Fottea, Olomouc, 24(1): 99–108, 2024
DOI: 10.5507/fot.2023.014

Description of *Azorothrix ramosa* gen. et sp. nov. (Tolypotrichaceae, Cyanobacteria), a new Tolypotrichaceae from Atlantic oceanic islands

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Abstract: The Tolypotrichaceae is a well–defined family within the filamentous heterocyst–forming cyanobacteria. However, the morphological and genetic similarity of some of its genera is very high, making the taxonomic assessment and the description of new taxa in this family challenging. Here we describe six strains of *Tolypothrix*–like cyanobacteria that were isolated from freshwater lakes and streams from São Miguel Island (Azores Archipelago, Portugal), and deposited in the BACA collection. All strains showed morphological similarities, restricting them to the same taxa, with the phylogenetic analysis revealing a distinct position in the Tolypotrichaceae clade. A deeper analysis of the morphological, ecological, and genetic (16S rRNA and 16S–23S rRNA internal transcribed spacer) data, and comparison with known genera in the Tolypotrichaceae, allowed the description of the taxon as *Azorothrix ramosa* gen. et sp. nov. in the Tolypotrichaceae family.

Key words: Atlantic Ocean, Azores, Freshwater, New genus, Nostocales, Oceanic islands, Phylogeny, 16S rRNA, 16S–23S rRNA ITS

Introduction

Cyanobacteria in the North Atlantic islands have been studied since the 19th century (Cordeiro et al. 2020a), starting in 1874 in the Azores Archipelago by Moseley (1874). However, cyanobacteria diversity in the Azores is still poorly known despite the long history of cyanobacteria records and the habitat diversity of these islands (Luz et al. 2020; Luz et al. 2022). The recently created Bank of Algae and Cyanobacteria of the Azores (BACA) holds a significant collection of cultured cyanobacteria strains from different ecosystems (lakes, streams, terrestrial and thermal) of the Azores, allowing their detailed morphological and genetic characterization (Luz 2018; Xavier et al. 2018; Cordeiro et al. 2020b). Recent results by Cordeiro et al. (2020b) revealed that several strains in the BACA collection could be new genera, mostly for the Nostocales order, three of them forming a monophyletic clade phylogenetically related to the Tolypotrichaceae Hauer et al. (Cordeiro et al. 2020b).

The Tolypotrichaceae family was proposed by Hauer et al. (2014) based on morphological characters and phylogenetic analysis of several cultured strains. Their work addresses a previously known problem of wrongly identified cyanobacteria such as *Tolypothrix* Kützing ex Bornet et Flahault, which are taxonomically problematic and misinformative in phylogenetic studies. Hauer et al. (2014) demonstrated that the Tolypotrichaceae could be distinguished as a unique family with several well–characterized genera, such as *Spirirestris* Flechtner et Johansen, *Rexia* Casamatta, Gomez et Johansen, *Coleodesmium* Borzì et Geitler, and more recently, *Dactylothamnos* Komárek et al. and *Kryptousia* Alvarenga et al. concerning morphology, ecology, and genetics. Nevertheless, *Tolyphothrix* is still a polyphyletic genus with high morphological polymorphism among identified strains (Hauer et al. 2013; Hauer et al. 2014).

As for all Cyanobacteria, the major challenge of the Tolypotrichaceae genera is their morphological polymorphism, making morphological identification

of both genera and species problematic. Also, the DNA similarity (p–distance) among morphologically close genera in the Tolypotrichaceae is quite high, making phylogenetic analysis more difficult (Flechtner et al. 2002). The same can be applied to *Dactylothamnos* (Komárek et al. 2015) or *Kryptousia* (Alvarenga et al. 2017). Indeed, Komárek et al. (2015) suggested that the strains used to describe *Kryptousia microlepis* could belong to *Dactylothamnos*, something that was never validly published and was later discarded by Alvarenga et al. (2017) and described as *Kryptousia* species. Alvarenga et al. (2017) supported this assessment on the phylogenetic position between *Kryptousia* strains and the distinctive ecology of *Dactylothamnos* and *Kryptousia* species. The role of *Streptostemon* Sant'Anna, Azevedo, Kaštovský et Komárek in the Tolypotrichaceae family is not considered here due to its problematic position in the family (Hentschke et al. 2016).

In this work, we applied a polyphasic approach, considering morphological, genetic, and ecological characteristics of six cultured cyanobacterial strains from freshwater ecosystems (lakes and streams) from the Azores to propose a new genus, *Azorothrix* gen. nov., and one species, *Azorothrix ramosa* sp. nov. All taxonomic treatment was made according to the International Code of Nomenclature for algae, fungi, and plants.

Materials and Methods

Site description and sampling. The Azores archipelago is an oceanic group of islands in the middle of the North Atlantic Ocean, roughly 1500 km from Europe and 1900 km from America (Fig. 1), with an oceanic temperate climate (Hernández et al. 2016). The six studied strains were isolated from five locations in São Miguel Island, four from lakes and two from a stream (Table 1).

Environmental data to characterize sampling sites was obtained during sampling or retrieved from previous studies (Table 1). Temperature (ºC), pH, conductivity (µS cm^{-1}), and dissolved oxygen ($mg.L^{-1}$) were recorded in situ using a multiparameter probe Horiba U–52 (Horiba, Pasadena, TX, USA). The trophic state of the lakes follows Cordeiro et al. (2020c), and hydromorphological data was retrieved from Pereira et al. (2014).

Isolation and culture. Field samples were grown on $BG–11_0$ (BG–11 medium without nitrogen) (Allen, 1968) for two weeks in 50 mL Erlenmeyer Flasks under 14:10 light:dark photoperiod. Growing colonies were then picked by pipette and transferred to $BG–11_0$ agar plates (1% agar). The target cyanobacteria were then isolated by repeating streaks in new agar plates (Rippka, 1988). Isolated strains were deposited in BACA, maintained in a 14:10 light:dark cycle (under 10–40 µmol photons m^{-2} s^{-1}) photoperiod at 19 °C.

