*Phytotaxa* 109 (1): 36–44 (2013)
www.mapress.com/phytotaxa/


Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

http://dx.doi.org/10.11646/phytotaxa.109.1.4

# *Calochaete gen. nov.* (Cyanobacteria, Nostocales), a new cyanobacterial type from the "páramo" zone in Costa Rica

TOMÁŠ HAUER[1,2*], MARKÉTA BOHUNICKÁ[1,2] & RADKA MÜHLSTEINOVÁ[1]

[1]*Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice, Czech Republic, CZ-37005.*
[2]*Centre for Phycology, Institute of Botany, Czech Academy of Sciences, Dukelská 135, Třeboň, Czech Republic, CZ-37982.*
** Corresponding author: hauer@butbn.cas.cz*

## Abstract

A new tapered and false branched morphotype of filamentous heterocytous cyanobacterium was isolated from soil material collected on a massif of Chirripó Mountain, Costa Rica. The strain was analyzed morphologically and a sequence of its 16S rRNA gene was compared with available 16S rDNA sequences of organisms with similar morphology, especially those with heteropolar tapering filaments. Phylogenetic analyses revealed that the strain was significantly different from Rivulariaceae, but was closely related to several strains designated as *Tolypothrix.* However, according to the original descriptions in the literature, members of the genus *Tolypothrix* possess only very slightly tapering filaments. With regard to all these differences, we decided to describe a new genus—*Calochaete gen. nov.* with type species *C. cimrmanii.*

**Key words:** 16S-23S ITS, 16S rRNA gene, Central America, Cyanoprokaryota, Microchaetaceae, morphology, new genus, taxonomy

## Introduction

Mesoamerica and the Caribbean region are listed among the world's biodiversity hotspots and are known for their large number of endemic vertebrates and plant species (Myers *et al.* 2000). However, there is no reason to not extend this biodiversity to organisms much smaller and not so easily visible such as algae and Cyanobacteria. In this sense, the monograph by Gardner (1927), or more recent studies (e.g. Komárek & Komárková-Legnerová 2007, Kaštovský *et al.* 2011) that described previously unrecognized cyanobacterial diversity in the tropical regions of Central and South America, are very valuable.

Within the group of filamentous cyanobacteria with heterocytes (order Nostocales *sensu* Hoffmann *et al.* 2005), there are three groups of false branched types. The family Scytonemataceae has isopolar filaments and tapering can be found in two of the five genera belonging to this group. The families Rivulariaceae and Microchaetaceae are both characterized by heteropolar filaments; tapered types are included in the family Rivulariaceae, as tapering was traditionally considered the fundamental difference between these two families (Komárek & Anagnostidis 1989). The only known exception to date is *Godleya alpina* Novis et Visnovsky (2011: 14), which is distinctly tapered, but was classed to Microchaetaceae based on molecular markers.

In the course of a broader study on the floristics of aerophytic cyanobacteria of the San Gerardo de Rivas region, San José, Costa Rica (Mühlsteinová 2011), a new morphotype of a heterocytous cyanobacterium was found and isolated into a unicyanobacterial strain. The aim of this study was to characterize this unusual type using a combination of morphological and molecular data (referred to as the polyphasic approach). By recognizing and describing our isolate as a species new to science, our study contributes to knowledge about the species richness of one of the planet's biodiversity hotspots. Concurrently, we discuss the heterogeneity of the family Microchaetaceae in the current concept.

 *Accepted by Saúl Blanco Lanza: 23 May 2013; published online in PDF: 10 June 2013*

## Material and methods

A soil crust in the "páramo" ecosystem was collected in March 2010 at 9° 29' 58" N, W 83° 30' 24" W along the trail from San Gerardo de Rivas to the summit of Chirripó Mountain (3820 m a.s.l.), Costa Rica, at an elevation around 3600 m a.s.l. The sample was dried in silicagel in the field and dilution plated on agar solidified Bold's Basal Medium (BBM, Bischoff & Bold 1963) upon return to the laboratory. A single clonal colony was then isolated and cultivated on BBM and Z8 (Kotai 1972) agar slants at 16 and 22 °C in a 12:12 light:dark cycle. To record the phases of the life cycle, the culture was examined at 14 days, 7 months, and 15 months after inoculation. Observations were performed using an Olympus BX 51 microscope equipped with Nomarski DIC optics; photographs were taken using an Olympus DP 71 digital camera.

