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The extremophile *Eurychoronema bolivianum* gen. et sp. nov. (Nodosilineales, Cyanobacteria) and *Leptolyngbya aquatica* comb. nov.

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ABSTRACT

Extreme environments exhibit conditions that are beyond the tolerances of most species. They are characterized by extremes in temperature, pressure, gas concentrations, salinity, radiation, pH or water availability. Despite their harsh conditions, extreme environments are a rich source for discovering new cyanobacterial taxa. The Bolivian Altiplano is an extreme environment characterized by sub-freezing temperatures, high salinity and some of the lowest precipitation rates on Earth. From this environment, the homocytous filamentous strain LEGE 231228 was isolated. Our 16S rRNA gene phylogenetic analyses showed that this strain was positioned within a clade, sister to a cluster containing *Toxifilum* and *Sodaleptolyngbya* strains, in the Nodosilineales clade. The 16S rRNA gene identity analysis revealed that when comparing LEGE 231228 with *Toxifilum* and *Sodaleptolyngbya*, the maximum values reached only 94.3% and 93.6%, respectively. Morphologically, LEGE 231228 differed from *Sodaleptolyngbya* and *Toxifilum* primarily by forming fascicles and having sheaths that sometimes envelop more than one trichome. Based on these findings, we propose *Eurychoronema bolivianum gen. & sp. nov*. Additionally, the 16S rRNA gene phylogenies and identity, alongside morphological and 16S-23S ITS analyses permitted us to transfer the Leptolyngbyaceae genus *Radiculonema* into *Leptolyngbya*. *Radiculonema* was nested within the *Leptolyngbya* clade and both genera shared 95.1% 16S rRNA identity. There was also no morphological difference between these two genera. We also demonstrate that conclusions based on secondary structures of the 16S-23S ITS region alone can be interpreted in different ways.

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INTRODUCTION

Extreme environments are a rich source for discovering new cyanobacterial taxa (Genuário *et al*., 2019). These environments exhibit conditions beyond the tolerances of most known species, including extremes in temperature, pressure, gas concentrations, radiation, pH, water availability and solute compositions (Van Den Burg, 2003). Historically, extreme environments were perceived as lifeless until the late nineteenth century, when the isolation of organisms from salted fish challenged this notion (Farlow, 1880). Subsequently, microbial isolation from the highly saline waters of the Dead Sea in 1936 further altered this perception (Rampelotto, 2013). Currently, it is known that these habitats are biologically rich due to the adaptations of organisms to their challenging conditions over evolutionary timescales (Rampelotto, 2013).

Organisms capable of thriving in such extreme conditions are classified as extremophiles (Genuário *et al*., 2019; Horikoshi *et al*., 2011). Research into extremophilic organisms has predominantly focused on taxonomic and physiological investigations, leading to the discovery of novel species and a deeper comprehension of their metabolic adaptations to environmental stresses (Genuário *et al*., 2019). Accordingly, recent studies have described numerous extremophile cyanobacteria taxa from various parts of the world, such as *Copelandiella* J.R. Johansen, Kaštovský & M.U.Akagha, and *Reofilinostoc* G.S. Hentschke, G.Garduño-Solórzano & M.Martínez-García (Kastovský *et al*., 2023; Solórzano *et al*., 2024).

In this context, Bolivia has a wide diversity of extreme environments, such as the Altiplano, and stands out as one of 20 countries with the highest biodiversity on the planet (Ibisch & Mérida, 2003). This great biodiversity is primarily due to the varied landscapes, which includes high mountain plateaus (Altiplano), sub-Andean dry forest, cloud forest (Yungas), Amazon-influenced lowlands and the Chaco basin. These regions are also characterized by diverse climatic conditions, making them suitable for the thriving and diversification of cyanobacteria (Navarro & Maldonado, 2004).

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The Bolivian Altiplano is an extreme environment for life, located at high elevation and low latitude. These conditions result in significant solar irradiation. Consequently, cyanobacteria in this environment, being obligate phototrophs, are exposed to potentially harmful levels of ultraviolet radiation (Fleming & Bebout, 2010). Moreover, the Bolivian Altiplano experiences sub-freezing temperatures and some of the lowest precipitation rates on Earth (Fleming & Bebout, 2010). Despite these harsh conditions, cyanobacteria thrive in small, isolated refugia such as snowmelt-fed lakes. In these high-elevation environments, cyanobacteria face additional stresses, including severe seasonal temperature shifts with periodic freezing, especially during winter. Additionally, dissolved salts from the surface of ice expose benthic cyanobacterial communities to high salinities (Fleming & Bebout, 2010; Vincent, 2000).

These unique conditions make the Bolivian Altiplano a distinctive environment. Therefore, the cyanobacterial communities within these lakes may include many undiscovered taxa that have evolved under such harsh conditions. Although cyanobacterial communities have flourished in these environments (Rouchy *et al.*, 1996), our understanding of this biodiversity remains largely neglected.

The Bolivian phycological literature is very limited and does not cover the wide range of ecosystems characteristic of the Bolivian territory. Currently, the phytoplankton/tychoplankton from the Altiplano and Amazon has received some attention, with literature primarily focusing on taxonomic studies. However, this taxonomy is based mainly on morphological analysis and covers only a small fraction of Bolivia's algal biodiversity (Morales *et al.*, 2008). The few studies that have been conducted on Bolivian cyanobacterial biodiversity mostly focus on the Altiplano region. Daga-Quisbert *et al.* (2023) reported the presence of '*Microcoleus*' populations as a part of a metagenomic study on the microbiome of the salt-lake Pastos Grandes. Another study by Morales *et al.* (2017), examined the Alalay Lagoon in Cochabamba, reporting a Harmful Algal Bloom (HAB) dominated by *Limnospira fusiformis* Nowicka-Krawczyk, Mühlsteinová & Hauer, the toxic cyanobacteria *Anabaenopsis milleri* Woronichin and *Aphanocapsa* sp., which killed fish and birds in this ecosystem in 2016.