Morphological characterization. Strains were characterized using a Leica DM4 B microscope with Digital Camera Leica MC 190 HD (Leica, Germany). At least 50 trichomes and three cells per trichome were examined for cell dimensions and descriptions. The cultures were examined at two weeks and two months of growth for morphology and branching characteristics description. Two–month agar streak cultures were photographed and examined using a Stemi 2000–C with an Axiocam 105 color using the ZEN 3.0 (blue edition) (Carl Zeiss™, Germany).

DNA extraction, 16S rRNA and 16S–23S rRNA ITS gene amplification, and sequencing. Fresh cultures were used for DNA extraction with the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the protocol recommended by the manufacturer for gram–negative bacteria. For the 16S rRNA and 16S–23S rRNA ITS region amplification, the primers 27F and 23S30R (Table 2) were used in a Polymerase chain reaction (PCR) with a total volume of 25 µL containing 1× PCR Buffer, 2 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (Thermo Fisher, Waltham, MA, USA), 0.5 pmol of each primer, 5–10 ng of DNA and 1.5 U of Supreme NZYTaq II DNA polymerase (Nzytech, Portugal). PCR conditions followed Taton et al. (2003), and thermal cycling was carried out in a ProFlex™ 3 × 32–well PCR System (Thermo Fisher, USA).

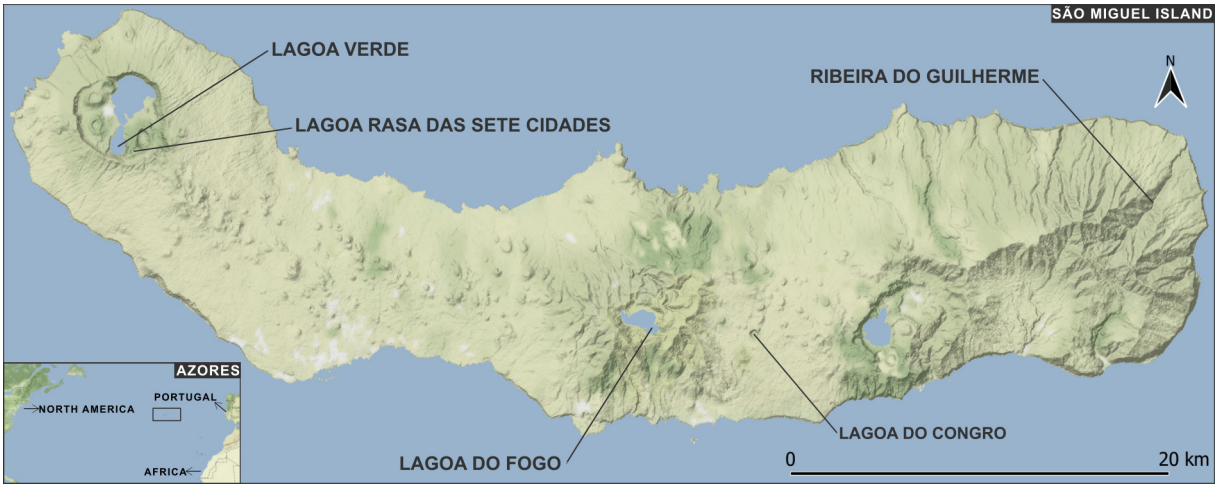

Fig. 1. Sample site locations of the six isolated strains in São Miguel Island in the Azores Archipelago, Portugal. Lagoa Verde (BACA0043), Lagoa Rasa das Sete Cidades (BACA0028), Lagoa do Fogo (BACA0147), Lagoa do Congro (BACA0066), Ribeira do Guilherme (BACA0093 and BACA0098). Base map retrieved from OpenStreetMap®, licensed under the Open Data Commons Open Database License by the OpenStreetMap Foundation.

PCR amplification products were visualized by electrophoresis in 0.8% agarose gel, in 0.5 × TBE (Tris–Borate–EDTA) buffer and stained with SYBR™ SAFE. Amplified bands were removed from the gel using a clean scalpel and then purified using NZYGelpure (Nzytech, Portugal). Sequencing of the 16S rRNA and 16S–23S rRNA ITS region was done by Macrogen Ltd. (Madrid, Spain) using the 27F, 781F, 781R, NITS_F, and 23S30R primers (Table 2).

Phylogenetic analysis of the 16S rRNA. The sequences of the new genera were aligned with 198 sequences retrieved from GenBank using BLAST, with the addition of reference strains of cyanobacterial genera. The sequences were aligned using MAFFT v7.520 with the G–INS–i method (Katoh & Standley 2013), with a final alignment containing 1107 informative sites. The best–fit nucleotide model was assessed using ModelFinder (Kalyaanamoorthy et al. 2017), according to BIC, with the selection of the K3P+I+G4.

Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.2.7a on XSEDE (Ronquist et al. 2012) through the CIPRES Science Gateway, and Maximum likelihood (ML) using IQ–Tree online version v1.6.12 (Trifinopoulos et al. 2016), respectively, using *Chroococcidiopsis thermalis* PCC 7203 and *Oscillatoria princeps* CCALA 1115 as outgroups. The BI was carried out with 1.0×10^7 generations, with two runs of four Markov chains, with custom parameters (temp=0.0175), sampling every 1000 generations and a 0.25 burn–in rate (the final average standard deviation of split frequencies was 0.037568) using the GTR+GAMMA+I model. The ML analysis was carried out using the K3P+I+G4 model with 1000 ultrafast bootstrap replicates (Hoang et al. 2017). Trees were visualized using FigTree v1.4.4 (Rambaut, 2007), and the final composite tree from Maximum likelihood with posterior probabilities values for BI was re–drawn using Inkscape v1.2.