**Molecular analyses**:—For phylogenetic analyses, a gene for the small subunit of ribosomal RNA (16S rRNA) was chosen. This marker, traditionally used for prokaryotic organisms, is convenient for comparisons on various taxonomic levels due to it containing both highly conserved and variable regions (e.g. Woese & Fox 1977, Nelissen *et al.* 1996, Chakravorty *et al.* 2007). Moreover, compared to other phylogenetic markers, a sufficient number of 16S rDNA sequences of various cyanobacteria are available in public databases, providing a good foundation for placement of unusual taxon in the tree of life. Approximately 1 $mm^3$ of the strain biomass was dried for 48 hours in silicagel and pulverized with wolfram carbide beads in a Retsch MM200 mill (Retsch GmbH, Haan, Germany) for 3 minutes. Total genomic DNA was isolated following the protocol of Yilmaz *et al.* (2009). A ~1600 bp segment of the rRNA operon containing partial SSU rRNA gene and the ITS region was amplified using primer 1 (5'-CTC TGT GTG CCT AGG TAT CC-3') after Wilmotte *et al*. (1993), and primer 2 ( 5'-GGG GAA TTT TCC GCA ATG GG-3') after Nübel *et al*. (1997), as previously described in detail by Boyer *et al*. (2001). 10 ng of template DNA were mixed with 6 pmol of each primer in a commercial PCR mix with *Taq* polymerase (Plain PP Master Mix, Top Bio, Czech Republic), and amplified with an initial denaturation step (5 minutes at 95 °C) followed by 35 cycles of denaturation (1 min at 94 °C), primer annealing (45 s at 55 °C), elongation (2 min at 72 °C), and final extension for 10 min at 72 °C. The PCR product was cloned using the standard pGEM-T Easy vector system (Promega Corp., WI, USA) according to the supplied manual. Plasmids containing insert were purified with Zyppy Plasmid Miniprep kit (Zymo Research Corp., CA, USA). The clones were sequenced using standard plasmid primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6r (5'-TAT TTA GGT GAC ACT ATA G-3') on ABI PRISM 3130 XL (Applied Biosystems, Life Technologies Corp., CA, USA) in the Laboratory of Genomics, Biology Centre of the Academy of Sciences of the Czech Republic. The sequence was deposited in the European Nucleotide Archive (EMBL/ENA, https://www.ebi.ac.uk/ena/) under accession number HF912386.

**Phylogenetic analyses:**—Obtained sequences were aligned using MAFFT v. 6 (Katoh *et al*. 2009) together with 67 selected OTUs representing the heterocytous cyanobacteria obtained from GenBank (February 2013), and two outgroup taxa (*Blennothrix* sp. AQS and *Chroococcidiopsis thermalis* Geitler (1933: 625) strain PCC 7203). Phylogenetic calculations were run employing Bayesian inference in MrBayes 3.2 (Ronquist *et al.* 2011), maximum likelihood analysis, and neighbor-joining BioNJ algorithm in SeaView 4.4 (Gouy *et al*. 2010). For the Bayesian analyses, two runs of eight Markov chains were executed for ~1 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01), and the first 25% of sampled trees were discarded as burn-in. The maximum likelihood calculation was executed on the generalized time-reversible (GTR) substitution model with discrete gamma distribution in six categories. The gamma shape parameter $\alpha$ as well as the proportion of invariable sites were estimated from the data set (GTR + $\Gamma$+ I model), and 1000 bootstrap replicate searches were conducted to evaluate the relative support of branches. The neighbor-joining analysis was run on the Hasegawa, Kishina and Yano (HKY) substitution model with 1000 bootstrap replicates. The secondary structures of ITS helices were folded using sFold (Ding *et al*. 2004).

