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*Pantanalinema* gen. nov. and *Alkalinema* gen. nov.: two novel pseudanabaenacean genera  
(Cyanobacteria) isolated from saline-alkaline lakes

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Running title: *Pantanalinema* gen. nov. and *Alkalinema* gen. nov.

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Note: The GenBank accession numbers for the 16-23S rRNA of the six strains of *Pantanalinema* gen. nov. and four strains of *Alkalinema* gen. nov. are, respectively, KF246483, KF246484, KF246488, KF246501, KF246503, HM105583 and KF246494- KF246497.

### Abstract

The genus *Leptolyngbya* Anagnostidis & Komárek (1988) was described from a set of strains identified as “LPP-group B”. The morphology within this group is not particularly informative and underestimates the group’s genetic diversity. In the present study, two new pseudanabaenacean genera related to *Leptolyngbya* morphotypes, *Pantanalinema* gen. nov. and *Alkalinema* gen. nov., are described under the provisions of the International Code of Nomenclature for algae, fungi, and plants, based on a polyphasic approach. *Pantanalinema* gen. nov. has sheaths and trichomes with slight gliding motility, which distinguish this genus from *Alkalinema* gen. nov., that possesses trichomes arranged in an ornate (interwoven) pattern. *Pantanalinema* and *Alkalinema* 16S rRNA gene sequences exhibited low identity compared to each other ( $\leq 91.6\%$ ) and compared to other sequences from known pseudanabaenacean genera ( $\leq 94.3$  and  $93.7\%$ , respectively). In a phylogenetic reconstruction, six *Pantanalinema* and four *Alkalinema* sequences formed two separate and robust clades (99% of bootstrap value), with the genera *Oculatella* and *Phormidesmis*, respectively, as the closest related groups. Intergenic spacer (ITS) sequences and secondary structures of *Pantanalinema* and *Alkalinema* did not correspond to any previous descriptions. The *Pantanalinema* and *Alkalinema* strains were able to survive and produce biomass at a range of pH values (4–11) and were also able to alter the culture medium to pH values ranging from 8.40–9.9. These data indicate that cyanobacterial communities in underexplored environments, such as the Pantanal wetlands, are promising sources of novel taxa.

**Keywords:** 16S rRNA, ITS secondary structures, *Leptolyngbya*-like, pH tolerance, Pantanal wetlands.

Cyanobacteria constitute a bacterial phylum with great morphological and metabolic diversity and are ubiquitous on Earth, including extreme environments (Castenholz & Waterbury, 1989). The classification of this microbial group has long been based on morphological traits, which are currently insufficient to delimit genera and species (Taton *et al.*, 2003; Taton *et al.*, 2006; Turicchia *et al.*, 2009;

Zammit *et al.*, 2012; Genuário *et al.*, 2013; Silva *et al.*, 2014). In attempting to clarify cyanobacterial classification, many studies have applied a combination of morphological, ecological and molecular data (Perkerson III *et al.*, 2011; Hašler *et al.*, 2012; Zammit *et al.*, 2012; Andreote *et al.*, 2014; Silva *et al.*, 2014), and phylogenies based on the 16S rRNA gene have been widely used for generic definitions (Fox *et al.*, 1992; Jonhansen & Casamata, 2005; Perkerson III *et al.*, 2011). Phylogenies based on this gene have demonstrated that some morphologically described genera are well defined in terms of evolutionary relationships (Komárek & Kaštovský, 2003; Willame *et al.*, 2006; Komárek, 2010). However, data that have been obtained from 16S rRNA phylogenies have also led to the separation and definition of new genera, such as *Desmonostoc* (Hrouzek *et al.*, 2013), *Halotia* (Genuário *et al.*, 2014), *Oxynema* (Chatchawan *et al.*, 2012), *Nodosilinea* (Perkerson III *et al.*, 2011) and *Oculatella* (Zammit *et al.*, 2012), which emerged from strains of the genera *Nostoc*, *Phormidium* and *Leptolyngbya*, respectively.

