along with *Nunduva*, *Kyrtuthrix*, *Phyllonema* and one sequence of *Microchaete* clustered together, whereas *Calothrix* and *Macrochaete* formed a separate cluster (Fig. 4). Similar results have also been observed in different phylogenetic trees represented in different studies (Berrendero *et al.* 2016; Shaligyn *et al.* 2017; Saraf *et al.* 2018). In the phylogenetic tree represented in Villanueva *et al.* (2019) it can be observed that the sequences corresponding to *Rivularia* were phylogenetically more related to *Scytonematopsis* and *Tolypothrix* rather than *Calothrix* and *Macrochaete* (Villanueva *et al.* 2019). Alternatively, in two instances the members of Rivulariaceae formed a monophyletic clade but the node was not supported in both the cases (León–Tejera *et al.* 2016; González–Resendiz *et al.* 2018). In the case of *Dichothrix* there is only a single 393 bp sequence available in the database, whereas at present there is no sequence data for *Gardnerula*, *Isactis* and *Sacconema*. To make the situation even more complex a few sequences of *Microchaete* and *Calothrix* were phylogenetically in proximity to the members of Nostocaceae rather than Rivulariaceae (Fig. 4: All problematic clusters have been colored). Two strains of *Calothrix brevissima* MBDU613 and IAM M249 are repeatedly clustered together with the members of Nostocaceae and this observation is consistent with the earlier study (Berrendero *et al.* 2016). Also, *Calothrix* sp. UAM 342 and *Calothrix* sp. CYN89 are clustered with five sequences of *Scytonematopsis contorta* with strong support. This pattern was also observed in our previous study (Saraf *et al.* 2018). We strongly recommend the revision of the taxonomic status of all the *Calothrix* and *Microchaete* strains that are phylogenetically distant from Rivulariaceae. These strains may possibly indicate a new evolutionary lineage. The polyphyletic nature of Rivulariaceae has been under constant debate and Berrendero *et al.* (2016) in their study discussed the possibility of separating *Calothrix* and *Macrochaete* from the existing Rivulariaceae. However, no formal reclassification was made in their study. It is necessary to note that similar revisions at the family level have been made in recent times with the creation of Gloeotrichiaceae from Rivulariaceae and Tolypothrichaceae and Godleyaceae from Microchaetaceae. Considering the earlier recommendations and the current trends observed in this study we propose re-erection of the family Calotrichaceae. At present, the family would include *Calothrix*, *Macrochaete* and *Dulcicalothrix*, with *Calothrix* being the type for the family. Rivulariaceae would include *Rivularia*, *Microchaete*, *Phyllonema*, *Kyrtuthrix*, *Nunduva*, *Dichothrix*, *Gardnerula*, *Isactis* and *Sacconema*. Creation of *Dulcicalothrix* and Calotrichaceae would serve to resolve some of the taxonomic confusion surrounding the tapering heterocytous cyanobacteria.

Through this study, we formally reclassify *Calothrix desertica* to *Dulcicalothrix desertica*. However, we would like to mention that even though *Calothrix parietina* 2T10 and *Calothrix parietina* SRS-BG14 fall within the *Dulcicalothrix* clade we have not reclassified *Calothrix parietina* to *Dulcicalothrix* due to the following reasons. From the phylogenetic tree it can be observed that there are in total three strains of *Calothrix parietina*, of which two strains 2T10 and SRS-BG14 clustered within the

