Cephalothrix gen. nov. (Cyanobacteria): towards an intraspecific phylogenetic evaluation by multilocus analyses

Camila Francieli da Silva Malone,¹ Janaina Rigonato,² Haywood Dail Laughinghouse IV,³,⁴ Éder Carlos Schmidt,⁵ Zenilda Laurita Bouzon,⁵ Annick Wilmotte,³ Marli Fátima Fiore² and Célia Leite Sant’Anna¹

¹Institute of Botany, Nucleus of Phycology, São Paulo, SP, Brazil
²Center for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil
³Laboratory of Bacterial Physiology and Genetics, Centre for Protein Engineering, University of Liège, Sart Tilman B6, Liège, Belgium
⁴Department of Arctic Biology, University Centre in Svalbard, Longyearbyen, Norway
⁵Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, SC, Brazil

For more than a decade, the taxonomy of the Phormidiaceae has been problematic, since morphologically similar organisms represent phylogenetically distinct entities. Based on 16S rRNA gene sequence analyses, the polyphyletic genus Phormidium and other gas-vacuolated Oscillatorioidae appear scattered throughout the cyanobacterial tree of life. Recently, several studies have focused on understanding the Oscillatorioida taxa at the generic level. At the specific level, few studies have characterized cyanobacterial strains using combined datasets (morphology, ultrastructure and molecular multilocus analyses). Using a multifaceted approach, we propose a new, well-defined genus, Cephalothrix gen. nov., by analysing seven filamentous strains that are morphologically 'intermediate' between gas-vacuolated taxa and Phormidium. Furthermore, we characterize two novel species: Cephalothrix komarekiana sp. nov. (strains CCIBt 3277, CCIBt 3279, CCIBt 3523, CCALA 155, SAG 75.79 and UTEX 1580) and Cephalothrix lacustris sp. nov. (strain CCIBt 3261). The generic name and specific epithets are proposed under the provisions of the International Code of Nomenclature for Algae, Fungi, and Plants.

INTRODUCTION

Cyanobacteria are widespread and morphologically distinct, dominant in several environments (Whitton, 1992). To characterize ‘true’ cyanobacterial diversity better, systematic studies should include a polyphasic or multifaceted approach, combining both phylogenetic relationships and phenotypic characterizations. Over the past few decades, new technologies have emerged that allow researchers to study and understand these organisms in an evolutionary context (Komárek, 2005; Marquardt & Palinska, 2007). Though polyphasic evaluation of diversity is sometimes very difficult, this approach is essential for the systematic community to reach a consistent and natural classification of this group (Komárek, 2006). Cyanobacterial systematics is still under revision and in a state of transition. Over the last few decades, studies have focused heavily on generic delimitation using 16S rRNA gene phylogenetic analyses. However, this gene can be too conservative in resolving species-level relationships, and not appropriate for this evaluation (Fox et al., 1992; Boyer et al., 2002). Thus, the primary and secondary structures of the more variable 16S–23S rRNA internal

Abbreviations: ITS, internal transcribed spacer; ML, maximum-likelihood; NJ, neighbour-joining; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene + ITS, rpoC1 and rbcLX sequences obtained in this study are detailed in Table 1.

A supplementary figure is available with the online Supplementary Material.
transcribed spacer (ITS) have been used increasingly to assess the relationships between closely related species and populations (Boyer et al., 2001, 2002; Ernst et al., 2003; Kling et al., 2012). Several studies (Casamatta et al., 2005; Siegsmund et al., 2008; Johansen et al., 2011) have described novel taxa based on morphological and ITS autapomorphies. More recently, Sciuto et al. (2012) showed that a new approach, based on phylogenetic multilocus analyses using the ‘classical’ 16S rRNA gene, the ITS and other molecular markers (such as the rpoC1 gene), was useful for evaluating infraspecific cyanobacterial diversity. This approach is particularly promising for analyses of the heterogeneous genera in the family Phormidiaceae, including the polyphyletic genus Phormidium Kützing and gas-vacuolated oscillatorioids.

Some authors (Suda et al., 2002; Komárek, 2005; Thu et al., 2012) have already demonstrated that genera of gas-vacuolated oscillatorioids such as Planktothrix (Gomont) Anagnostidis & Komárek, Planktothricoides (Wolszyńska) Suda & Watanabe and Aerosakkonema Thu et al. are not monophyletic. According to Thu et al. (2012), the presence of aerotopes in natural material does not provide robust phylogenetic information for the phylogenetic reconstruction of cyanobacteria at the generic or specific levels. In this context, strains with or without aerotopes can be phylogenetically closely related (Thu et al., 2012).