Likewise, Fleming & Bebout (2010) studied the cyanobacterial composition of a variety of microbial mats present in three lake systems: Laguna Blanca, Laguna Verde (altitude 4,300 m), and a lake at the top of the cone of the Licancabur volcano (altitude 5,970 m). These lakes and their adjacent geothermal springs had a diversity of environments within a geographically small region (5 km^2). From these sites, they isolated 78 cyanobacterial cultures, and obtained 400 cyanobacterial 16S rRNA gene sequences from environmental genomic DNA. Based on microscopy, culture and molecular analyses, these communities contained many heterocytous nitrogen-fixing cyanobacteria (e.g. *Calothrix, Nostoc* and *Nodularia*), as well as a large number of cyanobacteria belonging to the genus *Leptolyngbya* Anagnostidis & Komárek. More than one-third (37%) of the taxa in this study were new species and 11% represented new genera (Fleming & Bebout, 2010).

Laguna Chalviri, a saline and alkaline lake located near the towns of Mina Illimani and Lacachaca in Potosí, Bolivia, is another example of an extreme environment where cyanobacteria thrive. Situated in the Bolivian Altiplano, the lagoon lies at an altitude of 4,419 m. In this article, we propose the description of a new extremophile cyanobacteria isolated from Laguna Chalviri. Additionally, our analyses allowed us to review the taxonomic status of the genus *Radiculonema* R.F.S.Luz, Kaštovský, J.R.Johansen & V. Gonçalves (Leptolyngbyales).

MATERIAL AND METHODS

Sampling isolation and morphological analysis

A sample of a *Nostoc*-like biomass was collected from the margin of Laguna Chalviri, a saline alkaline lagoon in the Bolivian Altiplano (22°32.1167'S, 67°38.7833'W). At the time of sampling the pH was 8.53, the salinity was 0.7%, the conductivity >19.99 mS/cm^2 and the temperature 5°C. The sample was immediately enriched in Falcon tubes, with Z8 liquid medium (Kotai, 1972). Upon arrival at the laboratory, the *Nostoc* biomass was inoculated in a tissue culture flask with the same medium. From this culture, homocytous filaments grew. Subsequently, a single filament was picked using a tapered Pasteur pipette and inoculated into a new flask also with Z8 medium.

This isolated strain was included in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) under the code LEGE 231228 and is currently maintained at 19°C, with cycles of 12:12 hours light/dark cycle (25 µmol photons m^{-2} s^{-1}).

For the morphological analysis, the arrangement of the filaments, the shape, content and dimensions of vegetative cells were assessed. Additionally, the quantity of trichomes per sheath, the existence or lack of sheaths, constrictions at the cross-walls and the presence of hormogonia were documented. Twenty cells were measured from various positions on the slide. Finally, an aliquot of these culture was lyophilized and preserved in the University of Porto Herbarium under the code PO-T5170.

DNA extraction, PCR amplification and sequencing

The trichomes were harvested from the culture, and genomic DNA was extracted using the NZY Microbial gDNA Isolation Kit (NzyTech), according to the manufacturer's protocol. For 16S rRNA gene amplification, specific cyanobacteria primers 27SF (Neilan *et al.*, 1997), and 23SR (Taton *et al.*, 2003) were used.

The PCR mix consisted of 4× Reaction Buffer, 2 mM MgCl2, 150 µM of each deoxynucleotide triphosphate, 20 pmol of each primer, 0.1 U of GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), 5 mg ml^{-1} of bovine serum albumin (BSA) and 2 µl of DNA template. PCR amplification followed these conditions: initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 2 min, then an additional 25 cycles of

denaturation at 92°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 7 min.

The DNA bands of the expected size (approximately 2000 nucleotides) was excised from the gel and purified using the NZYGelpure kit (Nzytech, Portugal). Finally, the purified product was sequenced with primers 27SF, 359 F, 781 R, 1114 F and 23SR (Lane, 1991; Neilan *et al.*, 1997; Nübel *et al.*, 1997; Taton *et al.*, 2003). Sanger sequencing was conducted commercially (GATC Biotech, Ebersberg, Germany). The nucleotide sequences obtained were manually inspected for quality and assembled using the Geneious Prime 2023.2.1 software (https://www.geneious.com). Before phylogenetic analyses, the sequences were checked for possible chimera formation using the DECIPHER software 2.27.2 (Firtrh *et al.*, 2009). The sequence was deposited in GenBank under the accession number PP918021.

Phylogenetic analysis

For the phylogenetic analysis, the 16S rRNA gene sequence of LEGE 231228 was aligned with 154 sequences of Pseudanabaenales, Acaryochloridales, Nodosilineales, Oculatellales, Leptolyngbyales and other sequences retrieved from GenBank using BLAST. The final alignment consisted of 1,152 nucleotides, with 962 informative sites.