## Results

Class **Cyanophyceae**
Order **Nostocales**
Family **Microchaetaceae**

***Calochaete*** Hauer, Bohunická & Mühlsteinová, *gen. nov.*

Colony on agar dark green or brown, amorphous. Filaments usually very long with basal heterocyte, swollen at the base, then gradually tapering towards the end, without terminal hair, often with false branching, single or double. Sheath 0.5–1.5 µm thick, unstructured, colorless. Trichomes notably constricted at cross-walls, 7–9 µm wide at the base and 3.0–4.3 µm wide at well-developed ends. Cells olive green, brown to violet, cylindrical or barrel shaped, shorter or longer than wide, 3–8 µm long, basal:apical ratio 1.8–2. Terminal cell longer than wide, cylindrical or bluntly conical. Heterocytes basal or intercalary, green to yellowish, hemispherical or cylindrical, rarely conical, 4–7 µm wide, 4–8 µm long. Reproduction by hormogonia with almost spherical cells. No akinetes were observed.

**Type species:** *Calochaete cimrmanii* Hauer, Bohunická & Mühlsteinová.

**Etymology**:—From the Greek *Kalos* (beatutiful) and *chaete* (long hair), refers to one of the morphological features.

***Calochaete cimrmanii*** Hauer, Bohunická & Mühlsteinová, *sp. nov*. (Figs 1A–P)
The description corresponds to that of the genus.

**Type**:—COSTA RICA. Chirripó Mountain slope, 9° 29’ 58” N, 83° 30’ 24” W, surface soil in páramo, dried specimen from culture of sample, colle. *M. Bohunická* and *J. Mareš*, 6 March 2010 (holotype: CBFS! A–015). Type strain: CCALA 1012.

**Habitat:**—Surface soil in “páramo” (tropical high montane vegetation).

**Etymology**:—The species is named after the mythical Czech scientist, dramatist, poet, and writer Jára Cimrman.

**Observations**:—The life cycle contains several morphologically distinct stages (Fig. 1). One was described above, i.e long, gradually tapering, heteropolar filaments (Figs 1A, B, P). The second consists of hormogonia composed of short, isodiametric, barrel-shaped to spherical cells resembling in morphology *Nostoc* Vaucher ex Bornet et Flahault (1888: 181) types lacking the sheath (Fig. 1E). Some cells within the hormogonia grow in size and change their shape to spherical (Figs 1D, O). Cells may sometimes divide in more than one plane (Figs 1L, M, O), but never form a true branch. A new filament then germinates from the hormogonia (Fig. 1C). The first cell of the filament becomes a heterocyte. The hormogonia with large spherical cells (e.g. Fig. 1D) occur in young cultures only.

Our strain differs from the morphologically similar genus *Calothrix* Agardh ex Bornet et Flahault (1886: 345) by the absence of a terminal hair and frequent false single or double branching (Figs 1G, H, N). As notable from Fig. 2, the 16S rRNA sequence of *Calochaete cimrmanii* is very distant from members of Rivulariaceae and is more related to Microchaetaceae. The most similar are sequences of *Tolypothix* strains UAM 332, 334, 337 and PCC 7504, but the degree of tapering is significantly lower in these strains. The low degree of tapering itself is one of the key diacritical features of the genus *Tolypothrix* Kützing ex Bornet et Flahault (1887: 118), which is accepted in both the botanical (Komárek & Anagnostidis 1989) and bacteriological (Castenholz 2001) literatures. From the above mentioned *Tolypothrix* strains, *Calochaete cimrmanii* differs also by living in a significantly different –non-aquatic, high mountain– habitat.