Similarly, Phormidium sensu lato represents a taxonomically complex group, since morphologically similar organisms may represent phylogenetically distinct entities (Sciuto et al., 2012). Inside the eight morphological groups delineated by Komárek & Anagnostidis (2005), several new genera (e.g. Phormidesmis, Oxyenna and Wilmottia) have been described based on morphological features and 16S rRNA gene sequences (Komárek et al., 2009; Strunecky et al., 2011; Chatchawan et al., 2012). At the specific level, Sciuto et al. (2012) were the first authors to characterize Phormidium-like strains using a polyphasic approach based on morphology, ultrastructure and multilocus molecular sequencing and phylogeny. In the present study, we propose a new, well-defined genus, Cephalothrix gen. nov., based on seven filamentous strains morphologically similar to gas-vacuolated taxa and also to Phormidium, using a multifaceted approach. In this context, we confirmed that previously reported cyanobacterial genera were unsuitable to accommodate the studied strains (of Brazilian and European origin). In addition, based on the results of this study, we propose two new species: Cephalothrix komarekiana sp. nov. (for strains CCIBt 3277, CCIBt 3279, CCIBt 3523, UTEX 1580, CCALA 155 and SAG 75.79) and Cephalothrix lacustris sp. nov. (for strain CCIBt 3261). The generic name and specific epithets are proposed under the provisions of the International Code of Nomenclature for Algae, Fungi, and Plants.

METHODS

Cultured strains. Seven strains from distinct Brazilian environments and different culture collections [the Culture Collection of Auto-trophic Organisms (CCALA), the Culture Collection of Algae at Göttingen University (SAG) and the Culture Collection of Algae at the University of Texas at Austin (UTEX)] were investigated. All strains are maintained at the Institute of Botany Culture Collection (CCIBt), São Paulo, Brazil, including those acquired from other culture collections. The Brazilian strains were isolated from an extremely alkaline lake (CCIBt 3277), a freshwater aquarium (CCIBt 3279 and CCIBt 3253) and a freshwater lake (CCIBt 3261). Strain CCIBt 3277 can be considered alkaliphilic, since it grows in an alkaline lake (pH 9–11) in the Brazilian Pantanal wetland region (19° 34’ 32.6” S 57° 00’ 51.3” W). This ecosystem, located in the centre of South America, is one of the world’s largest wetlands and has hundreds of alkaline lakes (Barbiero et al., 2002; Malone et al., 2012). The freshwater lake (pH 7–8) where CCIBt 3261 was collected is located in a botanical garden (30° 03’ 2.48” S 51° 10’ 46” W) in south-eastern Brazil. Strain UTEX 1580 was isolated from a goldfish aquarium. Strains CCALA 155 and SAG 75.79, isolated in 1961 from concrete in Mallorca (Spain), are duplicates of the same strain deposited in different culture collections. In this study, the results for these two strains will be reported under one accession number (CCALA 155), since the two specimens are genetically identical. Subsamples of the Brazilian strains and field material were preserved in 4% (v/v) formaldehyde and deposited in the Maria Eneyda P. Kauffman Fidalgo Herbarium at the Institute of Botany, São Paulo, Brazil. CCIBt 3261 (culture aliquot SP428.686), CCIBt 3277 (field sample SP400.862 and culture aliquot SP427.779), CCIBt 3279 (culture aliquot SP427.780) and CCIBt 3523 (field sample SP427.932 and culture aliquot SP427.792). Furthermore, replicates of the Brazilian strains were deposited in the Belgian Coordinated Collections of Microorganisms (BCCM/ULC): CCIBt 3261 (ULC 715), CCIBt 3277 (ULC 718), CCIBt 3279 (ULC 719) and CCIBt 3523 (ULC 733).

The Brazilian strains were isolated following standard techniques (Jacinavicius et al., 2012). After unialgal isolation, the strains were maintained under the following conditions: temperature 23 ± 1 °C, irradiance 40–50 μmol photons m–2 s–1 (provided by daylight fluorescent lamps and measured with a Li-COR quantum metre sensor), a photoperiod with a 14 h:10 h light–dark cycle and liquid BG11 medium (Rippka, 1988). The strains acquired from the other culture collections were maintained under the same conditions.

Morphological evaluation. Morphological evaluations of all strains were based on the diacritical traits proposed by Komárek & Anagnostidis (2005) and Hoffmann et al. (2005). The analyses were carried out using a Zeiss Axioplan light microscope and identification was undertaken based on population analyses (n = 30). Photomicrographs were taken of each strain with a Zeiss Axioscam MRc digital camera. To characterize the proposed genus better, a life-cycle analysis was carried out daily on strain CCIBt 3277 for 30 days. The initial inoculum included only trichomes at the same life stage, with aerotopes and without a mucilaginous sheath.