The genus *Leptolyngbya* Anagnostidis & Komárek (1988) was described from a set of strains identified as “LPP-group B” by Rippka *et al.* (1979). This group includes strains of *Lyngbya*, *Phormidium* and *Plectonema* characterised by straight trichomes with isodiametric or cylindrical cells, variable degrees of constriction between adjacent cells, reproduction by trichome breakage, presence or absence of a sheath and facultative motile trichomes (Rippka *et al.*, 1979). Komárek and Anagnostidis (2005) defined the members of the genus *Leptolyngbya* as strains with thin trichomes (0.5–3.5 µm wide) that are rarely solitary, floating or attached to the substrate, usually not attenuated at the ends and not capitate, facultative with firm, thin, hyaline sheaths, rarely pseudobranched and with parietal thylakoids. However, the morphology of the genus *Leptolyngbya* is not taxonomically informative because some morphological traits can overlap among different species/genera (Johansen *et al.*, 2011). Several reports indicate that the genetic diversity within this group exceeds its morphological diversity (Casamata *et al.*, 2005; Jonhansen *et al.*, 2011; Silva *et al.*, 2014; Andreote *et al.*, 2014). Furthermore, *Leptolyngbya* is recognised as clearly polyphyletic (Johansen *et al.*, 2011; Perkerson III *et al.*, 2011; Zammit *et al.*, 2012), reinforcing the notion that this genus requires a taxonomic evaluation that takes into account more distinguish characters, such as molecular and ecophysiological data. Consequently, the description of new genera is expected (Albertano & Kováčik 1994, Taton *et al.* 2003, Casamata *et al.* 2005, Komárek, 2007; Johansen *et al.*, 2011).

Several saline-alkaline lakes in the Brazilian Pantanal wetland harbour cyanobacterial communities that have been underexplored (Andreote *et al.*, 2014). These researchers isolated a number of homocytous cyanobacterial strains that are genetically diverse based on 16S rRNA phylogeny, and some of these strains are unrelated to the *Leptolyngbya sensu stricto* cluster. These *Leptolyngbya*-like strains were thoroughly analysed in this study, and from this analysis, two new Pseudanabaenacean genera emerged. The descriptions of *Pantanalinema* and *Alkalinema* were based on a polyphasic approach taking into consideration morphological traits, the phylogeny of the 16S rRNA gene, ITS secondary structures and growth response to pH culture conditions.

The cyanobacterial strains investigated in this study were recovered from saline-alkaline lakes (Pantanal da Nhecolândia, municipality of Aquidauana, Mato Grosso do Sul State, Brazil). Nine strains were previously isolated and identified as Pseudanabaenaceae (Andreote *et al.*, 2014) (Table 1). These strains are maintained in the Center for Nuclear Energy in Agriculture Collection/University of São Paulo (CENA/USP), Brazil. One strain was isolated in the current study, and it is kept in the Culture Collection at the Institute of Botany (CCIBt), São Paulo, Brazil (Table 1). Unicyanobacterial cultures were maintained under white fluorescent light (40 µmol photons·m<sup>-2</sup>·s<sup>-1</sup>) with a 14:10 h light:dark (L:D) cycle at 25 °C ±1 °C. Cells from all strains were preserved in formaldehyde and deposited in the “Maria Eneyda P. Kauffman Fidalgo” Herbarium (SP) of the Institute of Botany, Brazil. Morphological descriptions were obtained according to the systematic scheme proposed by Komárek & Anagnostidis (1989, 2005) and revised by Hoffmann *et al.* (2005) and Komárek (2010). Additionally, recent studies dealing with the description of new genera were considered.

Total genomic DNA was obtained using a CTAB method, specific for cyanobacterial strains (Fiore *et al.*, 2000). The 16-23S rRNA gene sequence from the strain CCIBt1046 and the 16S-23S intergenic spacer (ITS) sequences from the remaining strains were PCR amplified using the 27F1/23S30R primers (Taton *et al.* 2003). PCR amplifications, cloning and sequencing were performed as described by Genuário *et al.* (2013). The sequenced fragments were assembled into one contig with the software Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, USA), and only bases with >20 quality were considered (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). The 16S rRNA nucleotide sequences available for the nine pseudanabaenacean strains (Andreote *et al.*, 2014), the generated sequence of the strain CCIBt1046 and related sequences retrieved from GenBank were aligned, refined (16S rRNA matrix with 1,491 base pair length) and used for phylogenetic reconstruction. The Maximum Likelihood (ML) method was used for phylogenetic inference with the MEGA program package, version 5 (Kimura 2-parameter model of sequence evolution) (Tamura *et al.*, 2011). Robustness of the phylogenetic tree was estimated by bootstrap analysis using 1,000 replications. The generated 16-23S ITS sequences were used for secondary structure folding analysis. The folding of conserved regions (D1-D1', Box B and V3 regions) was analysed using Mfold WebServer (Zuker, 2003). Default conditions were used, except for the use of structure draw mode untangle with loop fix. tRNA genes were found using tRNAscan-SE 1.21 (Schattner *et al.*, 2005).