For all analyses, the sequences were aligned using MAFFT (Katoh *et al.*, 2002) and the outgroup used was *Gloeobacter violaceus* PCC 8105 (AF132791). The phylogenetic trees were built using Maximum Likelihood (ML) and Bayesian Inference (BI) analysis. The GTR+G +I evolutionary model was selected using IQ-Tree online version v1.6.12 (Trifinopoulos *et al.*, 2016). The robustness of ML tree was estimated by bootstrap using 1000 replications in IQ-Tree. The Bayesian tree using MrBayes v.3.2.7a (Ronquist *et al.*, 2012) was constructed in two independent runs, with four chains each, for 5×10^6 generations, burn-in fraction set to 0.25, sample frequency 1000, in Cipres Gateway (Miller *et al.*, 2010). Visualization of these trees were performed using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

The 16S rRNA identity (*p*-distance) matrices were generated using MEGA11 (Tamura *et al.*, 2021). The 16S-23S ITS secondary structures of D1-D1', V2, Box B and V3 helices were folded using MFold (Zuker, 2003) as described by Lukesova *et al.* (2009).

RESULTS

The phylogenetic tree (Fig. 1) separated the orders Pseudanabaenales, Acaryochloridales, Nodosilineales, Oculatellales and Leptolyngbyales. These phylogenies also indicated that within the Nodosilineales lineage, our isolated strain LEGE 231228 formed a cluster with six other strains, which are currently either unidentified or identified as '*Leptolyngbya*' or '*Pseudanabaena*'. This clade was strongly supported (ML = 100%, BI = 1.0) and was sister to a cluster containing *Toxifilum* and *Sodaleptolyngbya* strains. This positioning of the clade containing LEGE 231228 suggested that it should be described as a new genus.

The 16S rRNA gene identity analysis supported the description of this new genus and species. Within the LEGE 231228 clade, the 16S rRNA identity values were high, ranging from 97.0% to 99.6%. When comparing this clade with *Toxifilum* and *Sodaleptolyngbya* clades, the maximum values did not surpass 94.3% and 93.6%, respectively (Table S1).

Regarding the 16S-23S ITS secondary structures, the D1-D1' helices of both LEGE 231228 and *Toxifilum* had seven residues in the 3' side of the basal lateral bulge, with no opposing residues in the 5' side. However, their sequences differed by the substitution from an 'U' in LEGE 231228 (5'CAUCCCA3') to an 'A' in *Toxifilum* (5'CAACCCA3'). After this bulge, LEGE 231228 exhibited a stem with four base pairs followed by a mismatched 'U' on the 3' side of the molecule. In *Toxifilum*, this region consisted of a stem with three base pairs and an 'A' forming the mismatch. The terminal loop was also different when comparing LEGE 231228 with *Toxifilum* (Fig. S1).

The Box B helices (Fig. S2) of LEGE 231228 had a different structure compared to that of *Toxifilum*. LEGE 231228 Box B helices had a mismatch consisting of a 'C' or an 'U' on the 3' side of the molecule after the basal stem. Additionally, another mismatch consisting of a 'G' on the 5' side of the molecule was present in LEGE 231228. In contrast, *Toxifilum* Box B helix did not exhibit these patterns.

In the V2 region, the strain LEGE 231228 had the sequence 5'CCTTCAGACT3' that could not be folded. The V3 helices showed significant variability among all strains, making them unsuitable for genus-level comparisons (Fig. S3).

Morphologically, strain LEGE 231228 differed from *Sodaleptolyngbya* in several aspects. LEGE 231228 forms fascicles, has firm sheaths, lacks aerotopes and its trichomes are tapered. Additionally, LEGE 231228 hormogonia typically consists of single cells. In contrast, *Sodaleptolyngbya* has entangled filaments, diffluent mucilage, aerotopes, cylindrical trichomes and the hormogonia composed of multiple cells (Fig. 2–7). LEGE 231228 also differed from *Toxifilum* in its ability to form fascicles and the occasional presence of more than one trichome enveloped in a single sheath. *Toxifilum*, on the other hand, does not form fascicles and its sheaths envelop only one trichome (Table 1).

Based on the phylogenetic analyses, 16S rRNA gene identity and morphological analyses, LEGE 231228 does not fit in the circumscriptions of *Toxifilum* or *Sodaleptolyngbya*, the phylogenetically closest related genera. Consequently, we propose here the new genus and species: *Eurychoronema bolivianum gen.* & *sp. nov.*, in the order Nodosilineales.

Eurychoronema G.S.Hentschke *gen. nov.*

TYPE SPECIES: ***Eurychoronema bolivianum* G. S.Hentschke**

Forming a well-supported clade sister to the genera *Toxifilum* and *Sodaleptolyngbya*

ETYMOLOGY: *Eurychoronema* is from the Greek 'eurychoro-', meaning 'widely spread' and '-nema', meaning 'filament'.

Fig. 1. 16S rRNA gene ML phylogeny. ML bootstrap values/BI posterior probabilities are indicated at the branches, values below 70% for ML and 0.90 for BI are not shown. The type strain of the newly described taxon is in bold. The genera *Eurychoronema, Leptolyngbya, Tapinothrix* and *Sodaleptolyngbya* are highlighted. Clade not essential for discussion are collapsed.