Transmission electron microscopy (TEM). TEM was used to study the cell ultrastructure (predominantly thylakoid and gas vesicle position) of strain CCIBt 3277 that had previously been grown for 2 weeks. Cells were fixed overnight with 2.3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose (Schmidt et al., 2009). The material was post-fixed with 1% osmium tetroxide for 4 h, dehydrated in an acetone gradient series (30, 50, 70, 90 and 100%) and embedded in Spurr’s resin (Spurr, 1969). Ultrathin cross-sections were stained with aqueous uranyl acetate followed by lead citrate. The samples were examined using a TEM JEM 1011 (JEOL) at 80 kV.
DNA extraction, PCR and sequencing. Genomic DNA was extracted from the Brazilian organisms and the strains acquired from culture collections using the UltraClean Microbial DNA Isolation kit (MoBio). The rbcLX and rpoC1 loci were amplified as described previously by Rudi et al. (1998) and Rantala et al. (2004), respectively. The full 16S rRNA gene plus ITS from the genomic DNA was amplified by PCR using primers 27F (Neilan et al., 1997) and 23S30R (Taton et al., 2003). Thermal cycling was performed in a Techne TC-412 Thermal Cycler (Bibby Scientific) with the following settings: initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 57°C and extension for 1 min at 72°C, and finally the reaction was completed with an extension for 7 min at 72°C. The PCR products were sequenced directly, except the 16S rRNA gene plus ITS fragments, which were cloned into the pGEM-T Easy vector system (Promega) according to the supplier’s manual. Competent cells of Escherichia coli DH5α were transformed and recombining plasmids were purified from white colonies by the alkaline lysis method (Birnboim & Doly, 1979). The cloned PCR product was sequenced using Big Dye Terminator 3.0 (Applied Biosystems) with the pGEM-T Easy vector-anchored primers M13F and M13R and internal primers 357F/357R, 1979). The cycle-sequencing reaction was performed with a Techne TC-412 (Bibby Scientific) with the following settings: initial denaturation at 95°C for 1 min and 36 cycles of 95°C for 15 s, 50°C for 15 s and 60°C for 2 min. After completion of the reaction, the DNA was precipitated using sodium acetate buffer (1.5 M sodium acetate, pH 9.0, and 250 mM EDTA) followed by the addition of 100 and 70% ethanol. The purified reaction mixtures were reconstituted in HiDi formamide (Applied Biosystems/Life Technologies), and the samples were analysed in an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems/Life Technologies). The sequenced fragments were assembled into contigs using the Phred/Phrap/Consed software package (Ewing et al., 1998; Ewing & Green, 1998; Gordon et al., 1998), and only bases with a quality >20 were considered. The gene sequences obtained in this study were deposited in the NCBI GenBank database under the accession numbers reported in Table 1.

Phylogenetic analyses. All sequences obtained in this study and reference sequences retrieved from the NCBI were aligned using CLUSTAL W (Thompson et al., 1994), refined visually and used to reconstruct phylogenetic trees. Phylogenetic trees for separate loci and concatenated sequences were reconstructed using the maximum-likelihood (ML), neighbour-joining (NJ) and Bayesian analysis methods. The best-fitting evolutionary models were selected using jModelTest (Darriba et al., 2012) and are detailed in the legend of each figure. NJ and ML methods were implemented with the MEGA program package version 5.05 (Tamura et al., 2011). The robustness of the trees was estimated by bootstrap percentages using 1000 replicates. Bayesian analysis was run in MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003) in two independent runs, with four chains each, for 5 x 10⁶ generations. Concatenated analyses were conducted using the 16S rRNA gene, rpoC1 and rbcLX loci and the ITS region. The best-fitting evolutionary model GTR+G + I was selected for all four cases. The evolutionary history of strains was reconstructed in the same manner as described above.

Secondary structure models of ITS regions. The ITS region (550 bp) was analysed by determining the secondary structure of the D1–D1’, Box B and V3 helix domains. Each helix was located by its position in the ITS as well as through common sequences at the base of the helix. Once the sequence fragment was identified, it was folded individually using Mfold 3.2 (Zuker, 2003). Except for the use of the structure draw mode ‘untangle with loop fix’, default conditions in Mfold were used.