P–distance calculations. For the 16S rRNA p–distance, Tolypotrichaceae genetic information from type species was selected (only one sequence was chosen if more than one was available, choosing the first made available in GenBank). Some OTUs outside the Tolypotrichaceae were selected for a broader analysis of the 16S rRNA p–distance delimitations values currently used in the Nostocales. Sequences were aligned using MAFFT v7.520 with the G–INS–i method (Katoh & Standley 2013), and p–distance calculation for both regions was done using MEGA 11.0.13 (Tamura et al. 2021).

Table 2. Primer sequences used for PCR and sequencing.

Primer	Sequence (5'-3')	Reference
27F	AGA GTT TGA TCC TGG CTC AG	Neilan et al. 1997
781F[a]	GGG ATT AGA TAC CCC TGT AGT C	This study
781R	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. 1997
NITS_F	GAA TTC GTT CCC GGG CCT TG	This study
23S30R	CTT CGC CTC TGT GTG CCT AGG T	Lepère et al. 2000

[a]Direct reverse complement of the 781R primer by Nübel et al. (1997).

Table 1. Sampled locations with hydromorphological and physicochemical characterization of the studied strains of *Azorothrix ramosa*.

Strain	Location	Coordinates	Elev. (m)	Type	Sampling	T (°C)	pH	C (µS.cm^{-1})	O_2 (mg.L^{-1})	Trophic state
BACA0028 *A. ramosa*	Lagoa Rasa das Sete Cidades	37°50'33.72"N, 25°46'48.04"W	545	Lake	2016-05-19	18	6.8	45	9.4	Oligotrophic
BACA0043 *A. ramosa*	Lagoa Verde	37°50'34.41"N, 25°47'19.37"W	260	Lake	2016-09-06	23	9	130	9	Eutrophic
BACA0066 *A. ramosa*	Lagoa do Congro	37°45'22.31"N, 25°24'29.55"W	420	Lake	2017-01-31	13	7.3	100	9.5	Eutrophic
BACA0093 *A. ramosa*	Ribeira do Guilherme	37°48'43.09"N, 25°10'27.76"W	260	Stream	2017-05-25	15	7.7	93	10.4	-
BACA0098 *A. ramosa*	Ribeira do Guilherme	37°47'54.50"N, 25°12'11.38"W	573	Stream	2017-05-12	13	7.4	89	10.5	-
BACA0147 *A. ramosa*	Lagoa do Fogo	37°45'53.31"N 25°28'26.79"W	574	Lake	2017-07-31	22	7.9	36	8.7	Mesotrophic

Analyses of 16S–23S rRNA ITS region. The 16S–23S rRNA ITS secondary structures of D1–D1′, Box–B, and V3 helices were identified following Iteman et al. (2000), and secondary structures were predicted using M–fold (Zuker 2003). Final composite images were re–drawn in Inkscape v1.2.

Results

***Azorothrix* R.F.S.Luz, G. S. Hentschke et V.Gonçalves gen. nov.**

Description: Thallus fasciculated forming irregular mats when old, cushion–like with erect filaments. Filaments solitary or fasciculated, later irregularly entangled, up to 1.5 mm. Trichomes heteropolar, unconstricted or constricted at cross walls, repeatedly false branched. False branches unilateral and/or bilateral, never more than one per filament, divaricated or ± parallelly arranged. False branches of the "scytonematoid" type are very rare. Sheaths are thin, firm, colourless, open at the apex. Cells isodiametric to shorter than wide. Terminal cells rounded. Heterocytes basal, hemispherical, cylindrical, conical rounded or discoid, solitary, or seriated up to 5 in a row, rarely intercalary (before disintegration?). Akinetes not observed. Reproduction by hormogonia and disintegration of trichomes (necridia present).

Type species: *Azorothrix ramosa* R.F.S. Luz, G.S. Hentschke et V. Gonçalves

Etymology: *Azorothrix* = Azores (site of origin) + thrix (for its filamentous structure and resemblance to *Tolypothrix*).

Remark: Phylogenetic distinct and placed in the Tolypotrichaceae family by the 16S rRNA. Morphologically similar to *Tolypothrix*¸ but with a distant phylogenetic placement.

***Azorothrix ramosa* R.F.S.Luz, G.S. Hentschke et V.Gonçalves sp. nov. (Fig. 2)**

Description: Thallus fasciculate, forming erect cushion–like aggregations when old. Filaments false branched, unilateral, and bilateral, later growing in a predominantly parallel orientation. Filaments commonly very long, reaching more than 1 mm in length (1.5 mm), 10.2–18.4 µm in diameter. Trichomes uniseriate, cylindrical slight constricted at cross–walls, more visible in the trichome terminal part, not tapering. Trichomes with isolated heterocytes or in rows up to five, one or two pored, very rarely without basal heterocytes. Sheaths are thin, firm, colourless, and distinct. Cells isodiametric to shorter than wide, 8.1–12.1 µm wide and 2.4–10.7 µm long, blue–green or olive green, end cells rounded and sometimes larger. Necridia present. Basal heterocytes variable in shape, hemispherical, cylindrical, conical rounded or discoid 6.7–14.2 µm wide 4.4–13.2 µm long. Intercalary heterocytes are very rare, probably developing before disintegration. Akinets not found in culture, reproduction by trichome fragmentation.

Holotype: Dried material preserved in a permanently inactive state at Herbário Ruy Telles Palhinha, University of Azores, Portugal, under the AZB 3833 code.

Type locality: Lagoa do Congro, São Miguel Island, Azores, Portugal (37°45' 22.3092"N, 25°24'29.5488"W).

Habitat: Found in lakes with very different trophic states (oligotrophic, mesotrophic, and eutrophic) and freshwater streams on the surface of submerged stones (epilithon).

Etymology: ramosa = having many branches.

Reference strain: BACA0066 (Bank of Algae and Cyanobacteria of the Azores, Azores, Portugal).

Gene Sequences: GenBank accession number MT176722 for the 16S rRNA and 16S–23S rRNA ITS region.