**Table 1.** Origin and morphological features of strains of novel pseudanabaenacean genera (*Pantanalinema* and *Alkalinema*) isolated from water of saline-alkaline lakes of the Pantanal

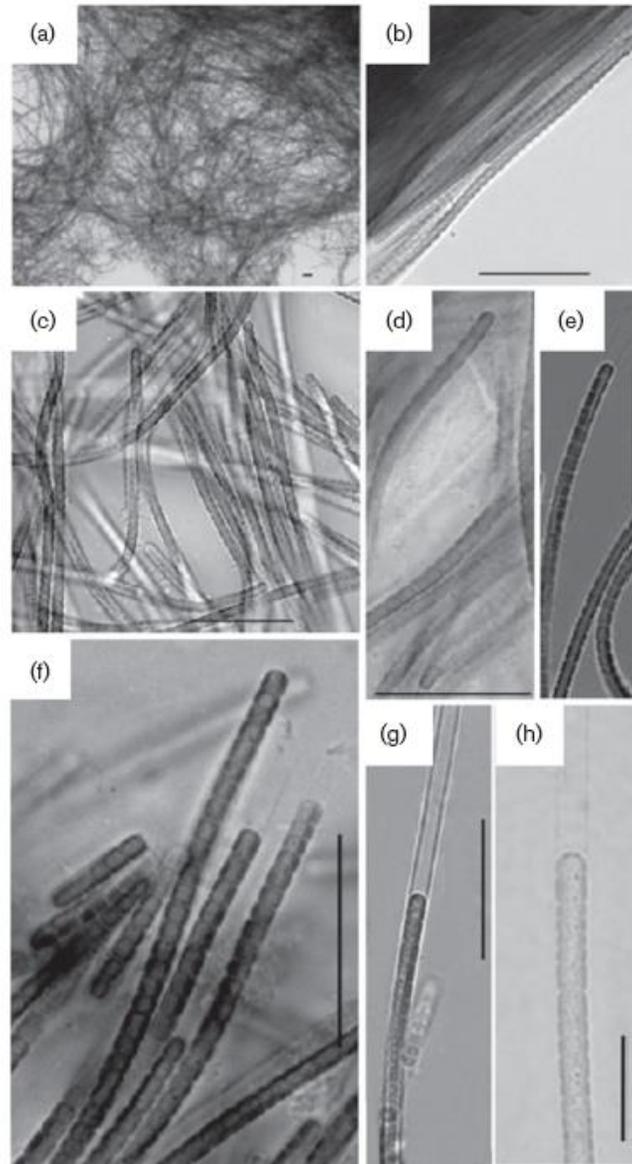
Strain	Origin	Length (µm)	Width (µm)	N	S	FB	M
<i>Pantanalinema rosaneae</i>							
CENA516	Salina Verde (19° 28' 13" S 56° 3' 22" W)	1.2–2.3	1.9–2.1	–	+	–	+
CENA517	As above	1.6–2.4	1.5–2.3	–	+	–	+
CENA521	As above	1.4–2.1	1.6–2.1	–	+	–	+
CENA537	Salina Grande (19° 26' 56" S 56° 7' 45" W)	1.8–3.1	1.9–2.5	–	+	–	+
CENA539	Salina Verde	1.3–2.9	1.9–2.5	–	+	–	+
CCIBt1046	Salina da Ponta (18° 58' 58" S 56° 39' 36" W)	1.4–2.9	2.3–3.1	–	+	–	+
<i>Alkalinema pantanalense</i>							
CENA528	Salina Preta (19° 26' 56" S 56° 7' 55" W)	2.0–4.1	1.7–2.2	–	–	–	–
CENA529	As above	1.6–2.2	1.2–1.7	–	–	–	–
CENA530	As above	1.7–3.0	1.0–1.7	–	–	–	–
CENA531	As above	1.5–2.8	1.5–2.2	–	–	–	–

The presence or absence of the following morphological features is shown: N, necridial cells; S, sheaths; FB false branching; M, motility.