RESULTS AND DISCUSSION

All studied strains (of Brazilian and European origin) had a number of morphological features similar to those of well-defined and distinct traditional oscillatioran genera, such as Planktothrix, Trichodesmium Ehrenberg ex Gomont and Phormidium. The morphological analysis showed that trichomes with and without gas vesicles or in an intermediate stage (with some segments having gas vesicles whereas others did not) occurred in a single strain, with mucilaginous sheaths facultatively present. Except for CCALA 155, the studied strains were similar to the genera Planktothrix and Trichodesmium in having gas vesicles in one part of their life cycle, in contrast to the genus Phormidium. In addition, all of the strains had characteristics resembling those of Phormidium, such as a conspicuous mucilaginous sheath (absent in Planktothrix and Trichodesmium) and a variety of apical cells. As in Phormidium, strains of Trichodesmium can have a slightly capitate apical cell, similar to some of the analysed strains. However, Trichodesmium usually forms colonies with parallel or radially arranged fascicles or flocculent masses in marine environments (Capone et al., 1997; Komárek & Anagnostidis, 2005), and is therefore very different from our strains. Therefore, morphologically, the studied strains represented an intermediate state between Planktothrix and Phormidium.

Additionally, the analysed strains have aerotopes, just like the newly erected genus Aerosakkonema described by Thu et al. (2012). According to the original description, the diagnostic features of Aerosakkonema are its twisted trichomes with small and inconspicuous gas vacuoles. These features differentiate Aerosakkonema from the other freshwater gas-vacuolated oscillatoriodi, including Cephalothrix gen. nov. Besides, our results also demonstrated a difference in cell form: Aerosakkonema has discoid cells and the new genus Cephalothrix has trichomes with subquadrate cells.

To study the presence of aerotopes in the studied strains better, we performed a daily analysis of strain CCIBt 3277.

Table 1. GenBank/EMBL/DDBJ accession numbers of sequences acquired in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S rRNA gene + ITS</th>
<th>rpoC1</th>
<th>rbcLX</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. komarekiana sp. nov.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIBt 3277</td>
<td>KJ994514</td>
<td>KJ994519</td>
<td>KJ994525</td>
</tr>
<tr>
<td>CCIBt 3279</td>
<td>KJ994515</td>
<td>KJ994520</td>
<td>KJ994526</td>
</tr>
<tr>
<td>SAG 75.79</td>
<td>KJ994517</td>
<td>KJ994522</td>
<td>KJ994528</td>
</tr>
<tr>
<td>CCIBt 3523</td>
<td>KJ994516</td>
<td>KJ994521</td>
<td>KJ994527</td>
</tr>
<tr>
<td>UTEX 1580</td>
<td>AY218830</td>
<td>KJ994523</td>
<td>KJ994529</td>
</tr>
<tr>
<td>C. lacustris sp. nov.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIBt 3261</td>
<td>KJ994513</td>
<td>KJ994518</td>
<td>KJ994524</td>
</tr>
</tbody>
</table>
The data enabled us to confirm two distinct phases in the life cycle: from an initial inoculum of trichomes with aerotopes and without a mucilaginous sheath (Fig. 1a, b), we observed that, after 6 days, there was an initial loss of aerotopes (Fig. 1e) and formation of conspicuous hyaline sheaths (Fig. 1h). The mucilaginous sheaths then became wider (Fig. 1i) and the trichomes broke repeatedly, forming hormogonia (Fig. 1k, l). Afterwards, the hormogonia were released from the sheath and aerotopes began to appear again (Fig. 1m). This cycle indicates that, in nature, these trichomes may have a planktonic phase with aerotopes and a benthic phase without aerotopes and with a sheath. The gas vesicle structure was clearly confirmed using TEM (Fig. 2a, b). We also observed these two distinct phases in strains CCIBt 3279 (Fig. 3a–e), CCIBt 3523 (Fig. 3f–j), UTEX 1580 (Fig. 3k–o) and CCIBt 3261 (Fig. 4a–e). However, aerotopes have not been observed in CCALA155 (isolated from a concrete surface) (Fig. 4f–j), which may be explained by the different environmental conditions of its original habitat or a subsequent mutation under culture conditions (Werner & Laughinghouse, 2009). In favour of the first possibility, we assume that the presence of aerotopes and a two-stage life cycle are useful only in aquatic biotopes. In contrast to CCALA 155, its replicate SAG 75.79 had a mucilaginous sheath (Fig. 4j), similar to the other five gas-vacuolated strains analysed. We hypothesize that the sheath plasticity in these replicates may be related to their different cultivation history, since they have been maintained under different culture conditions for a long time.