Morphological analysis

Azorothrix is positioned in the Tolypotrichaceae, according to its characteristic isolate false branching (Fig. 2A–C and Fig. 3A–C) and its phylogenetic position (Fig. 4). This new genus is phylogenetically closely related to *Kryptousia*, from which it differs in morphological traits and ecology. *Azorothrix* presents fasciculate or parallel–oriented thallus, with erect filaments when older, while *Kryptousia* presents interwoven filaments, which are never parallel–oriented or erect. These two genera also differ in their ecology. *Azorothrix* is found in the epilithon of freshwater lakes and streams, while *K. macronema* is from mangroves and is epiphytic on *Avicennia schaueriana*, a plant known for salt–secreting glands in its leaves (Fitzgerald et al. 1992).

Compared to *Coleodesmium*, *Azorothrix* presents only one trichome per sheath, whereas *Coleodesmium* always has more than one. *Dactylothamnos* differs from *Azorothrix* by presenting narrowed branches, while *Azorothrix* trichomes are cylindrical. Morphologically, *Azorothrix* presents isodiametric to shorter than wide cells, while in *Hassallia* Berkeley ex Bornet et Flahault cells are always shorter than wide. *Azorothrix* presents morphological features common to *Tolypothrix* from which can only currently be separated by molecular analysis.

16S rRNA phylogenetic and 16S–23S rRNA ITS analysis

All six strains showed only one band in the electrophorese gel following PCR. Sequencing of the obtained bands showed high quality when using the new designed primer (NITS_F) together with the remaining used primers (Table 2), with the presence of both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the sequenced operons.

The ML and BI trees were built with 198 operational taxonomic units (OTUs) and 1107 analysed positions of the 16S rRNA gene. The new genus is positioned in the Tolypotrichaceae clade, along with *Tolypothrix* (HG970652), *Hassallia* (AM905327), *Spirirestis* (AF334690), *Rexia* (KF934181), *Dactylothamnos* (KM199732), and *Coleodesmium* (AF334703). The new genus *Azorothrix* is supported as a sister clade of a cluster containing identified *Hassallia* strains, including the type species *Hassallia byssoidea.* Other Tolypotrichaceae genera are closely placed, namely *Kryptousia, Rexia, Tolypothrix, and*

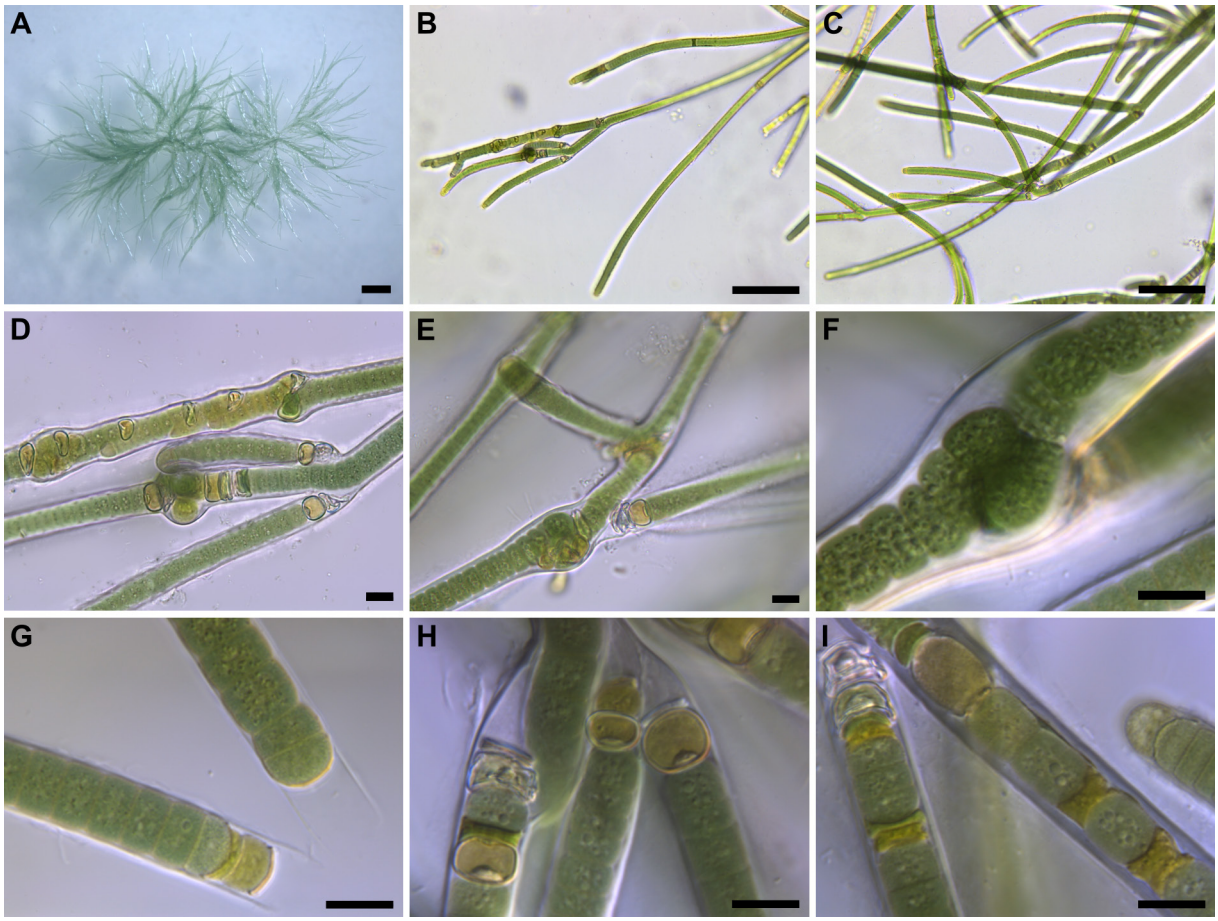

Fig. 2. *Azorothrix ramosa* BACA0066 morphology: (A) colony grown in agar; (B-F) filaments ramification; (G) end cells, (H-I) heterocytes and necridic cells in liquid media. Scale bars 100 µm (A-C), 10 µm (D-I).