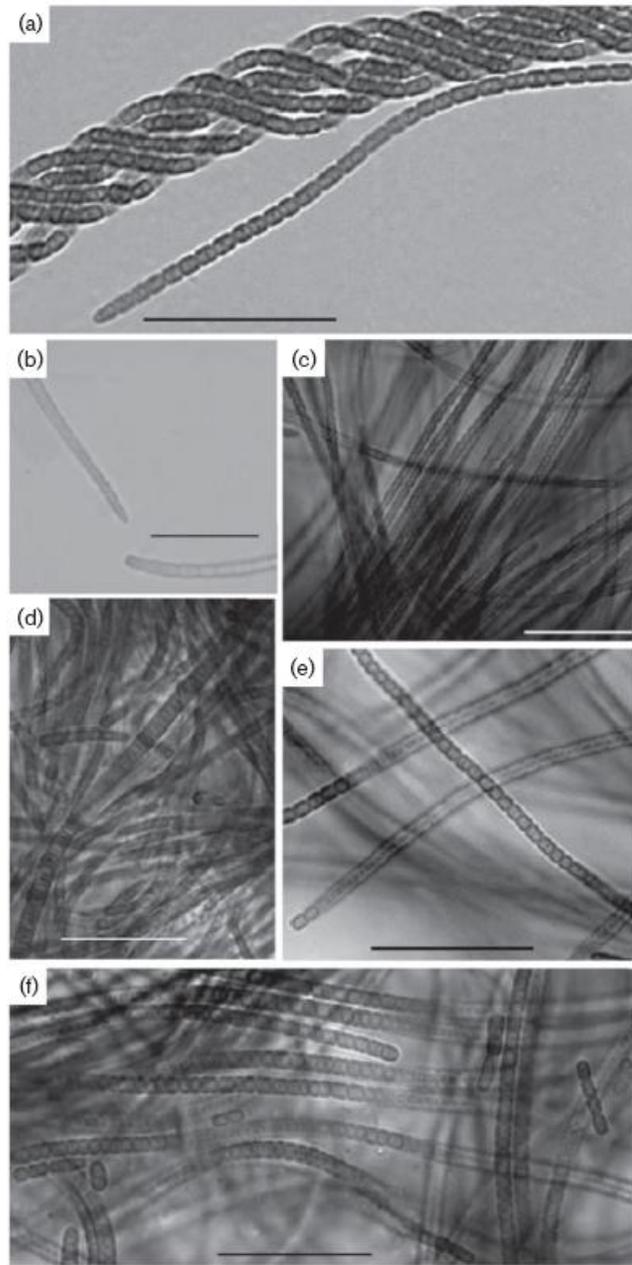
All strains were tested for their ability to survive and/or grow under different pH conditions. pH values were adjusted using 1 M HCl or 2 M NaOH solutions. Initially, each strain was grown for fifteen days in 250 mL glass flasks containing 50 mL of BG-11 medium under the temperature and light conditions described above. The cyanobacteria trichomes were fragmented via syringe flows and used as pre-inoculum (1 % of biomass v/v) for the assays. The experiments were conducted in 15 mL glass flasks containing 5 mL of liquid BG-11 with different pH values: 4, 5, 7, 9 and 11. Each strain was grown for 14 days, and the final pH value was measured at the end of the experiment. The effects of pH were analysed by determining dry weight (DW) after cultivation. To determine DW, flasks were dried for seven days or more to obtain a constant mass. All experiments were conducted in duplicate. Two flasks containing 5 mL of liquid BG-11 (pH 7.2) without inoculum were used as controls. The DW data were analysed, and the values were compared using the Kruskal-Wallis non-parametric test with a significance level of 5 %. All calculations were performed using the R program (2012).

According to morphological analyses, the ten strains possessed traits related to morphotypes of *Leptolyngbya sensu lato*. The cells were slightly cylindrical, generally isodiametric, and longer/shorter (1.2–4.1 µm) than wide (1.1–3.1 µm). The colours of the trichomes were olive-green or reddish-brown

and could form mats. Necridial cells, false branches and aerotopes were not observed, but irregular scarifying cells (false necridial cells) were observed in some cases. These strains were distinguishable as two groups based on the apical cell, the presence/absence of sheaths and motility. A total of six strains (CENA516, CENA517, CENA521, CENA537, CENA539 and CCIBt1046) had a rounded to slightly conical apical cell, motile trichomes with sheaths and hormogonia formation via cell disintegration (Figure 1; Table 1). The remaining strains (CENA528, CENA529, CENA 530 and CENA 531) exhibited a narrowed or rounded-conical apical cell, no sheath and non-motile (Figure 2; Table 1). In addition, the trichomes of the second group of strains exhibited an ornated mat (interwoven) pattern and hormogonia formation via trichome fragmentation (Figure 2).