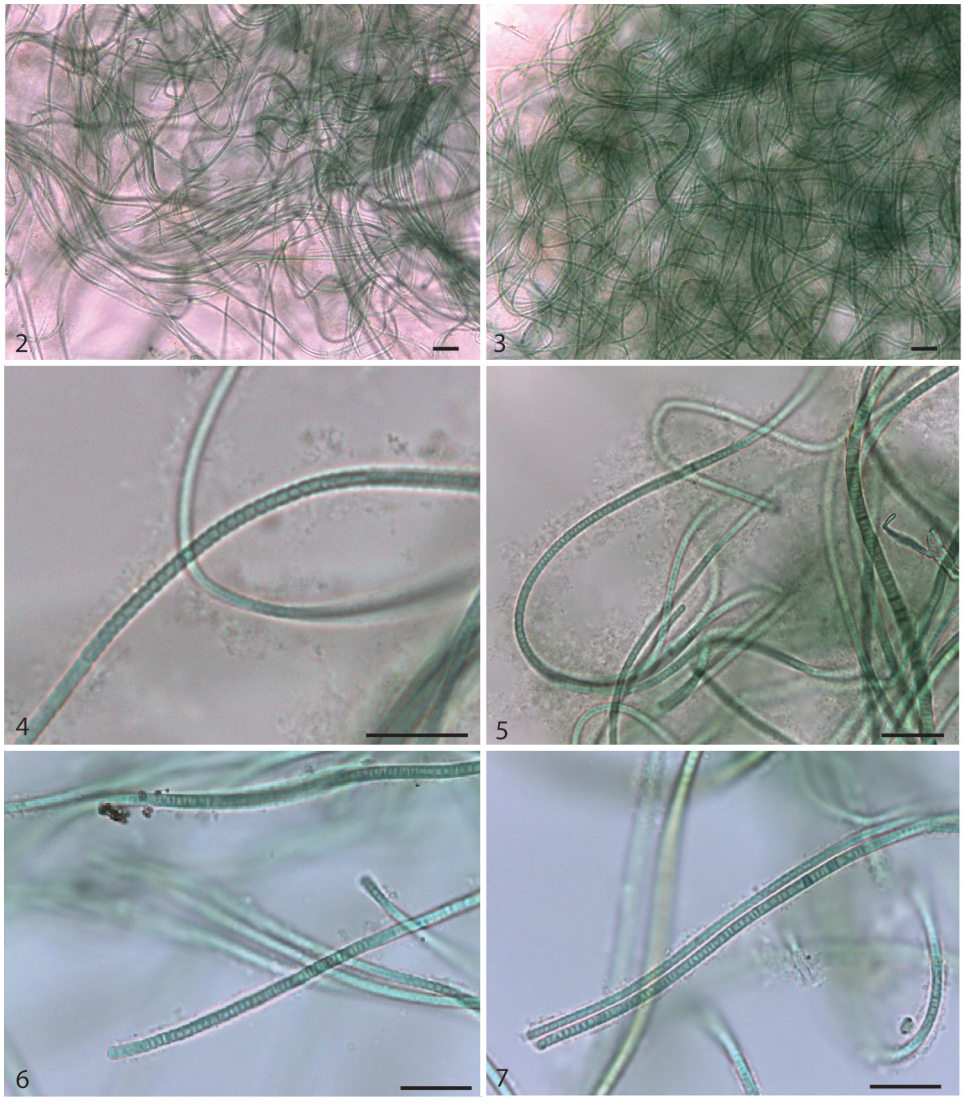
Eurychoronema bolivianum G.S.Hentschke sp. nov.

Figs. 2–7

DESCRIPTION: In culture, filaments long, solitary, entangled (Fig. 2, 3) and sometimes forming fascicles. Trichomes isopolar, constricted (Fig. 4), usually cylindrical or sometimes slightly tapered towards the ends. Sheaths firm, thin, colourless, joined to the trichome, opened at the apical end, usually enveloping only one, very rarely two trichomes (Fig. 7). Apical cells rounded (Fig. 6). Cells isodiametric, shorter or longer than wide; sometimes compressed, becoming almost discoid, while the trichome becomes wider (up to 3um; Fig. 5). Cell dimensions 1.5–2.3 um long, 1.5–2.2 (3um) wide. Cell content homogenous (Fig. 4). Hormogonia usually single celled or rarely in doubles.

HOLOTYPE: PO-T5170, collected August 2023 by Vitor M. Vasconcelos, preserved in a metabolically inactive state (lyophilized) in the University of Porto herbarium (PO), Portugal.

TYPE LOCALITY: 22°32.1167'S, 67°38.7833'W, margin of Laguna Chalviri, a saline alkaline lake. Altitude 4,419 metres. pH = 8.53, salinity = 7.1 ppt (0.7%), temperature = 5°C



Figs. 2–7. *Eurychoronema bolivianum* gen. et sp. nov.
Figs. 2, 3. General habit showing entangled filaments and fascicles of type culture.
Fig. 4. Constricted trichome between cells.
Fig. 5. Detail of trichomes. The arrow indicates a trichome with compressed cells.
Fig. 6. Apex of trichome within sheath.
Fig. 7. Two trichomes within one sheath. Scale bars = 10 µm.

TYPE STRAIN: LEGE 231228, maintained at the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), Matosinhos, Portugal.

HABITAT: Isolated from a *Nostoc* microcolony growing at the margin of a saline alkaline lake.

ETYMOLOGY: The specific epithet *bolivianum* is derived from 'Bolivia' where the sample was collected.

OBSERVATIONS: In the new genus clade, the entries '*Pseudanabaena*' SCSIOS3, Uncultured cyanobacterium TDNPwbc972511123, Pseudanabaenaceae cyanobacterium CENA319, Oculatellaceae cyanobacterium N15MA1Bcl1 and '*Leptolyngbya*' HA4233MV11co2 are probably distinct species from *Eurychoronema bolivianum gen. & sp. nov.* Considering that we do not have access to these strains, we cannot describe them as new taxa.