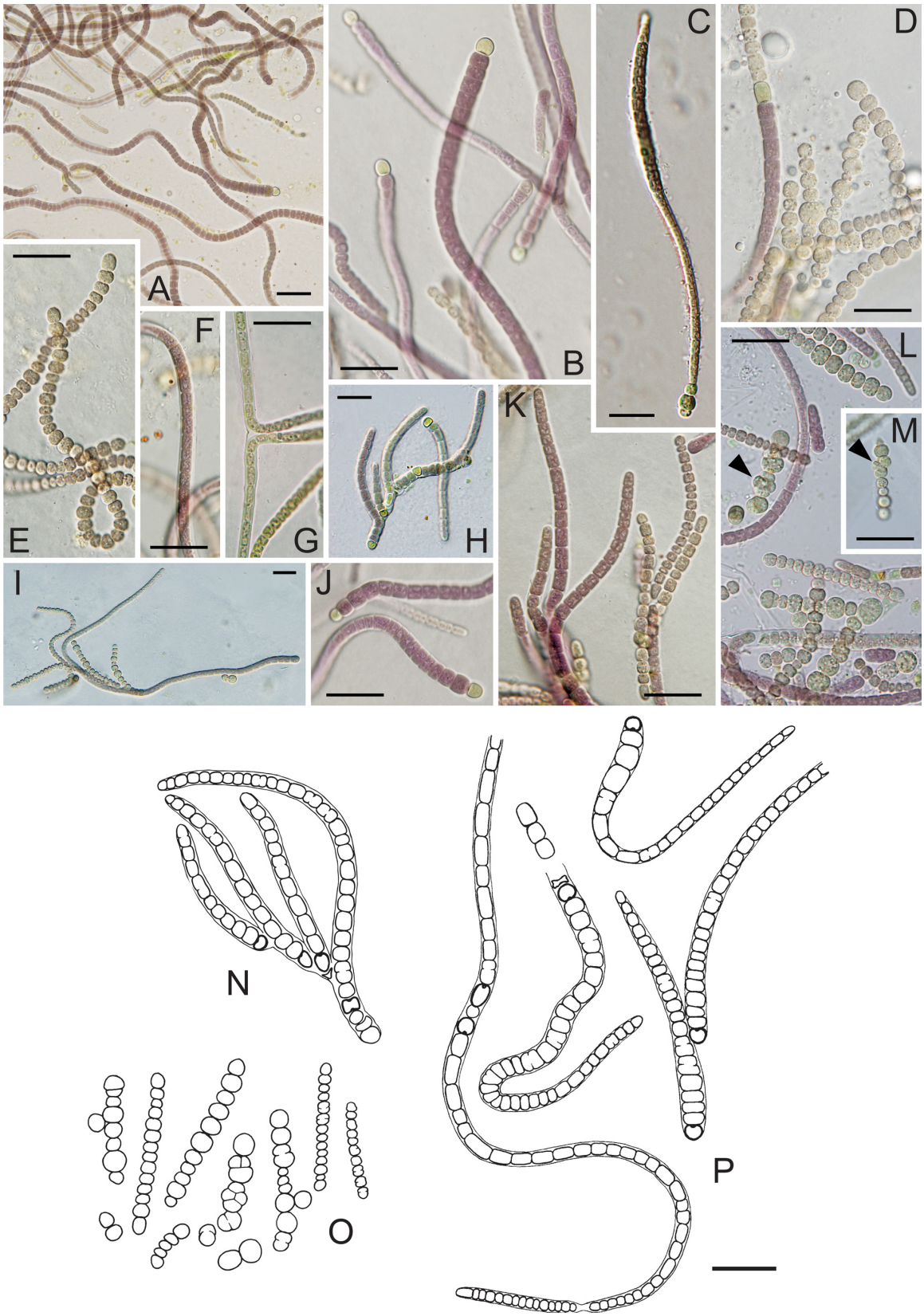


**FIGURE 1**. Morphological variability of the strain CCALA 1012. A. Mature heteropolar filaments. B. detail of well-developed filament with terminal heterocyte. C. A filament germinating from hormogonium with enlarged cells. D, E. Formation of a hormogonium in a filament and hormogonia with enlarging cells. F. Middle part of a mature filament with thin colorless sheath. G. Double branching. H. Single branching. I. Young filament with distinct tapering. J. Swollen bases of mature filaments. K. Shape of the terminal cells. L, M. Enlarged cells in hormogonia dividing in two planes (marked with arrowheads). N. Young growing thallus with single branching. O. Hormogonia and their development after release from mother sheath. M. Well-developed filaments. Bar length = 20 µm.