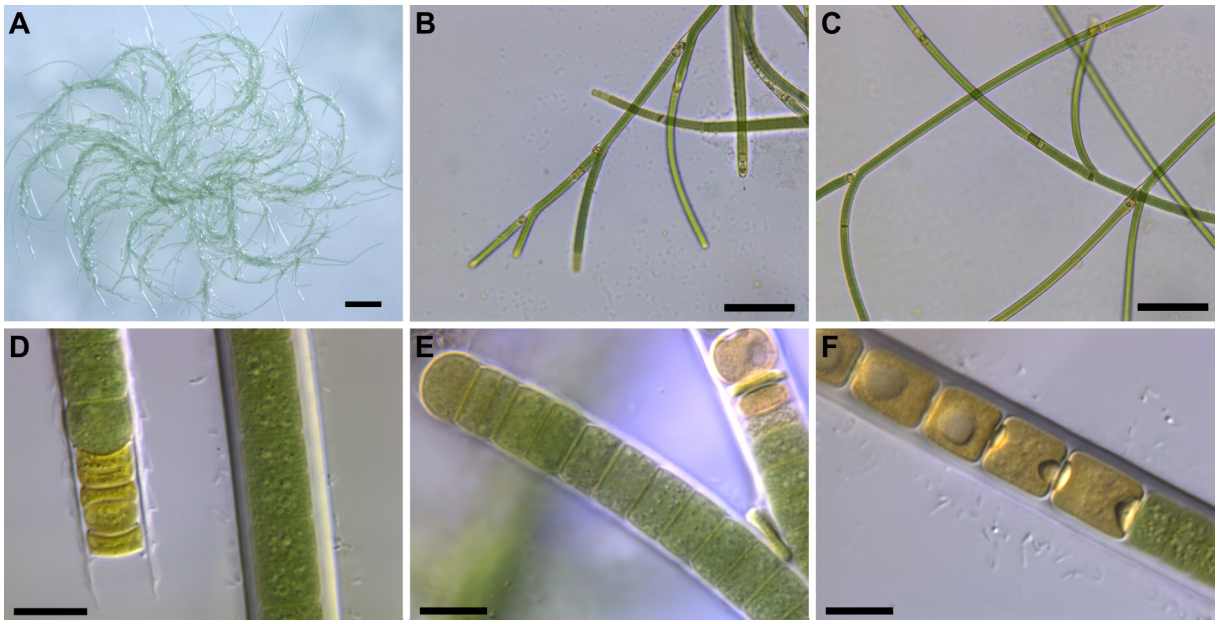

Fig. 3. *Azorothrix ramosa* BACA0028 morphology: (A) colony grown in agar; (B-C) filaments ramification; (D-E) end cells and necridic cells; (E-F) heterocytes in liquid media. Scale bars 100 µm (A-C), 10 µm (D-F).