Our phylogenies (Fig. 1), also indicated that within the Leptolyngbyales, the *Leptolyngbya* cluster is phylogenetically well supported (ML = 100, BI = 1) and consisted of six distinct clades that have unsupported relationships to each other. The genus '*Radiculonema*' (type strain BACA0731, clade 2) was positioned within this *Leptolyngbya* cluster.

The 16S rRNA gene identity analysis showed that '*Radiculonema*' had a minimum identity of 95.1% when compared to other *Leptolyngbya* strains. (Table S2). Specifically, '*Radiculonema*' shared 95.2% and 95.1% 16S rRNA identity with the representative strains of *Leptolyngbya: Leptolyngbya boryana* CCALA 1076 and PCC 6306, respectively. Also, the closest phylogenetically related genus to the *Leptolyngbya* cluster was *Tapinothrix*, but both genera shared only 91.6–93.3% of 16S rRNA gene identity.

Comparing both genera, the secondary structures of 16S-23S ITS analyses (Figs. 8, 9, Figs. S4 and S5) revealed that the D1-D1' helix region was the most conserved among all the studied sequences. The D1-D1' helix of '*Radiculonema*' exhibited a structure similar to *Leptolyngbya* helices, and all of them were markedly different from *Tapinothrix*. Specifically, the D1-D1' helices of *Leptolyngbya* cluster (including '*Radiculonema*') featured a lateral bulge without opposing residues at the base. This lateral bulge consisted of a sequence of five or six residues, comprising either the sequences 5'CAACU3' (CCALA 1076, LEGE 18517, ANTLPR22) or 5'CAUCUC3' ('*Radiculonema*', FBCCA 1479 and 1478). In contrast, the lateral bulge of *Tapinothrix* also contained the sequence 5'CAUCU3', but with the addition of three other residues, totalling eight. Furthermore, the lateral bulge of *Tapinothrix* was opposed by three other residues (5'AAU3').

Table 1. Morphological and habitats comparisons between *Eurychoronema bolivianum gen. et sp. nov.*, *Toxifilum* and *Sodaleptolyngbya*.

	Eurychoronema bolivianum gen. et sp. nov. LEGE 231228	*Toxifilum* (Zimba *et al.*, 2017)	*Sodaleptolyngbya* (Cellamare *et al.*, 2018)
Thallus	Filaments solitary, entangled or forming fascicles	Filaments rarely solitary, usually forming mats	Filaments solitary or entangled
Trichomes	Slightly tapered towards the ends or not	Tapered towards the ends	Cylindrical, not tapered towards the ends
Trichomes constriction	Yes	Yes	Slightly constricted
False branching	No	No	No
Sheaths	Firm, thin, enveloping only one or very rarely two trichomes	Thin, enveloping one trichome	Diffluent, thin, enveloping one trichome
Cell shape	Isodiametric, shorter or longer than wide; sometimes compressed, becoming almost discoid while the trichome becomes wider	Cylindrical	Longer than wide
Cell content	Homogenous	Granulose	With aerotopes. Refractive granule in apical cells.
Cell measurements (um)	1.5–2.3 long x 1.5–2.2 (3) wide	Up to 3.1 long x 0.5–2.5 wide	1.5–2 wide
Hormogonia	1–2 cells	Not described	of many cells
Habitat	Saline-alkaline (pH 8.5, Salinity 7.1 ppt 0.7%)	Salt marshes	Saline-alkaline