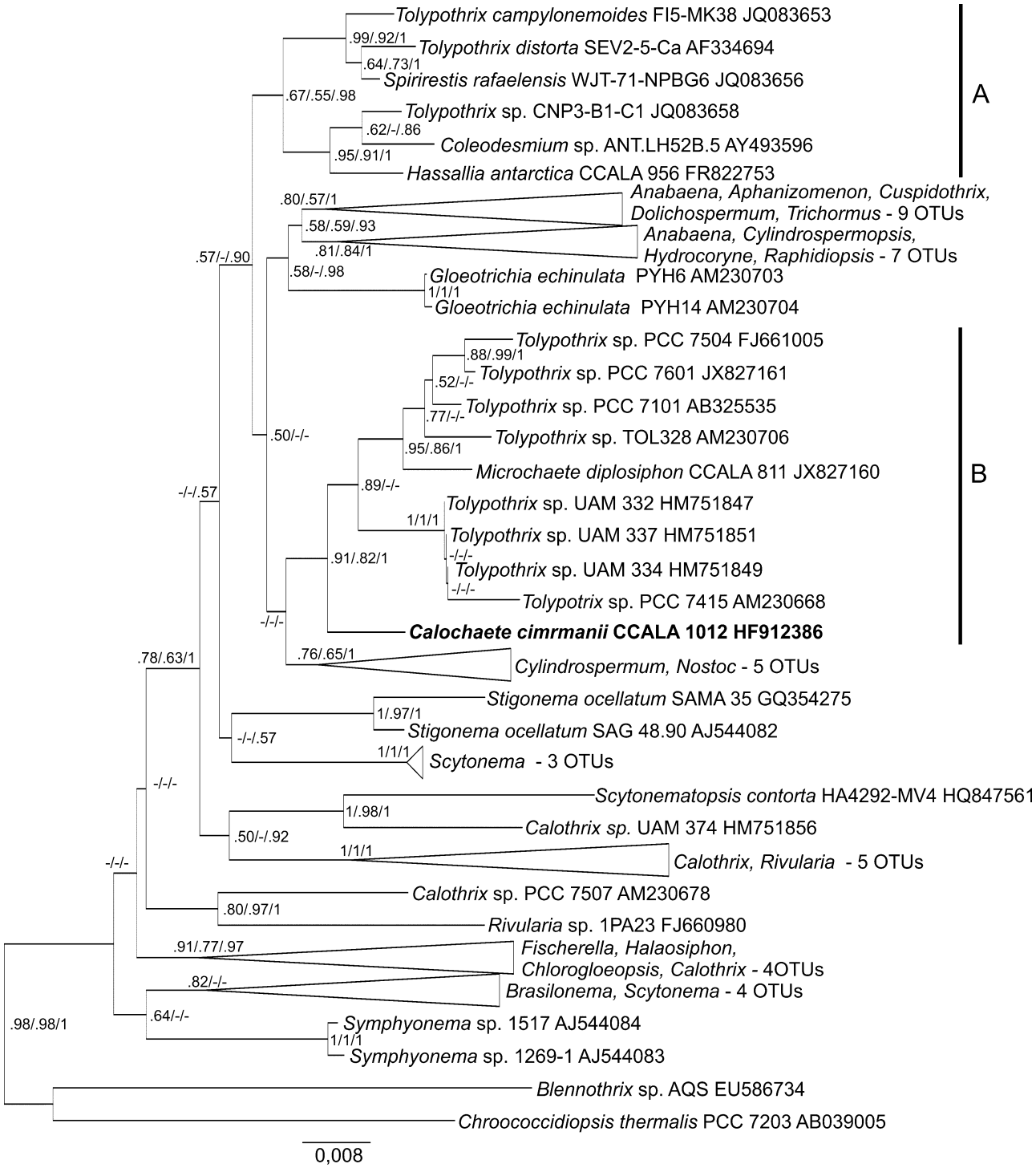


**FIGURE 2**. Phylogenetic analysis based on 16S rDNA sequence with 70 OTUs, using neigbor-joining topology. The support values are given for Neighbor-joining, Maximum likelihood, and Bayesian posterior probabilities. The cut-off values for bootstrap and probability are 50 (0.5 resp). Our reference strain CCALA 1012 (accession number HF912386) is printed in bold. Clusters A and B represent clades with members of the Microchaetaceae family demonstrating heterogeneity of the genus *Tolypothrix* and the whole family Microchaetaceae.