**Fig. 1.** Light photomicrographs of strains of *Pantanalinema rosanae* gen. nov., sp. nov. (a–c) *P. rosanae* CENA516; (d) *P. rosanae* CENA517; (e) *P. rosanae* CENA521; (f) *P. rosanae* CENA539; (g) *P. rosanae* CENA537; (h) *P. rosanae* CCIBt1046. Bars, 20  $\mu\text{m}$  (a–g), 10  $\mu\text{m}$  (h).



**Fig. 2.** Light photomicrographs of strains of *Alkalinema pantanalense* gen. nov., sp. nov. (a) *A. pantanalense* CENA530; (b) *A. pantanalense* CENA528; (c, e) *A. pantanalense* CENA529; (d, f) *A. pantanalense* CENA531. Bars, 20  $\mu$ m.

Nearly complete 16S rRNA gene sequences (1,412–1,415 bp) for nine strains were obtained by Andretote *et al.* (2014), and one was generated in this study (strain CCIBt1046), which was 1,415 bp. Additionally, the 16-23S ITS regions of all ten strains were sequenced (474–570 bp), characterised and folded (Table 2, Figure 4).

The 16S rRNA gene sequences of the six strains (which had motile trichomes and sheaths) exhibited low identities (<94.3 %) compared to related sequences available in GenBank. Comparative analysis within these six sequences indicated nucleotide identities  $\geq 99.5$  %. In the phylogenetic

reconstruction based on 16S rRNA gene sequences (Figure 3), the studied strains grouped together in a robust clade (99 % of bootstrap values), to which two sequences of uncultured bacteria from saline-alkaline soil also belonged (Valenzuela-Encinas *et al.*, 2009). This clade was related to *Oculatella*, *Leptolyngbya sensu lato* and *Geitlerinema* sp. PPC8501 (Figure 3). The 16-23S ITS sequences from these six strains, ranging from 474 to 478 bp, and the tRNAs were absent. Four different secondary structures were observed for D1-D1', three for Box-B and two for the V3 region (Table 2 and Figure 4).

**Table 2.** Lengths of 16S–23S rRNA ITS regions of strains of *Pantanalinema* and *Alkalinema*

Strain	Length of 16S–23S rRNA ITS region (nt)				
	D1–D1'	tRNA(Ile)	tRNA(Ala)	Box-B	V3
<i>Pantanalinema rosanaeae</i>					
CENA516	81	Absent	Absent	44	58
CENA517	81	Absent	Absent	44	58
CENA521	81	Absent	Absent	44	58
CENA537	82	Absent	Absent	43	57
CENA539	82	Absent	Absent	44	57
CCIBt1046	82	Absent	Absent	44	57
<i>Alkalinema pantanalense</i>					
CENA528	64	74	73	48	56
CENA529	64	74	73	48	56
CENA530	64	74	73	48	56
CENA531	64	74	73	48	56

The remaining four strains shared 16S rRNA sequence identities lower than 93.5 % when compared with those retrieved from GenBank. These four sequences shared identities  $\geq 99.6$  % and formed a stable clade (99 % of bootstrap values). This clade had an external branch consisting of an orphan sequence of cf. *Leptolyngbya* sp. Greenland-9, which was isolated from alkaline soil (Roeselers *et al.*, 2007). In addition, this clade was related to *Phormidesmis*, *Leptolyngbya frigida* ANT.L53B.2 and *Phormidium* sp. SAG 37.90 (Figure 3). The ITS sequences of these four strains were 570 bp in length, and both tRNAs (Ile and Ala) were identified (Table 2 and Figure 4).

The low identities of the 16S rRNA gene sequences, the distinct phylogenetic positions and the dissimilarities between the ITS sequences strengthen the differences between these two new groups and other known genera, such as *Leptolyngbya*, *Oculatella*, *Phormidesmis* and *Nodosilinea*. Morphological, molecular and ecological data permitted the proposal of two new genera, *Pantanalinema* and *Alkalinema*, as well as the description of the type-species for each genus, *Pantanalinema rosanaeae* and *Alkalinema pantanalense*, respectively. No morphological or molecular differences were observed among the strains of each genus, and therefore, they were named at the same species level; the diagnoses are presented below.