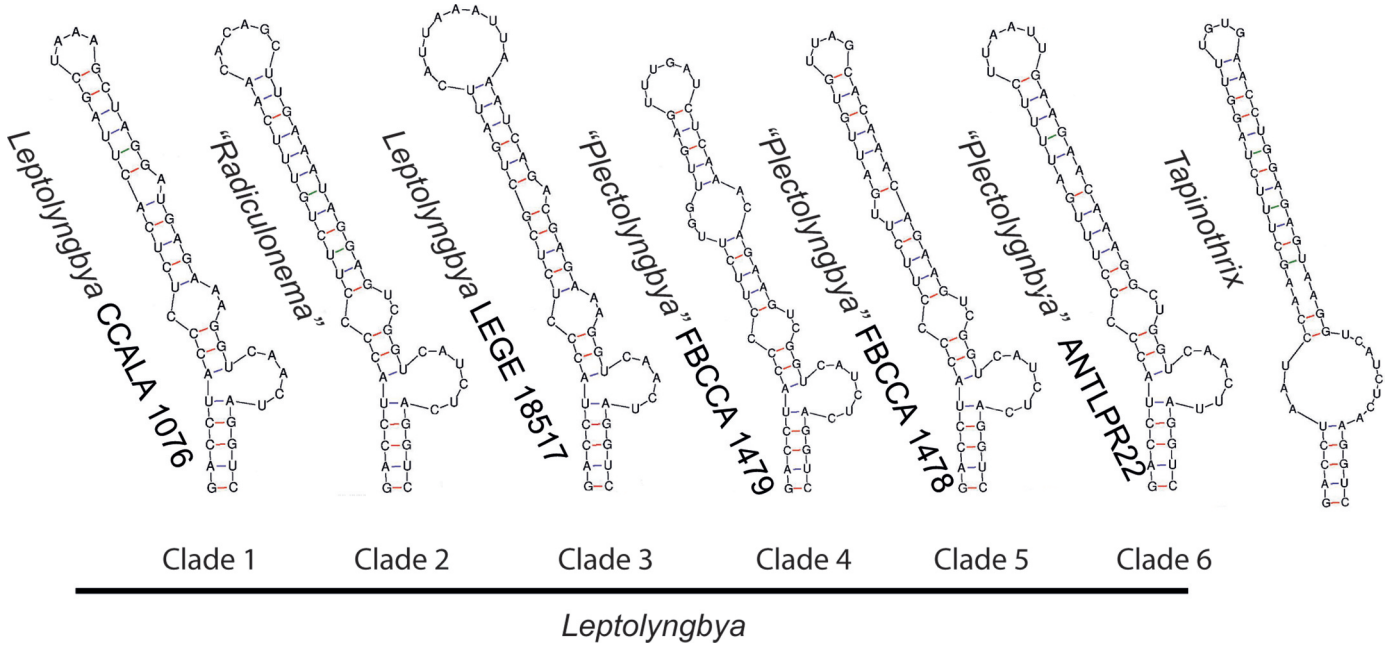


Fig. 8. 16S-23S ITS D1-D1′ helices of *Leptolyngbya* (clades 1, 3–6) *"Radiculonema"* (clade 2), and *Tapinothrix*.

Additionally, for all *Leptolyngbya* D1-D1′ helices, following the lateral bulge, the sequence 5'ACC 3' at the 5' side of the molecule was succeeded by a mismatch characterized by a 'C'. On the opposite side (3' side) of the molecule, this mismatch region comprised 'AA' (CCALA 1076, LEGE 18517) or 'UC' ('*Radiculonema*', FBCCA 1478, 1479 and ANTLPR22). In *Tapinothrix*, however, this mismatch region differed, consisting of two 'A' on each side of the helix. The Box B helices of '*Radiculonema*' and *Leptolyngbya* were also highly similar to each other, and both differed from *Tapinothrix* (Fig. 9). In both '*Radiculonema*' and *Leptolyngbya* helices, a mismatch was consistently observed at the base, consisting of an 'A' on the 5' side and 'CC' on the 3' side. However, in *Tapinothrix*, this mismatch region comprised 5'CU3' on the 5' side and lacked opposing residues on the 3' side of the helix. Following this region, the *Leptolyngbya* and *'Radiculonema'* helices displayed similar sequences such as 5'CUCA3' (CCALA 1076, '*Radiculonema*', and FBCCA 1478), 5'CUAA3' (LEGE 18517), 5'CUCG3' (FBCCA 1479), or 5'UUCA3' (ANTLPR22) on the 5' side. In these sequences, the final 'A' or 'G' invariably formed mismatches. These sequences were absent in *Tapinothrix*. The V2 and V3 helices were highly distinct among all the studied sequences, and therefore could not be used to compare genera (Figs. S4, S5). However, the V2 helix of the strain *'Plectolyngbya'* ANTLPR22 was very similar in structure to *Tapinothrix*.

Morphologically, '*Radiculonema*' is identical to *Leptolyngbya* with entangled filaments, false branching, isopolar and constricted trichomes, with firm sheaths. Both '*Radiculonema*' and *Leptolyngbya* differ from *Tapinothrix*, primarily by the heteropolar nature of the latter genus (Table 2).

Considering the 16S rRNA gene phylogeny, 16S sequence identity, 16S ITS rRNA secondary structures and morphological analyses, we