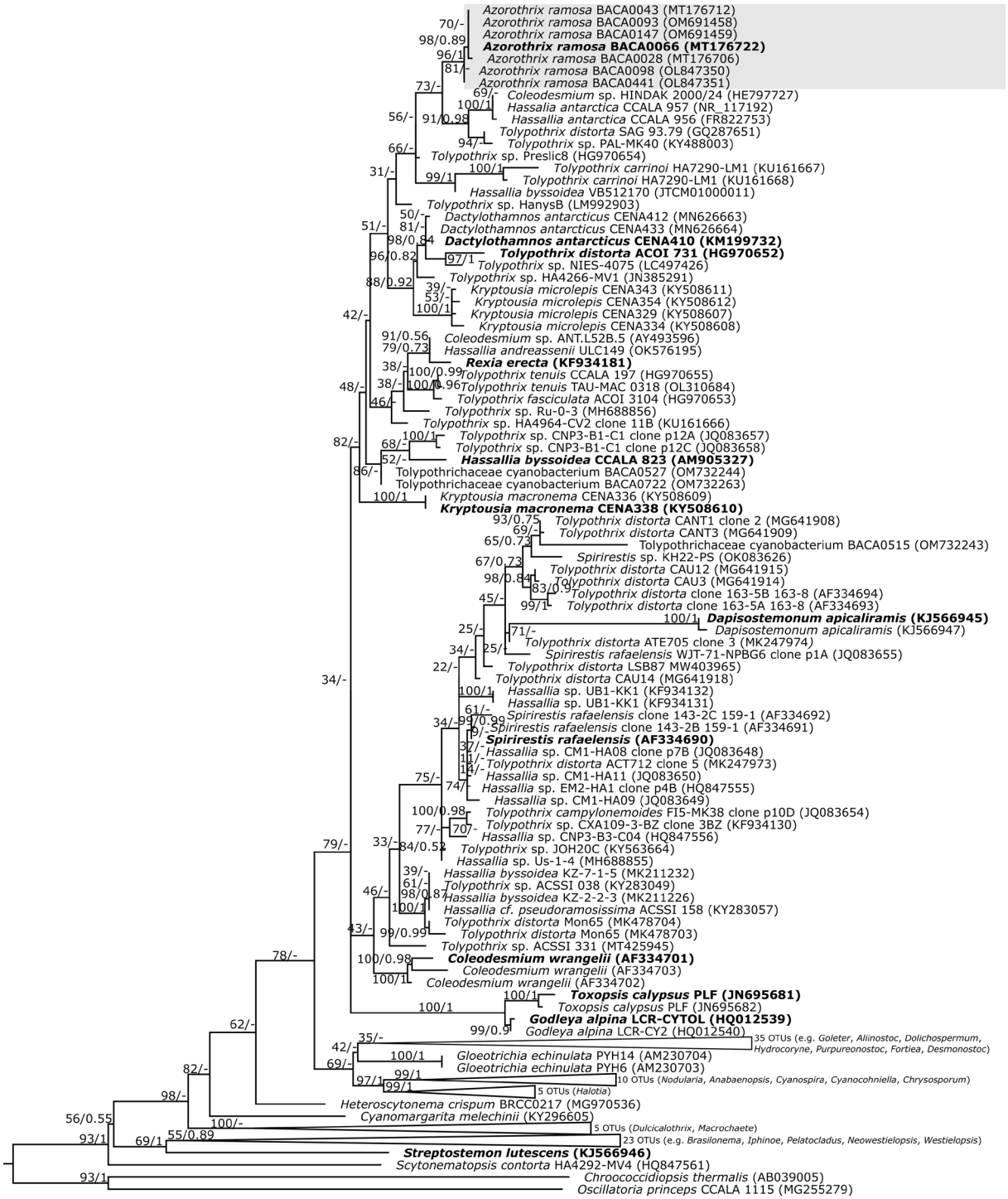

Fig. 4. Maximum Likelihood (ML) phylogenetic tree based on 16S rRNA sequences (1107bp) of *Azorothrix* and other cyanobacterial strains. Bootstrap values for maximum likelihood and posterior probabilities for Bayesian Inference are indicated on the tree. The novel genus is in grey shade and type species of the Tolypotrichaceae are in bold font. Outgroups: *Chroococcidiopsis thermalis* (AB039005) and *Oscillatoria princeps* CCALA 1115 (MG255279).

Dactylothamnos. The genus *Kryptousia* is polyphyletic as *Kryptousia microlepis* is positioned close to *Dactylothamnos* with good support (ML 93, BI 0.90) and far from the type species *K. macronema*. In both ML and BI trees, the family Tolypotrichaceae is formed by a monophyletic clade, although the placement of OTUs within the family is not the same. Among the few clades that seem more stable is the one formed by *Dactylothamnos*, *K. microlepis* and *T. distorta,* where a good support value is observed (ML 93, BI 0.90), while the remaining OTUs placement changes frequently (Fig. 4; Fig S1).

Regarding the 16S rRNA, there is a high similarity among some Nostocales genera, with a p–distance as low as 2.18% among phylogenetic distant species such

as *Goleter apudmare* HA4356–MV2 and *Tolypothrix fasciculata* ACOI 3104. These occur between almost all Tolypotrichaceae genera, with p–distance close to 2%. Although all these genera present high 16S rRNA gene similarity (Table S1), the obtained phylogeny supports the new genus as an independent and monophyletic taxon in the Tolypotrichaceae.

The 16S–23S rRNA ITS region was aligned among Tolypotrichaceae genera and 16S–23S rRNA ITS secondary structures folded after the identification of the D1–D1' (Fig. 5), Box–B (Fig. 6) and V3 helix (Fig. 7). It is possible to identify conserved motifs among all compared genera, such as the basal stem and the lateral bulge in the D1–D1'. Indeed, the D1–D1' region presents similar sequences and structures among *Azorothrix, Dactylothamnos, Tolypothrix, Coleodesmium, Rexia,* and *Kryptousia microlepis*. The differences among these genera are found only in a few nucleotide substitutions in the 3' side of the molecule between the lateral bulge and the mid internal loop. *Kryptousia macronema* helices present very distinct stems, on both sequence and structure, above the basal lateral bulge. The Box–B (Fig. 6) and V3 helix (Fig. 7) have a higher variability than the D1–D1', showing high variability in the sequence and structure among them. The Box–B has a common basal stem and common internal loop, except for *Azorothrix,* which significantly differs from the remaining by a much larger internal loop. All analysed strains share a terminal hair pin, although the sequence and structure differ among all. The V3 helix has the most significant difference among strains where the common structure is the terminal hair loop, except for *Azorothrix ramosa* and *Dactylothamnos antarcticus* that share the same V3 helix sequence and structure.

Discussion

The six studied BACA strains, previously identified as *Tolypothrix*, present a distinct phylogenetic position, which, combined with morphological and ecological features, allowed the description of a new genus. Morphologically very similar to the *Tolypothrix* and *Hassallia* genera (*Tolypothrix distorta* ACOI 731, *Hassallia byssoidea* CCALA 823) but distant phylogenetically and in p–distance (Table S1), *Azorothrix* is a new genus genetically distinct from *Tolypothrix* and *Hassallia*. Although the current known geographic distribution of *Azorothrix* is restricted to the Azores Islands, it will

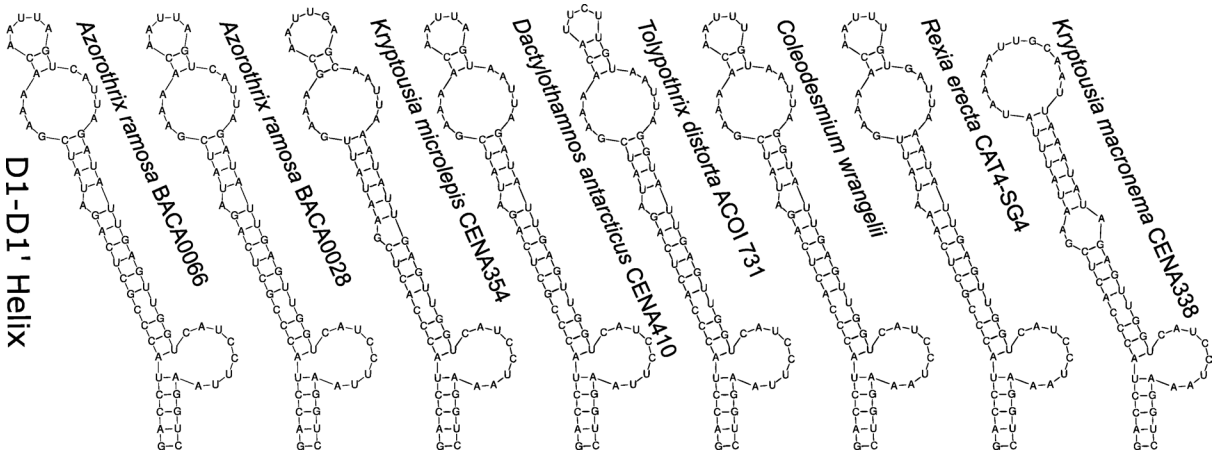

Fig. 5. Secondary structure of the D1–D1' helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