The ten strains tolerated the pH values tested (4, 5, 7, 9 and 11) and were able to grow. Significant differences were not observed in terms of biomass production for the different treatments (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 1.3848, df = 4, p-value = 0.8468). After fourteen days, pH values of the BG-11 culture medium became 8.40–9.9, demonstrating that these strains were able to induce alkalinity of the culture medium. The pH values of the BG-11 culture medium used as a control did not change significantly, ranging from 7.2 to 7.4 (mean values).

#### **Diagnosis for *Pantanalinema* gen. nov. (Figure 1)**

***Pantanalinema* M.G.M.V. Vaz et al. gen. nov.** (Pan.ta.na.li.ne´ma. N.L. n. *Pantanal*, a Brazilian wetland; Gr. neut. n. *nema* thread, filament; N. L. neut. n. *Pantanalinema* a filament from Pantanal).

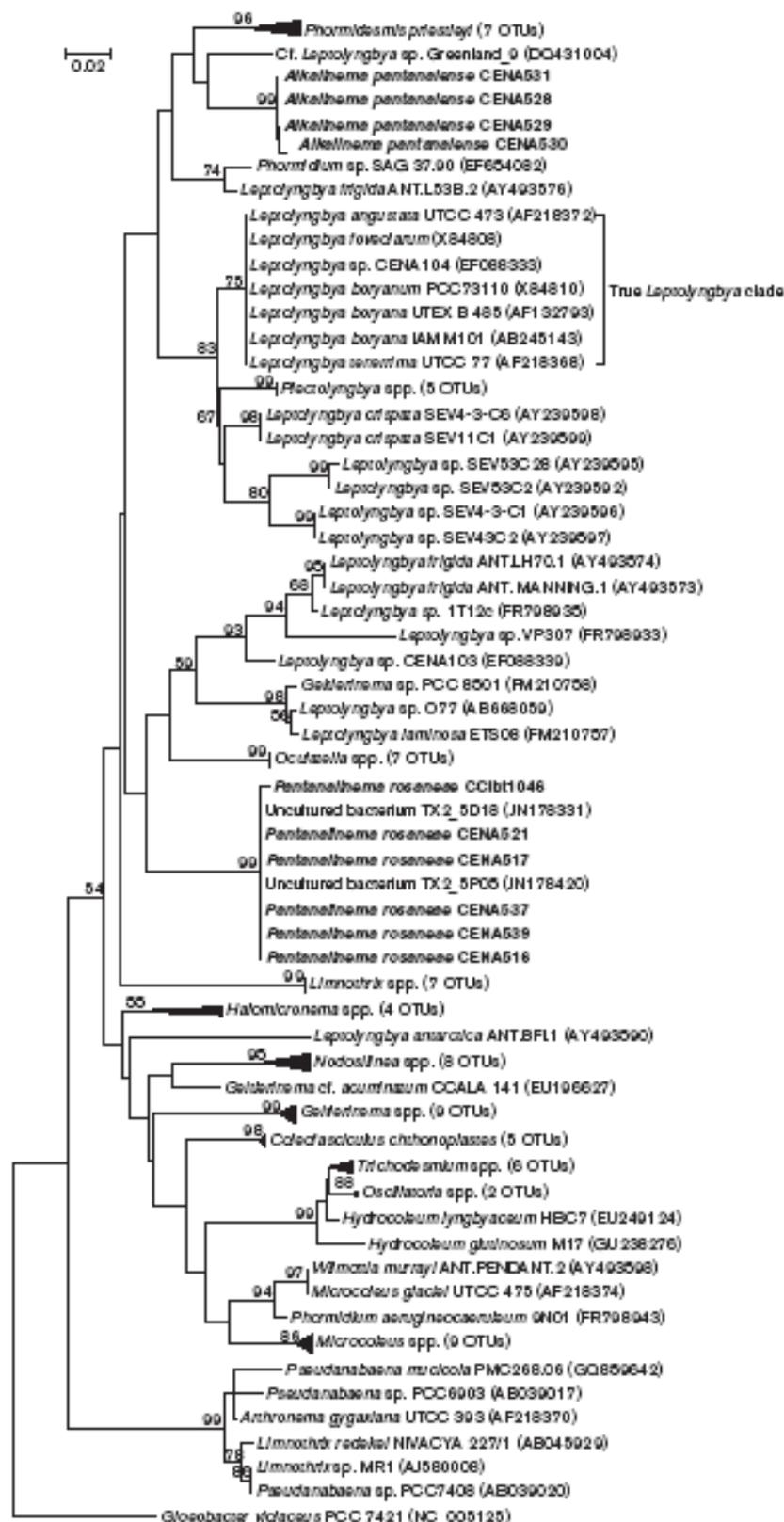
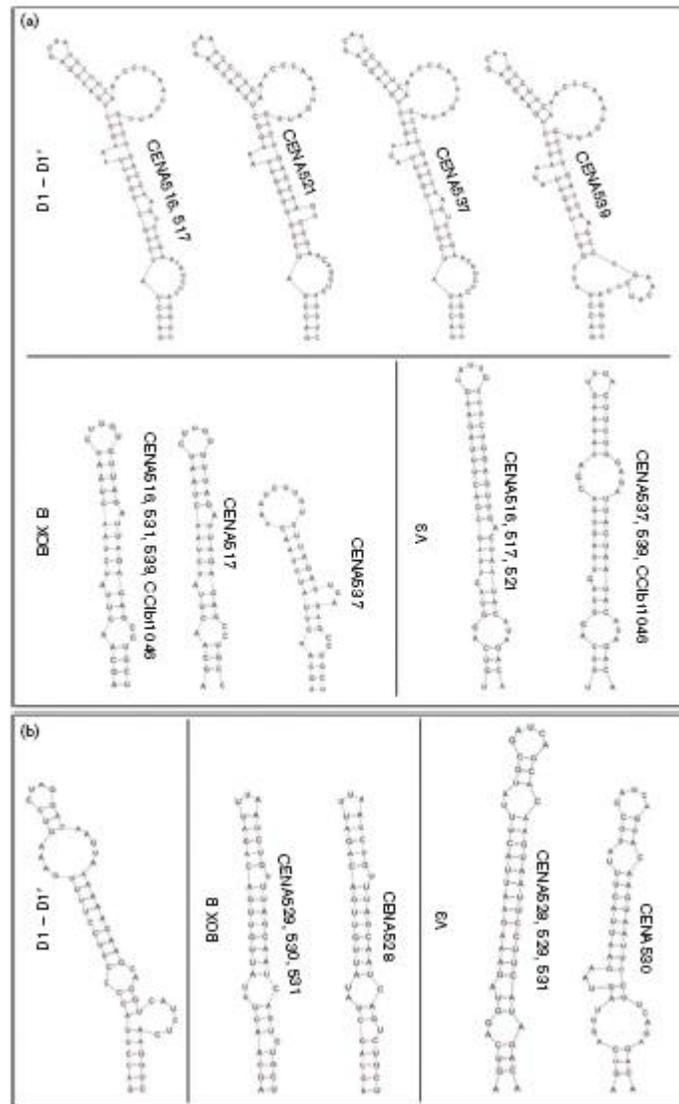