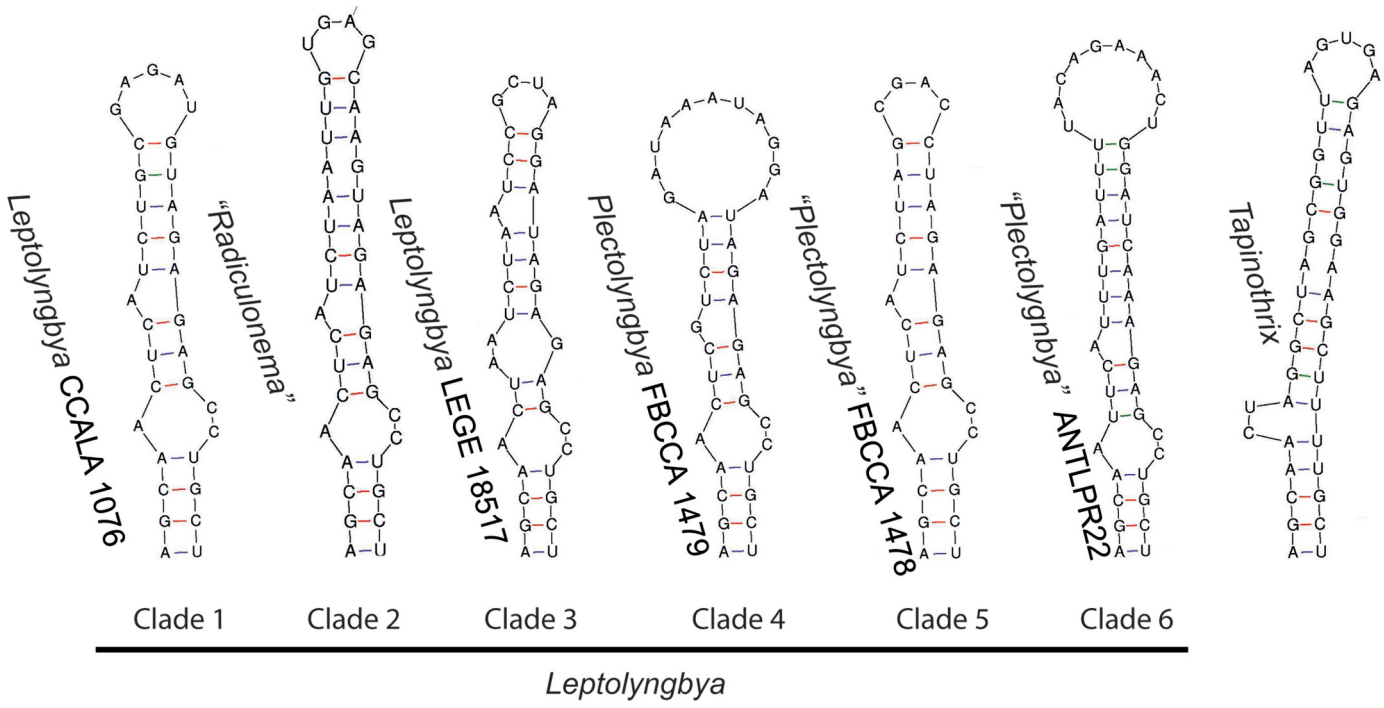


Fig. 9. 16S-23S ITS Box B helices of *Leptolyngbya* (clades 1, 3–6) *"Radiculonema"* (clade 2), and *Tapinothrix*.

Table 2. Morphological and habitat comparisons between *'Radiculonema'*, *Leptolyngbya* and *Tapinothrix*.

	Leptolyngbya (Kastovský *et al.*, 2023)	*"Radiculonema"* (Luz *et al.*, 2023)	*Tapinothrix* (Bohunická *et al.*, 2011)
Thallus	Solitary filaments or entangled	Entangled filaments	Spreading
Trichomes	Isopolar, not or slightly attenuated towards the ends.	Isopolar, not tapered towards the ends	Isopolar and tapering to both apices when young. Fragmenting centrally to become heteropolar with maturity, swollen at the base, tapered towards the ends. Ending in a hair
Trichomes constriction	Constricted or not	Yes	Constricted in basal and median regions. Not constricted towards the apices
False branching	Yes	Yes	No
Sheaths	Firm	Firm	Firm, rough
Cell shape	Isodiametric, shorter or longer than wide. Sometimes irregularly swollen ('Arhtronema')	Isodiametric, shorter or longer than wide	At the base, shorter than wide. At the apices cylindrical
Cell content	Without aerotopes, rarely granulated	Not described	Without aerotopes, granulated, often with a large central granule
Cell measurements (um)	1.5–3.2 wide	1.2–7.4 long x 2.5–3.8 wide	0.75–1.5 long x 0.75–4 wide
Habitat	Freshwater, marine, thermal springs, terrestrial or endogloeic.	Freshwater	Terrestrial

believe there is no reason to diffirententiate the genus '*Radiculonema*' from *Leptolyngbya*. Consequently, we transfer the type species *R. aquatica* R.F.S.Luz, Kaštovský, J.R.Johansen & V.Gonçalves to *Leptolyngbya* as *L. aquatica comb. nov.*

Leptolyngbya aquatica (R.F.S.Luz, Kaštovský, J.R.Johansen & V.Gonçalves) G.S.Hentschke *comb. nov.*

BASIONYM: *Radiculonema aquaticum* R.F.S. Luz, Kaštovský, J.R. Johansen & V.Gonçalves 2023: pg. 6–9, Fig. 4.

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DISCUSSION

Eurychoronema bolivianum gen. & sp. nov. (type strain LEGE 231228) formed a clade with high sequence dissimilarity and strong phylogenetic supporting its new genus status. The genus *Eurychoronema* was sister to *Toxifilum* and *Sodaleptolyngbya*. This relationship between *Eurychoronema bolivianum*, *Toxifilum* and

Sodaleptolyngbya is interesting as these taxa are typical from alkaline and saline environments (Cellamare *et al.*, 2018; Zimba *et al.*, 2017).

Regarding the classification of the new genus at the order level, Strunecký *et al.* (2023) stated that *Toxifilum* and *Sodaleptolyngbya* are within the order Oculatellales, but in our tree, the three genera were within the Nodosilineales. Indeed, the three orders, Oculatellales, Nodosilineales and Leptolyngbyales, are all closely related (Strunecký *et al.*, 2023). In Strunecký *et al.* (2023), the 16S rRNA gene phylogeny, which separates these three orders, was built using topological constraints. We did not use a topological constraint in our 16S rRNA gene phylogeny, and because of these methodological differences, we would not expect an identical tree topology. Consequently, at this moment, we consider *Eurychoronema, Toxifilum* and *Sodaleptolyngbya* provisionally in the Nodosilineales, but clearly these orders need further analyses and possible revisions. Other genera that may need to be revised, regarding their phylogenetic positions, are *Metis* Konstantinou & Gkelis (Oculatellales) and *Heteroleibleinia* Hoffmann (Leptolyngbyales) that also appeared among the Nodosilineales in our analysis.

Beyond the unresolved relationships among Oculatellales and Nodosilineales, and Leptolyngbyales, our phylogeny also showed that within the Nodosilineales, the two existing families were intermixed. Because of this, it was not possible to precisely attribute *Eurychoronema bolivianum* to either the Nodosilineaceae or Cymatolegaceae. Probably, in order to know the precise taxonomical classification of the new taxon at the family level, a genomic study may be necessary. Despite that, *Eurychoronema bolivianum* proved to be a distinct taxon from any other cyanobacteria.

Within the *Eurychoronema bolivianum* clade, it was also evident that more than one species exists based on percent identity threshold for the 16S rRNA gene (Yarza *et al.*, 2014). The strain N7-MA2co4, for instance, shared only 97.2% of identity with the type *Eurychoronema bolivianum* and is probably a different species. Further studies are needed to describe the different *Eurychoronema* species.