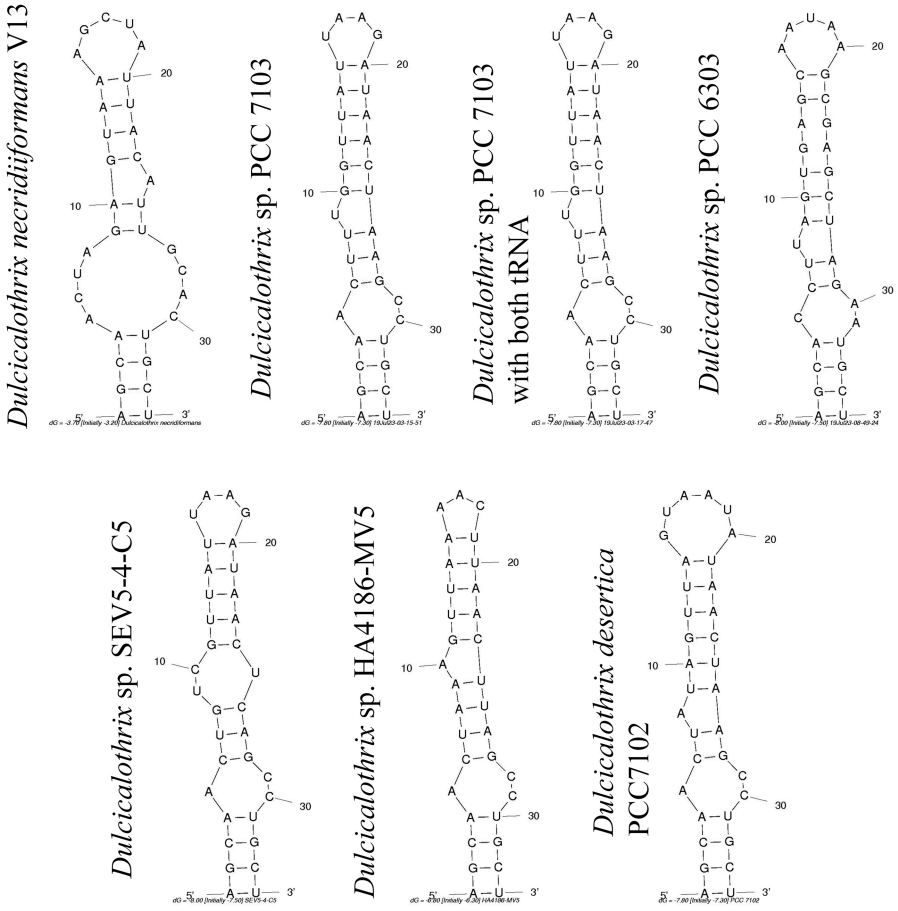

Figure 6. Comparative secondary structure analysis of boxB region of *Dulcicalothrix necridiiformans* with the phylogenetically related strains based on 16S rRNA gene phylogenetic analysis.

Dulcicalothrix clade (C1) whereas strain CCAP 1410/11 clustered within the clade C2 (Fig. 3). Also the 16S rRNA gene similarity among the three strains is < 98.7% indicating that they may represent different species (Table S3, Supporting Information). Furthermore, there is lack of morphological data for strains 2T10 and SRS-BG14 regarding the hair forming ability (Flechtner *et al.* 2014; Cuzman *et al.* 2010), which is one of the important morphological trait of *Calothrix parietina* (Bornet and Flahault 1886). It must be noted that Berrendero *et al.* (2016) in their study had mentioned that, *Calothrix parietina* SAG 1410–3 forms terminal hair, thus satisfying the morphological description of *Calothrix parietina*. Also in their phylogenetic tree, it can be observed that strain SAG 1410–3 clustered distantly from strains 2T10 and SRS-BG14. This suggests that strains 2T10 and SRS-BG14 may be identified incorrectly as *Calothrix parietina*. Therefore, we recommend that both strains should be further studied before making any conclusions.

Description and proposal for re-erection of the family Calotrichaceae (Bennet and Murray 1889)

In spite of an overall morphological similarity with the family Rivulariaceae, the proposal for the re-erection of this family is based on strong and exhaustive phylogenetic evidence along with clear support from previous published works by other taxonomists.

Type genus: *Calothrix* Agardh ex Bornet et Flahault, 1886. *Ann des Sci Nat Bot* Septième S:323–81.

Description of *Dulcicalothrix* Saraf *et al.* gen. nov.

Etymology: *Dulcicalothrix* (Dul.ci.ca'lo.thrix. L. adj. *dulcis* sweet; N.L. fem. n. *Calothrix* a cyanobacterial genus; N.L. fem. n. *Dulcicalothrix Calothrix* from freshwater).

Description: Tapering heterocytous cyanobacteria found in freshwater and terrestrial habitats. No hair formation evident even after reducing phosphorous concentration. Morphologically similar to the genus *Calothrix* in having tapering trichomes but differing very clearly in habitat. 16S rRNA gene phylogeny clearly separates *Dulcicalothrix* from the morphologically similar genera *Calothrix*, *Rivularia* and *Macrochaete*.

Type species: *Dulcicalothrix necridiiformans*

Description of *Dulcicalothrix necridiiformans* Saraf *et al.* sp. nov.

Etymology: *necridiiformans* (ne.cri.di.i.for'mans. N.L. neut. n. *necridium* nectridium; L. pres. part. *formans* forming; N.L. part. adj. *necridiiformans* producing necridia).

Description: Bluish green macroscopic appearance in nature, found submerged in shallow water. Trichomes prominently tapering at one end with the broad base having a basal heterocyte. The attenuation is continuous and regular with the apical/distal ends being distinctly attenuated. Sheath surrounding the filament is colorless and has clear lamellation. Cells usually constricted at the ends with the older filaments having more prominent constrictions. Bow shaped necridia

formation is the diagnostic feature of the species, often with hormogonia formation adjacent to the necridia. Vegetative cells are wider near the basal heterocyte and continue to narrow towards the distal end. In older filaments, width of the vegetative cells is prominently more than the length. In younger filaments the length of the vegetative cells is more than the width. Distal vegetative cells are 5.20–5.26 μm in length to 3.63–3.69 μm in width. Intercalary vegetative cells with sheath are 6.20–6.26 μm in length to 8.16–8.21 μm in width. Intercalary vegetative cells without sheath are 6.27–6.37 μm in length to 6.46–6.54 μm in width. Heterocytes are always basal and solitary with the shape being usually spherical although sometimes with a slightly elongated end. Basal heterocytes are 5.80–5.90 μm in length to 6.0–6.60 μm in width. Akinetes were not observed.