All seven strains were highly similar at the trichome tip and, therefore, a capitate apical cell can be considered as a diagnostic morphological feature of the proposed genus. This character also agrees with the phylogenetic analysis as described below. Inside the new genus, species delimitations were based on variations of the apical cell, phylogenetic analysis of several genetic markers and the secondary structure of conserved regions of the ITS (D1–D1'). Phylogenetic analyses of the 16S rRNA gene sequences placed the Brazilian strains in a robust novel clade that included isolates previously identified as Phormidium, such as CCALA 155, UTEX 1580 and Phormidium sp. KS (GenBank accession no. AB510147) (Fig. 5). Tree topology and the position of the proposed genus were highly supported by NJ, ML and Bayesian phylogenetic analyses (bootstrap values of 99 and 100 % and Bayesian posterior probability of 1.00, respectively), and the three phylograms were similar. This novel clade was distantly related to well-known gas-vacuolated oscillatorioids, such as Planktothrix, Planktothrixoides and Trichodesmium (pairwise similarity less than 90 %), and formed a sister group to the recently published gas-vacuolated genus Aerosakkonema, with a similarity between 94.3 and 94.7 %. Other genera distinguished from the traditional genus Phormidium, such as Wilmsotia and Oxynema, shared less than 92 % 16S rRNA gene sequence similarity with Cephalothrix. The new genus also had low similarity (around 90 %) to Phormidium cf. irrigum CCALA 759, which was considered by Sciuto et al. (2012) to belong to group VIII of Phormidium morphotypes (designated by Komárek & Anagnostidis, 2005), which includes the type species Phormidium lucidum Kützing ex Gomont. Therefore, these authors concluded that the clade that includes Phormidium cf. irrigum CCALA 759 should represent Phormidium sensu stricto (Sciuto et al., 2012). Chatchawan et al. (2012) stated that strain CCALA 155 should be considered within Phormidium sensu stricto. However, our molecular data (Fig. 5) indicate that this strain belongs to Cephalothrix and not to Phormidium sensu stricto. Furthermore, CCALA 155 has been identified as Phormidium tergestinum (Kützing) Anagnostidis et Komárek, but, based on both our morphological and molecular data, we believe that this strain is not related to P. tergestinum. Morphologically, P. tergestinum has round or hemispherical apical cells (Komárek & Anagnostidis, 2005), differing from strain CCALA 155 (Cephalothrix), which has slightly capitate apical cells. Besides, CCALA 155 had an ITS secondary structure (D1–D1', Box B and V3) and thylakoid arrangement similar to those of other analysed strains, as described below. Similarly, UTEX 1580 belongs to Cephalothrix and is not related to Phormidium autunnale (Agardh) Trevisan ex Gomont, as identified previously. The presence of gas vesicles (Fig. 3k) in this strain corroborates the placement of UTEX 1580 in our proposed genus.

In conclusion, the phylogenetic results confirm that the clade presented in this study represents the new genus Cephalothrix and that previously described cyanobacterial taxa were unsuitable to accommodate the studied strains (of Brazilian and European origin). Besides, the cellular ultrastructure of strain CCIB 3277 revealed the presence of radial thylakoids (Fig. 2c–f), which is typical of the family Phormidiaceae. Strain CCALA 155 presented a similar thylakoid arrangement to CCIB 3277 (see Fig. 5(d) of Marquardt & Palinska, 2007), confirming its phylogenetic position within Cephalothrix.

http://ijs.sgmjournals.org
Fig. 2. TEM of *C. komarekiana* CCIBt 3277. (a) General view of vegetative cells with aerotopes. (b) Detail of aerotopes (side view). (c) Radial arrangement of the thylakoids (longitudinal section). (d) General view of a cross-section of a cell. (e, f) Detail of thylakoids. Bars, 1 μm (a, c, d), 0.5 μm (e), 0.2 μm (f).
The use of single-gene trees to reconstruct phylogenetic history is heavily debated (Lipscomb et al., 2003; Tautz et al., 2003), and authors have demonstrated that the 16S rRNA gene is useful for generic placement in cyanobacteria but can be too conservative at the species level (Boyer et al., 2001, 2002). The 16S rRNA gene trees (NJ, ML and Bayesian analyses) indicated the potential for a wider diversity (several species) within the proposed genus (Fig. 5). Indeed, there were two well-supported groups in our genus, which we identified as the novel species Cephalothrix komarekiana sp. nov. and Cephalothrix lacustris sp. nov. The alignment of the ITS supports this hypothesis (Fig. S1, available in the online Supplementary Material). Both the conserved regions D1–D1’ (positions 16–56) and Box B (positions 411–451) demonstrated three or two clades in the alignment. Box B follows our initial hypothesis of the existence of two species, while D1–D1’ demonstrated that C. komarekiana has a more diverse ITS structure (higher intrapopulation diversity) than C. lacustris. Further analysis with rpoC1 sequences (Fig. 6) and the secondary structure of the ITS region (D1–D1’ helix; Fig. 7) showed a certain genetic diversity in the C. komarekiana group, where UTEX 1580 and CCIBt 3523 were slightly different from the other strains. The Box B secondary structure (Fig. 7) clearly showed only two distinct groups (C. komarekiana and C. lacustris), where C. komarekiana contains a 12 bp loop in the mid-region of the stem that is not found in C. lacustris. The V3 region is conserved between the two species (Fig. 7).