This proposal of the new genus *Eurychoronema* and the synonymizing of *'Radiculonema'* into *Leptolyngbya aquatica comb. nov.* were grounded on the monophyletic concept of genera (Johansen & Casamatta, 2005). If '*Radiculonema*' was considered as a distinct genus, *Leptolyngbya* would be paraphyletic. Also, we used the 16S rRNA gene identity parameters established by Yarza *et al.* (2014), which stated that strains with less than 94.5% identity should not be classified within the same genus. '*Radiculonema*' does not meet this threshold and share more than 95% of 16S rRNA gene identity with *Leptolyngbya. Eurychoronema* forms a distinct clade and shares less than 94.5% of identity with any other genera.

Eurychoronema bolivianum (LEGE 231228) was isolated from a *Nostoc* biomass sampled in a Bolivian alkaline saline lake. We do not have physical-chemical data from the environments of the remnant strains within the *Eurychoronema bolivianum* clade, but in a NCBI search it is possible to assign them to a variety of environments such as seagrass meadow ("*Pseudanabaena* SCSIOS3), wetlands (Uncultured cyanobacterium TDNPWbc972511123) and tree leaves (Pseudanabaenaceae cyanobacterium CENA319). Based on available data, we assign *Eurychoronema bolivianum* as associated with *Nostoc* microcolonies and tolerant to saline and alkaline environments, but we highlight the need for more studies on this genus, as well as on *Toxifilum* and *Sodalinema*, in order to elucidate their possible relation to various environments.

The geographical distribution, based on the strains of the genus *Eurychoronema*, appears to be remarkably widespread, found in the Bolivian Altiplano, the Brazilian Atlantic Rainforest, Spain, Nigeria and Hawaii (USA).

Regarding the proposed synonymizing of the genus *Radiculonema*, we found that the strain '*Radiculonema aquaticum*' BACA0731 fell within a *Leptolyngbya* clade with strong support. Furthermore, the 16S rRNA gene identity analysis supported the phylogenetic results. Morphologically, Luz *et al.* (2023) distinguished and separated '*Radiculonema*' from *Leptolyngbya* based on the presence of false branching in '*Radiculonema*' and its absence in *Leptolyngbya*. However, a revised description of *Leptolyngbya* (Kastovský *et al.*, 2023) included false branching as a characteristic. Therefore, this character cannot be used anymore as a distinguishing feature between '*Radiculonema*' and *Leptolyngbya*. Additionally, even if '*Radiculonema*' and *Leptolyngbya* exhibited any morphological difference, these differences could hardly override the phylogenetic results, where the description of '*Radiculonema aquaticum*' would make, at least, *Leptolyngbya* paraphyletic.

Recently, *Leptolyngbya* underwent a thorough revision, and one genus was already transferred into its circumscription. In Kastovský *et al.* (2023), the authors transferred '*Plectolyngbya*' into *Leptolyngbya* primarily based on phylogenetic analysis. Their phylogeny demonstrated that '*Plectolyngbya*' belonged to the same clade as the representative strains *Leptolyngbya* CCALA1076 and PCC6306. This conforms with our results. In the present paper, we employed the same logical arguments as used for the above new combinations. Consequently, the genus '*Radiculonema*' could not be sustained as a distinct taxon.

The use of 16S-23S ITS secondary structure analysis is currently widely employed in polyphasic descriptions of new cyanobacterial taxa and was used to separate *'Radiculonema'* from *Leptolyngbya* (Luz *et al.*, 2023). However, we have some reservations regarding the use of secondary structure analyses at the genus level. Our opinion is detailed below and is also based on evidence from previous studies (Oliveira *et al.*, 2024), but primarily on the subjective interpretation involved in evaluating secondary structures. In our comparisons, the 16S-23S ITS secondary structures of '*Radiculonema*' and *Leptolyngbya* can have distinct alternate interpretations. Luz *et al.* (2023), considered that the helices of '*Radiculonema*' and *Leptolyngbya* are different enough to produce a new genus. In our opinion, '*Radiculonema*' and *Leptolyngbya* had similar D1-D1' and Box B helices patterns, quite distinct from their sister genus *Tapinothrix*, which Luz *et al.* did not compare in their article.

This paradox was possible because current comparisons involving secondary structures do not rely on statistical methods, unlike phylogenetic, which uses statistical algorithms, and sequence identity analyses, which are measurable and

comparable. In our opinion, this makes the ITS secondary structures analysis subjective, and this leads to results that might be biased to agree with the phylogenies. The questions: 'how much secondary structures can vary within a genus? How different they must be to separate two genera? Is it possible for secondary structures to not be in agreement with the phylogenetic and identity results?' have no answer, and to answer that a detailed revision must be performed to check whether the 16S-23S rRNA ITS secondary structures are really important for taxonomy at the genus level. An example in which secondary structures are uninformative for distinguishing genera are the D1-D1' helices of *Acaryochloridopsis* (Acaryochloridales) and *Vasconcelosia* (Nodosilineales), which are very similar even with these taxa are placed in different orders (Hentschke et al. 2024; Oliveira *et al*., 2024).

In conclusion, this paper introduces the new taxon *Eurychoronema bolivianum gen. & sp. nov*. and proposes the transfer of '*Radiculonema*' into *Leptolyngbya*. Additionally, we discuss issues related to the use of 16S-23S ITS secondary structures in the description of new cyanobacterial genera, and emphasize the necessity for further studies on extreme environments.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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