Type locality: Shrirampur, Ahmednagar, Maharashtra, India (19.62°N, 74.65°E).

Habitat: In an oligotrophic water body as bluish green macroscopic colonies found submerged in shallow water. The water temperature at the time of collection was 38.2°C while the pH was recorded as 7.3.

Holotype (here designated): portion of a culture of *Dulcicalothrix necridiiformans* preserved in metabolically inactive form in National Centre for Microbial Resource (NCMR) formerly Microbial Culture Collection (MCC), National Centre for Cell Science, Pune, India and is available under the accession number MCC 3314.

Dulcicalothrix desertica (Schwabe) Saraf *et al.* comb. nov.

Basionym: *Calothrix desertica* Schwabe GH. Zur autotrophen Vegetation in ariden Böden. Blaualgen und Lebensraum IV. *Österreichische Botanische Zeitschrift* 1960;**107**:282, Figs 1–5.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

ACKNOWLEDGMENTS

We thank the Head, Department of Botany, BHU and Director, NCCS for providing the necessary facilities and encouragement. We thank the Principal, Ramniranjan Jhunjhunwala College, Mumbai for support. We thank Professor Aharon Oren for help in the scientific names and etymology. We thank Dr Esther Berrendero Gómez for the useful discussions. The authors thank the anonymous reviewers for the thorough critical review of the work. AS is thankful to Council of Scientific & Industrial Research (CSIR-HRDG) for providing the financial assistance.

FUNDING

PS is thankful to the Department of Science and Technology (DST), India for the project YSS/2014/000879. A part of the work was supported by the Department of Biotechnology (DBT; Grant No. BT/Coord.II/01/03 2016), Government of India, under the project 'Establishment of Centre of Excellence for National Centre for Microbial Resource (NCMR)'. The study was also supported by an internal grant of M. Akmullah Bashkir State Pedagogical University for the year 2019.

Conflict of interest. None declared.

REFERENCES

Alvarenga DO, Rigonato J, Henrique J *et al. Phyllonema avicenicola* gen. nov., sp. nov. and *Foliisarcina bertiogensis* gen. nov., sp. nov., epiphyllic cyanobacteria associated with *Avicennia schaueriana* leaves. *Int J Syst Evol Microbiol* 2016;**66**:689–700.

Bagchi SN, Dubey N, Singh P. Phylogenetically distant clade of *Nostoc*-like taxa with the description of *Aliinostoc* gen. nov. and *Aliinostoc morphoplasticum* sp. nov. *Int J Syst Evol Microbiol* 2017;**67**:3329–38.

Bennet A, Murray G. *A handbook of Cryptogamic Botany*. London: Longmans, Green and Co, 1889.

Berrendero E, Johansen JR, Kaštovský J *et al. Macrochaete* gen. nov. (Nostocales, Cyanobacteria), a taxon morphologically and molecularly distinct from *Calothrix*. *J Phycol* 2016;**52**:638–55.

Berrendero E, Perona E, Mateo P. Genetic and morphological characterization of *Rivularia* and *Calothrix* (Nostocales, Cyanobacteria) from running water. *Int J Syst Evol Microbiol* 2008;**58**:447–60.

Bohunická M, Pietrasiak N, Johansen JR *et al. Roholtiella*, gen. nov. (Nostocales, Cyanobacteria)- a tapering and branching cyanobacteria of the family Nostocaceae. *Phytotaxa* 2015;**197**:084–103.

Bornet E, Flahault C. Revision des Nostocacées hétérocystées contenues dans les principaux herbiers de France. *Ann des Sci Nat Bot* 1886;**Septième S**:323–81.

Cuzman OA, Ventura S, Sili C *et al.* Biodiversity of phototrophic biofilms dwelling on monumental fountains. *Microb Ecol* 2010; **60**:81–95. 10.1007/s00248-010-9672-z20446084

Darriba D, Taboada GL, Doallo R *et al.* JModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 2012;**9**:772.

Edwards U, Rogall T, Blöcker H *et al.* Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989;**17**:7843–53.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;**39**:783.

Flechtner VR, Boyer SL, Johansen JR *et al. Spirirestis rafaelensis* gen. et sp. nov. (Cyanophyceae), a new cyanobacterial genus from arid soils. *Nova Hedwigia* 2014; **74**:1–24.