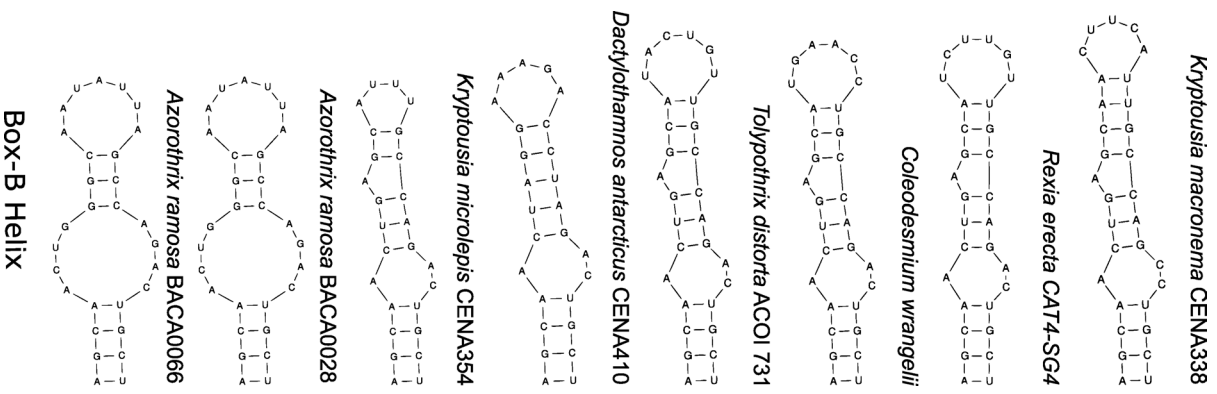

Fig. 6. Secondary structure of the Box-B' helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

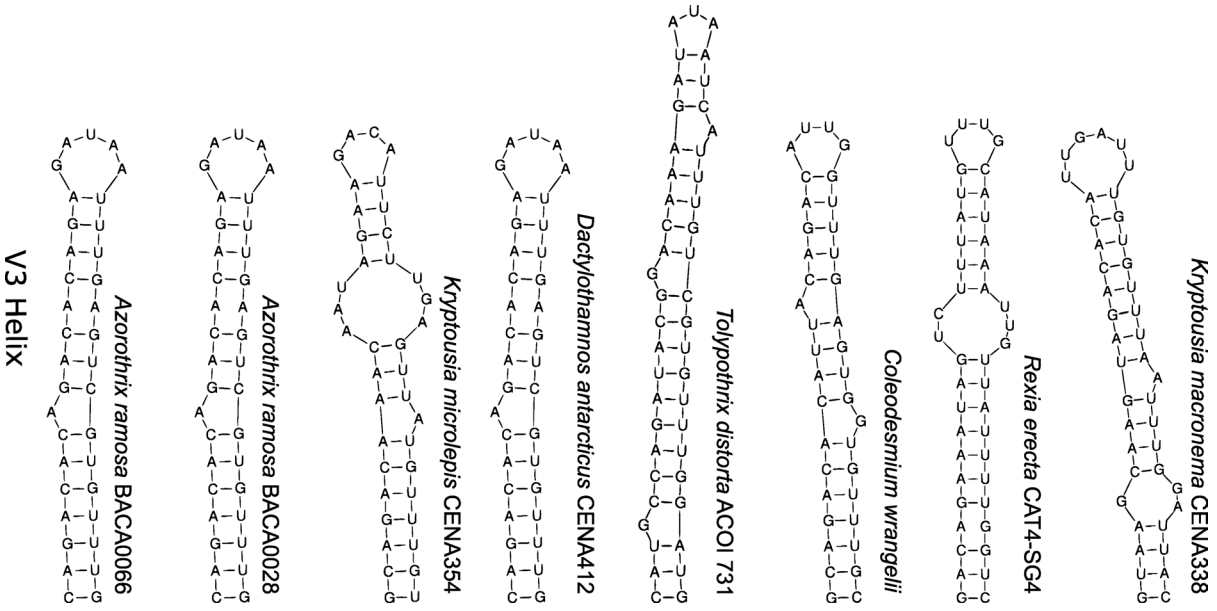

Fig. 7. Secondary structure of the V3 helix in *Azorothrix ramosa* and representative Tolypotrichaceae genera.

probably be found in other areas, and the description of new species or strains will help better characterize this genus. All strains are identical when compared with cell size and form along the trichome and hormogonia, but some differences were noticed, namely in the type of branching that influences the formation of the colonies in agar plates (Fig. 2 and Fig. 3). In BACA0066 (Fig. 2A), bilateral branching is more frequent than unilateral, with branches staying close to the main filament (later fasciculated), while in BACA0028 (Fig. 3A), false branches are widely divaricated and less frequent. These traits seem to be stable as they are maintained by each strain on long–term maintenance culture.

All six strains showed only one band in the electrophoresis gel following PCR, something unusual in the Nostocales as normally these present up to five operons (Kaštovský et al. 2014; Johansen et al. 2017). However, this seems common in the Tolypotrichaceae family, as only one operon was found in *Spirirestis*, *Tolypothrix* and *Coleodesmium* (Flechtner et al. 2002).

The *Azorothrix* strains are phylogenetically positioned close to *Kryptousia*, *Dactylothamnos,* and *Coleodesmium*. Together with the phylogenetic distinction, these genera can also be distinguished morphologically. Although similar to *Coleodesmium, Azorothrix* does not have more than two trichomes per sheath, which is a diacritical feature in *Coleodesmium* (Komárek 2013). Both *Kryptousia* and *Dactylothamnos,* possess similar colony formation with interwoven or flake–like groups (Komárek et al. 2015; Alvarenga et al. 2017), different from *Azorothrix* that follows a more organized (fasciculate) growth. A distinguishable trait is also the longer trichomes found in *Azorothrix* against the short trichomes found in *K. microlepis* and *Dactylothamnos* (Komárek et al. 2015; Alvarenga et al. 2017). In terms of ecology, these genera are all found in freshwaters, but they were described from very different geographic and climate zones. *Dactylothamnos* was originally found in the Antarctic (Komárek et al. 2015), *Kryptousia* from South American Atlantic Forest (Alvarenga et al. 2017), *Coleodesmium* from Europe (Bornet & Flahault, 1888), and *Azorothrix* from the Azores.