Comparison of the secondary structures of 16S-23S ITS is of taxonomic importance according to Johansen *et al*. (2011). To illustrate specificity and the rate of relatedness of the different morphologically similar taxa included in our study, we provide the two most conserved ITS domains, the D1-D1' and Box-B helices for *Calochaete cimrmanii*, *Tolypothrix* sp. PCC 7504 from cluster "B", *Spirirestis rafaelensis* Flechtner et Johansen in Flechtner *et al.* (2002: 6) WJT-71-NPBG6 from cluster "A", and *Calothrix* sp. PCC

7507 from a cluster phylogenetically distant from *Calochaete* (Fig. 3). It can be clearly seen that *Calochaete cimrmanii* and *Tolypothrix* PCC 7504 are significantly similar in both structures, especially in the basal parts and the bottom halves of the loops (Figs 3A, B, E, F). Concurrently, there are obvious differences in the top portions of the loops, supporting our decision to separate *Calochaete* from the traditional genus *Tolypothix*. The similarity of the secondary structures decreases when comparing *Calochaete* with *Spirirestis* as a representative of cluster "A" and the most phylogenetically but supposedly morphologically related *Calothrix.*

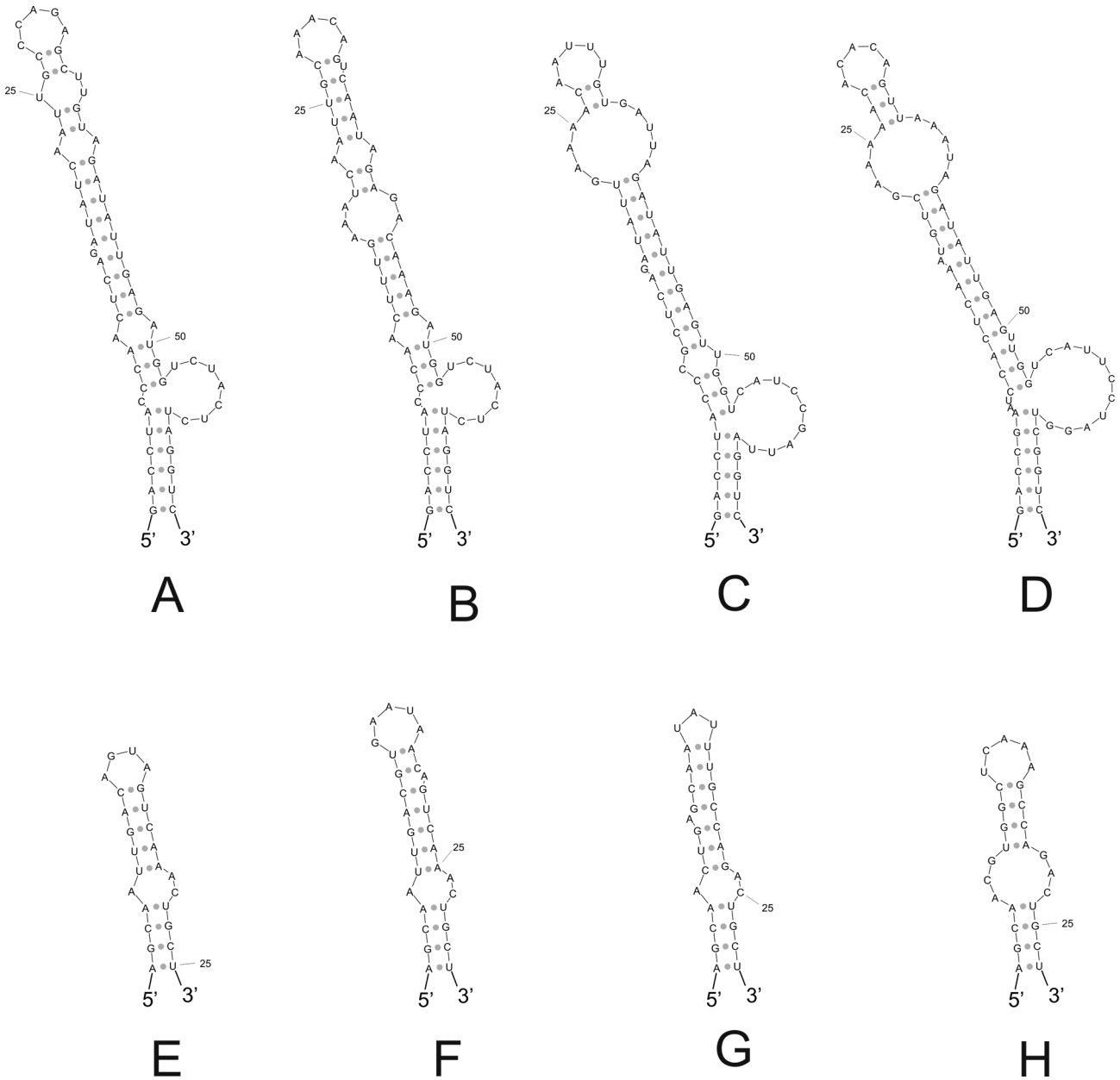


**FIGURE 3.** Secondary structures of the D1- D1'(row one) and Box-B helices (row two) of strain CCALA 1012 and related strains of types similar either morphologically (*Calothrix*) or molecularly (members of Microchaetaceae—*Tolypothrix* and *Spirirestis*). A, E. *Calochaete cimrmanii* CCALA 1012. B, F. *Tolypothrix* sp. PCC 7504. C, G. *Spirirestis rafaelensis* WJT-71-NPBG6. D, H. *Calothrix* sp. PCC 7507.

## Discussion

We performed a detailed study of an unusual strain isolated from a terrestrial habitat at high elevation in Costa Rica. Many authors, e.g. Berrendero *et al*. (2011), pointed out changes in morphology of cyanobacterial strains after transfer from nutrient-rich to nutrient-poor cultivation media. To document the variability in the strain morphology under different conditions, we used two different cultivation media—BBM and Z8. We also observed the culture in different stages of the life cycle from very young colonies 14 days after the inoculation growing in high light intensities and 22 °C (exponential growth phase), up to cultures 15 months old kept at 16 °C and dim conditions (stationary growth phase). We were not able to detect changes in morphology between clones cultivated in different media and the diacritical features remained stable also under different environmental conditions (temperature, light) and the age of the culture. As mentioned above, a stage of short isopolar hormogonia with several enlarged cells dividing in two planes occurred in young cultures of our strain. Similar structures were already reported in *Westiellopsis prolifica* Janet (1941: 170), and later in *Rexia erecta* Casamatta, Gomez et Johansen (2006: 23).