To verify the specific-level diversity, a molecular multilocus approach based on the concatenated sequences of the 16S rRNA gene, rpoC1 and rbcL loci and the ITS region was also applied, reconstructing the phylogenetic history of the isolates (Fig. 8). These loci have a smaller sampling size than the 16S rRNA gene due to the smaller numbers of sequences available for comparison, but the dataset was sufficient to understand the relationships among the strains. On this basis, we propose two novel species among our strains: C. komarekiana (for strains CCIBt 3277, CCIBt 3279, CCIBt 3523, CCALA 155 and UTEX 1580) and C. lacustris (for strain CCIBt 3261).

The clade formed by C. lacustris CCIBt 3261 and Phormidium sp. KS is separated in the 16S rRNA gene phylogeny (Fig. 5) in a well-supported subclade (bootstrap values of 94 % NJ and 95 % ML and Bayesian posterior probability of 0.85), and this separation was also confirmed in the multilocus analyses (Figs. 6 and 8). Indeed, the 16S rRNA gene sequence similarity between C. lacustris CCIBt 3261 and C. komarekiana CCIBt 3277 is 98.94 % (based on 1033 positions). Morphologically, CCIBt 3261 presented a unique feature: an apical cell with a pronounced calyptra (Fig. 4c–e), distinguishing it from the other strains. Additionally, the habitat of this organism, a freshwater lake, is different from that of the other studied strains (Table 2). According to our phylogenetic data, Phormidium sp. KS probably belongs to C. lacustris, as its 16S rRNA gene sequence shares 99.7 % similarity and the secondary structures of Box B and D1–D1’ in the ITS were identical (Fig. 7). Strain KS was isolated from a Japanese aquarium, and the mechanism of its mobility on solid agar has been studied (Sato et al., 2014). As we were not able to study this strain, it is still necessary to confirm its inclusion in the novel species C. lacustris.

Despite the overall morphological similarity, two subclades were visible in C. komarekiana (Figs 6 and 8; subclades A and B) based on the rpoC1 gene and the concatenated sequences. Interestingly, there was some morphological and ecological diversity in subclade A (CCIBt 3277, CCIBt 3279 and CCALA 155), though the branch length was short in both the rpoC1 and concatenated trees (Figs 6 and 8). Strain CCALA 155 is the most different, with the absence of gas vesicles, the slightly capitate end cell and the absence of a sheath. However, this strain has been maintained in culture since 1961, and some genetic modifications could have occurred, such as the loss of aerotopes and sheath and some modifications of the apical cell. Moreover, its duplicate strain, SAG 75.79, still shows a wide sheath, similar to those of other strains belonging to C. komarekiana. Inactivation of gas vesicle synthesis has been reported in other genera, such as Microcystis Küting ex Lemmermann, due to gene rearrangements with the insertion of sequences (a putative transposase) at different sites (Mlouka et al., 2004). Besides, the full development of the apical cell is difficult to observe in culture, due to intense fragmentation of trichomes.

Subclade B, formed by CCIBt 3523 and UTEX 1580, was well supported (Figs 6 and 8). However, morphologically, the two strains are similar to CCIBt 3277 and CCIBt 3279 (subclade A; Figs 6 and 8). The ecology of CCIBt 3523 and UTEX 1580 is similar, since both were isolated from an aquarium, as was CCIBt 3279. Thus, the reconstructed phylogeny, which includes data on the evolutionary history of different genes from the strains in subclades A and B (Figs 6 and 8), indicated two different populations of the same species. Some morphological differences among them can be related to the maintenance of material in culture, since cultivation has long been known to cause morphological alterations (Stanier et al., 1971; Palinska et al., 1996; Nadeau et al., 2001; Casamatta et al., 2005).