Frémy P. Les Nostocacées de la Normandie. *Not Mem Doc Soc Agric Archéol Hist nat Manche* 1929;**41**:197–228.

Geitler L. Cyanophyceae. In Rabenhorst L (ed). *Kryptogamenflora von Deutschland, Österreich und der Schweiz*. Leipzig: Akademische Verlagsgesells-chaft m. b. H., 1932, 1196.

Gkelis S, Rajaniemi P, Vardaka E *et al. Limnothrix redekei* (Van Goor) Meffert (Cyanobacteria) strains from Lake Kastoria, Greece form a separate phylogenetic group. *Microb Ecol* 2005;**49**:176–82.

González–Resendiz L, Johansen JR, Alba–Lois L *et al. Nunduva*, a new marine genus of Rivulariaceae (Nostocales, Cyanobacteria) from marine rocky shores. *Fottea Olomouc* 2018;**18**:86–105.

Hauer T, Bohunická M, Johansen JR *et al.* Reassessment of the cyanobacterial family Microchaetaceae and establishment of new families Tolypothrichaceae and Godleyaceae. *J Phycol* 2014;**50**:1089–100.

Hauer T, Bohunická M, Mühlsteinová R. *Calochaete* gen. nov. (Cyanobacteria, Nostocales), a new cyanobacterial type from the "páramo" zone in Costa Rica. *Phytotaxa* 2013;**109**:36–44.

Hentschke GS, Johansen JR, Pietrasiak N *et al. Komarekiella atlantica* gen. et sp. nov. (Nostocaceae, Cyanobacteria): a

new subaerial taxon from the Atlantic Rainforest and Kausi, Hawaii. *Fottea* 2017;**17**:178–90.

Hrouzek P, Lukešová A, Mareš J *et al*. Description of the cyanobacterial genus *Desmonostoc* gen. nov. including *D. muscorum* comb. nov. as a distinct, phylogenetically coherent taxon related to the genus *Nostoc*. *Fottea* 2013; **13**:201–13.

Komárek J, Anagnostidis K. Modern approach to the classification system of the cyanophytes 4.Nostocales. *Algol Stud* 1989;**56**:247–345.

Komárek J, Kaštovský J, Mareš J *et al*. Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia* 2014;**86**:295–335.

León-Tejera H, González- Resendiz L, Johansen JR *et al*. Phylogenetic position reevaluation of *Kyrtuthrix* and description of a new species *K. huatulcensis* from Mexico´s Pacific coast. *Phytotaxa* 2016;**278**:1–18.

Řeháková K, Johansen JR, Casamatta DA *et al*. Morphological and molecular characterization of selected desert soil cyanobacteria: three species new to science including *Mojavia pulchra* gen. et sp. nov. *Phycologia* 2007;**46**:481–502.

Rippka R, Deruelles J, Waterbury J *et al*. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Microbiology* 1979;**111**:1–61.

Ronquist F, Teslenko M, Van Der Mark P *et al*. Mrbayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 2012;**61**:539–42.

Saraf A, Dawda HG, Singh P. *Desikacharya* gen. nov., a phylogenetically distinct genus of Cyanobacteria along with the description of *Desikacharya nostocoides* sp. nov. and *Desikacharya soli* sp. nov. and reclassification of *Nostoc thermotolerans* to *Desikacharya thermotolerans* comb. nov. *Int J Syst Evol Microbiol* 2018;**69**:307–15.

Saraf A, Dawda HG, Suradkar A *et al*. Insights into the phylogeny of false-branching heterocytous cyanobacteria with the description of *Scytonema pachmarhiense* sp.nov. isolated from Pachmarhi Biosphere Reserve, India. *FEMS Microbiol Lett* 2018;**365**:fny160

Schwabe GH. Zur autotrophen Vegetation in ariden Böden. Blaualgen und Lebensraum IV. *Österreichische Botanische Zeitschrift* 1960;**107**:281–309.

Shalygin S, Shalygina R, Johansen JR *et al*. *Cyanomargarita* gen. nov. (Nostocales, Cyanobacteria): convergent evolution resulting in a cryptic genus. *J Phycol* 2017;**53**:762–77.

Suradkar A, Villanueva C, Gaysina LA *et al*. *Nostoc thermotolerans* sp. nov., a soil-dwelling species of *Nostoc* (Cyanobacteria). *Int J Syst Evol Microbiol* 2017;**67**;1296–305.

Tamura K, Peterson D, Peterson N *et al*. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731–9.

Villanueva C, Garvey A, Petr H *et al*. Descriptions of *Brasilonema geniculatum* and *Calothrix dumus* (Nostocales, Cyanobacteria): two new taxa isolated from cemetery tombstones. *Phytotaxa* 2019;**387**:001–20.

Yarza P, YilmaZ P, Pruesse E *et al*. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* 2014;**12**:635–45.

Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;**31**:3406–15.