The folding of the 16S–23S rRNA ITS shows a high similarity of the D1–D1' region among the phylogenetic cluster made by *Azorothrix*, *K. microlepis*, *Dactylothamnos*, *Coleodesmium,* and *Rexia,* in contrast with *K. macronema*. Their differences rely mainly on nucleotide sequence on the 3' side between the main lateral bulge and the first internal loop. In the Box–B and V3 Helix, a more diverse folding was found, as expected (Johansen & Casamatta 2005). In Box–B, although *Azorothrix* has a more distinct fold with a larger mid internal loop, the remaining analysed strains have a common secondary structure, varying mostly in the length and sequence of the hair pin. The V3 secondary structure is the most distinct among the studied strains, with the presence/absence of some internal loops, except for *Azorothrix* and *Dactylothamnos* where the V3 is equal in structure and sequence. The highly conserved D1–D1' structure among *Azorothrix*, *Kryptousia*, *Dactylothamnos*, *Coleodesmium,* and *Rexia,* and the similarity in the V3 region of *Azorothrix* and *Dactylothamnos* is an unusual feature when comparing so many different genera, however with such a conserved 16S rRNA as seen in the Tolypotrichaceae, is not surprising that all the 16S–23S rRNA operon would also be somehow conserved, and variability of the 16S–23S rRNA ITS is in concordance with the variability of the 16S rRNA.

Hauer et al. (2014) elevated the former subfa–mily Tolypothrichoidae Komárek and Anagnostidis to the family level. Our results support this classification with the monophyletic nature of the Tolypotrichaceae (Fig.

4). Nevertheless, some uncertainties remain due to the morphological polymorphism of *Tolypothrix* species and distant phylogenetic placement of some strains in phylogenetic trees (Hauer et al. 2014). In our tree (Fig. 4), the distant position between *Tolypothrix distorta* (type species) and the clade composed by *Tolypothrix fasciculata* and *Tolypothrix tenuis* strains suggests that the latter could be a new undescribed genus. The identification of close genetic strains that are either distinct morphologically, as seen in *Spirirestis* (Flechtner et al. 2002) and *Dactylothamnos* (Komárek et al. 2015), or ecologically, as seen in *Kryptousia* (Alvarenga et al. 2017), seems to be common in the Tolypotrichaceae.

The phylogenetic analysis is important for genera and species delimitation in the Tolypotrichaceae and in the Nostocales in general (Strunecký et al. 2022). The 16S rRNA in the Nostocales is highly conserved (Kaštovský et al. 2014), but at the same time, it shows a high phylogenetic divergence among the studied OTUs. This, combined with the high morphological similarities that many of the described genera and species have, is challenging for a correct taxonomic assessment. Although this work uses detailed morphological characterization, ecology, geographical distribution and genetics for genus and species distinction, it relies strongly on the phylogenetic analysis for the new taxa description, especially the current phylogenetic separation of previously described genera, such as *Tolypothrix*, *Coleodesmium, Rexia, Dactylothamnos* and *Kryptousia*. Taking into account the morphological similarities with *Tolypothrix*, *Azorothrix ramosa* could be described as a new *Tolypothrix* species, however this would only create more confusion in an already problematic clade. Besides, if that would be done, we would be recognizing only morphology, and ignoring genetic data and the existence of cryptic taxa, failing to follow the recommended polyphasic approach in the taxonomic assessment (Komárek et al. 2014). Furthermore, the adoption of such approach would mean that the broader clade containing *Tolypothrix* / *Hassallia* / *Rexia* / *Dactylothamnos* / *Kryptousia* / *Azorothrix* type species should be collapsed into a single genus.

Presently, Tolypotrichaceae is one of the most problematic cyanobacteria families, with a low 16S rRNA p–distance threshold (close to 2%) for genera delimitation. This situation extends to several other genera of the Nostocales (e.g. *Cyanocohniella, Goleter*, *Halotia*; Table S1) that are placed in distant phylogenetic clades and genomically recognized families (Kaštovský et al. 2014; Strunecký et al. 2022). Thus, phylogeny is of paramount importance in taxonomic studies in the Nostocales. Furthermore, in such clades as the Tolypotrichaceae, where 16S rRNA threshold values are low, and the morphology is similar, it is urgent to include genomic characterization to resolve their taxonomic situation. A taxonomic assessment using a much more robust analysis with genomes and phylogenomics will help clarify the status of these genera, supporting their distinction or collapse to the original *Tolypothrix* genus.

Acknowledgements
Rúben Luz was supported by a Ph.D. grant (M3.1.a/F/002/2020) from the Fundo Regional da Ciência e Tecnologia (FRCT). This work was funded by FEDER funds through the Interreg–MAC 2014–2020 Program under the projects REBECA—Red de excelencia en biotecnología azul (algas) de la región macaronesia (MAC1.1a/060) and REBECA–CCT—Red de Excelencia en Biotecnología Azul de la Región Macaronésica. Consolidación, Certificación y Transferencia (MAC2/1.1b/269), and by Portuguese National Funds through FCT - Foundation for Science and Technology under the projects UIDB/50027/2020 and UIDP/50027/2020. CIIMAR acknowledges funding by FCT through UIDB/04423/2020 and UIDP/04423/2020.

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Supplementary material

The following supplementary material is available for this article:

Table S1. 16S rRNA p-distance percentage of *Azorothrix* and close phylogenetic genera. (Red: < 2%; Yellow: >2% and <3%; Green: >3%). Type species of the Tolypotrichaceae genera are in bold.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

Received January 31, 2023
Accepted September 25, 2023