Several authors have proposed the ITS region as an adequate marker for species-level identification of taxa (Wilmutte, 1994; Boyer et al., 2001, 2002; Johansen et al., 2011; Kling et al., 2012). For further comparison between species and to resolve cryptic diversity, the secondary structure of different regions of the ITS has been used as an important marker (Bohunická et al., 2011; Johansen et al., 2011; Perkerson et al., 2011).

This study indicates that a single-gene genealogy does not necessarily match the true evolutionary history of a species, as the 16S rRNA gene did not show the existence of subclades in the species C. komarekiana. Therefore, we
recommend the combination of several genes for better-supported hypotheses in phylogenetic inference. Furthermore, it was confirmed that morphological analyses of strain material could lead to an underestimate of the ‘true’ biodiversity, as discussed by Rejmánková et al. (2004).

**Fig. 3.** (a–e) *C. komarekiana* CCIBt 3279. Apical cell fully developed (a); trichomes with initial loss of aerotopes (b); trichomes completely without aerotopes (c); detail of the sheath attached to trichome (d); wide sheath (e). (f–j) *C. komarekiana* CCIBt 3523. Apical cell fully developed (g); trichomes totally with aerotopes (h); trichomes with initial loss of aerotopes (i); detail of wide sheath (j). (k–o) *C. komarekiana* UTEX 1580. Trichomes with aerotopes and detail of apical cell strongly capitate (k); trichomes with initial loss of aerotopes (l); trichomes completely without aerotopes (m); wide sheath (n); hormogonia with aerotopes (o). Bars, 10 μm.

**Fig. 4.** (a–e) *Cephalothrix lacustris* CCIBt 3261. Trichomes totally with aerotopes and detail of apical cell development (a–c); apical cell strongly capitate with calyptra (fully developed) (d); detail of sheath (e). (f–h) *C. komarekiana* CCALA 155. Apical cell development (f, g); apical cell slightly capitate (fully developed) (h). (i, j) *C. komarekiana* SAG 75.79. Detail of trichome (i); wide sheath (j). Bars 10 μm.
Description of Cephalothrix gen. nov.

Cephalothrix (C. F. S. Malone et al.) (Ce.pha’lo.thrix. Gr. fem. n. cephalè head; Gr. fem. n. thrix hair; N.L. fem. n. Cephalothrix hair-like capitate organism).

Thallus fasciculated, blue–green. Cylindrical and straight trichomes, slightly attenuated, sometimes bent at the end, unconstricted or slightly constricted at the crosswalls, facultative hyaline and firm sheath, attached to trichome or wider. Cells wider than long, 2.0–3.4 (mean 2.6) μm long, 4.2–6.5 (mean 5.2) μm wide. Apical cell strongly or slightly capitate, sometimes with conical calyptra, cell content with facultative aerotopes. Hormogonia formation by biconcave necridic cells. Thylakoids radially oriented. The type species is Cephalothrix komarekiana.

Description of Cephalothrix komarekiana (C. F. S. Malone et al.) sp. nov.

Cephalothrix komarekiana (ko.ma.re.ki.a’na. N.L. fem. adj. komarekiana to honour Professor Jiří Komárek).

Cylindrical and straight trichomes, bent at the end, slightly attenuated, unconstricted or constricted at the crosswalls, facultative hyaline and firm sheath, narrow or wide. Cells wider than long, 2.0–3.5 μm long, 4.8–7.3 μm wide. Apical cell slightly or strongly capitate, cell content with facultative aerotopes. Hormogonia formation by biconcave necridic cells. Holotype: Brazil, Mato Grosso do Sul State, municipality of Corumbá, Brazilian Pantanal wetlands, (herbarium preparation of cultured material CCIBt 3277), Herbarium of the Institute of Botany, SP, Brazil (SP427.779).

Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences (1033 bp) and reconstructed using ML analysis of evolutionary distances determined by the Hasegawa–Kishino–Yano + G + I model. NJ/ML bootstrap values (above 50 %)/Bayesian posterior probabilities are provided for each node. Sequences determined in this work are indicated in bold. Bar, 0.02 substitutions per nucleotide position.

Fig. 6. Phylogenetic tree based on rpoC1 gene sequences (520 bp), reconstructed using ML analysis of evolutionary distances determined by the Tamura–Nei + G + I model. NJ/ML bootstrap values (above 50 %)/Bayesian posterior probabilities are provided for each node. Sequences determined in this work are indicated in bold. Bar, 0.05 substitutions per nucleotide position.
Reference strain: CCIBt 3277.

Type location: grows in an alkaline lake in the Brazilian Pantanal wetlands (18° 57’ 42” S 56° 37’ 26” W), Mato Grosso do Sul State, Brazil.