Discrepancies between morphological and molecular data, as shown above, were reported before. An example of the same phenomenon was noted by Novis & Visnovsky (2011) describing the cyanobacterial genus *Godleya* Novis et Visnovsky (2011: 14). The type strain possesses an important diacritical feature of Scytonemataceae—isopolar development of the trichomes, but the 16S rDNA sequence is more similar to heteropolar *Coleodesmium* Geitler (1942:154) strain ANT.LH52B.5.

The presented phylogenetic tree reveals also another fact: the obvious polyphyly in the genus *Tolypothrix* and consequently in the whole family Microchaetaceae. Two sequences of strains designated as *T. distorta* Kützing ex Bornet et Flahault (1887: 119), the type of the genus according to CyanoDB.cz (Komárek & Hauer 2013), and one as *T. tenuis* Kützing ex Bornet et Flahault (1887: 122) fall in a well-supported cluster (Fig. 2, cluster A) with *Spirirestis rafaelensis*, another member of the family Microchaetaceae. Other sequences of strains designated as *Tolypothrix*, e.g. *Tolypothrix* PCC 7415, PCC 7504, UAM 337, or *Microchaete diplosiphon* Gomont ex Bornet et Flahault (1886: 211) strain CCALA 811, form a distant and also well supported cluster (Fig. 2, cluster B), where *C. cimrmanii* is a basal group. This disunity of the family was denoted already in work of Berrendero *et al*. (2011), where the authors distinguished two subclusters of Microchaetaceae. We propose that the family Microchaetaceae as currently concieved does not exist and further analyses of strains of its members are necessary. Obviously, the same revision awaits also the Rivulariaceae family.

## Conclusions

We have described a new cyanobacterial genus from a “páramo” zone of Chirripó Mountain, Costa Rica. Our results clearly show the need for taxonomic revision of the families Microchaetaceae and Rivulariaceae.

## Acknowledgements

The research was supported by grants GA CR P506/12/1818, MŠMT/AMVIS LH12100, and a long-term research development project no. RVO67985939. Access to the MetaCentrum and CERIT-SC computing facilities provided under the programmes National Grid Infrastructure MetaCentrum (LM2010005) and Center CERIT Scientific Cloud (CZ. 1.05/3.2.00/08.0144) funded by the Czech Republic and the European Union is highly acknowledged. The authors would like to thank Professor Jiří Komárek for valuable consultations concerning strain morphology and also anonymous referees for their comments leading to improvement of the manuscript.

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