**Description of Cephalothrix lacustris (C. F. S. Malone et al.) sp. nov.**

*Cephalothrix lacustris* (la.cus’tris. N.L. fem. adj. lacustris inhabiting lakes).

Cylindrical and straight trichomes, bent at the end, slightly attenuated and constricted at the crosswalls, hyaline and firm sheath, attached to trichome or wide. Cells wider than long, 2.2–3.1 μm long, 5.6–6.7 μm wide. Apical cell strongly capitate with conical calyptra, cell content with facultative aerotopes. Hormogonia formation by biconcave necridic cells.

Holotype: Brazil, Rio Grande do Sul State, municipality of Porto Alegre, Botanical Garden, (herbarium preparation of cultured material CCIBt 3261), Herbarium of the Institute of Botany, SP, Brazil (SP428.686).

---

**Fig. 7.** Secondary structures of the D1–D1’, Box B and V3 helix regions of Cephalothrix and Phormidium sp. KS.
Table 2. Comparative morphology of strains belonging to the two novel morphospecies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Life cycle</th>
<th>Gas vesicles</th>
<th>Trichome apex</th>
<th>Apical cell</th>
<th>Calyptra</th>
<th>Mucilaginous sheath</th>
<th>Cell size (µm)</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diameter</td>
<td>Length</td>
</tr>
<tr>
<td><em>C. komarekiana</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIBt 3277</td>
<td>Two phases</td>
<td>Present</td>
<td>Slightly attenuated and constricted, bent</td>
<td>Strongly capitate</td>
<td>Absent</td>
<td>Attached to trichome or wide</td>
<td>4.8–6.6</td>
<td>2.0–3.4</td>
</tr>
<tr>
<td>CCIBt 3279</td>
<td>Two phases</td>
<td>Present</td>
<td>Slightly attenuated and constricted, bent</td>
<td>Strongly capitate</td>
<td>Absent</td>
<td>Attached to trichome or wide</td>
<td>5.8–7.3</td>
<td>2.4–3.5</td>
</tr>
<tr>
<td>UTEX 1580</td>
<td>Two phases</td>
<td>Present</td>
<td>Not attenuated and not constricted, slightly bent</td>
<td>Strongly capitate</td>
<td>Absent</td>
<td>Attached to trichome or wide</td>
<td>5.8–7.1</td>
<td>2.4–3.2</td>
</tr>
<tr>
<td>CCIBt 3523</td>
<td>Two phases</td>
<td>Present</td>
<td>Slightly attenuated and not constricted, bent</td>
<td>Strongly capitate</td>
<td>Absent</td>
<td>Attached to trichome or wide</td>
<td>5.8–6.8</td>
<td>2.3–3.2</td>
</tr>
<tr>
<td>CCALA155</td>
<td>One phase</td>
<td>Absent</td>
<td>Not attenuated and not constricted, straight</td>
<td>Slightly capitate</td>
<td>Absent</td>
<td>Thin (CCALA155) or wide (SAG 75.79)</td>
<td>4.8–6.4</td>
<td>2.1–3.0</td>
</tr>
<tr>
<td><em>C. lacustris</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCIBt 3261</td>
<td>Two phases</td>
<td>Present</td>
<td>Slightly attenuated and constricted, bent</td>
<td>Strongly capitate</td>
<td>Conical</td>
<td>Attached to trichome</td>
<td>5.6–6.7</td>
<td>2.2–3.1</td>
</tr>
</tbody>
</table>

Reference strain: CCIBt 3261.

Type location: grows in a freshwater lake in the Botanical Garden (30° 03’ 2.48” S 51° 10’ 46” W), municipality of Porto Alegre, Rio Grande do Sul State, Brazil.

**ACKNOWLEDGEMENTS**

This study was supported by grants from the National Council for Scientific and Technological Development (CNPq) (562213/2010-4). C.F.S.M. received a graduate fellowship from the State of São Paulo Research Foundation (FAPESP – 2010/51666-0 and 2012/18528-9). M. F. F. would like to thank CNPq for a research fellowship (306607/2012-3). H. D. L. would like to thank FRS-FNRS for a postdoctoral fellowship at the University of Liège (Belgium) and the Drouet Fund (Dept Botany, NMNH-SI) for support. A.W. is a Research Associate of the FRS-FNRS of Belgium. We are very grateful to Professor Dr J. Komárek for discussions of morphology and phylogenetics that have greatly improved our paper. We would like to thank Dr T. Filgueiras for help with Latin names.

**REFERENCES**