Fig. 3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains of the Pseudanabaenales and Oscillatoriales. The studied strains of *Pantanellinema* and *Alkalinema* are shown in bold. A bootstrap test involving 1000 resamplings was performed, and bootstrap values greater than 50% are noted at the corresponding nodes. Bar, 0.02 substitutions per site.

In nature, these strains grow in saline-alkaline lakes in the Brazilian Pantanal wetland. The trichomes are microscopic and in liquid media grow attached to the tube bottom and walls. Macroscopically, the colonies are an olive-green colour. Filaments are entangled and flexuous. The motile trichomes are characterised by a slight gliding motility. The cells are slightly constricted, and the cross wall is quite translucent. The sheath is hyaline, firm, attached to the trichome and always present. Cells are isodiametric or wider than longer, 1.2–3.1 µm (length), 1.5–3.1 µm (width). The apical cell is cylindrical with a rounded to slightly conical apex. Cell content is homogeneous and brownish-green or olive-green. Hormogonia formation occurs via cell disintegration (false necridial cells) or trichome fragmentation.



**Fig. 4.** Folded 16-23S ITS secondary structures of *Pantanalinema* (A) and *Alkalinema* (B) strains.

Type species: *Pantanalinema rosanae* (type strain: CENA516)

*Pantanalinema rosanae* **M.G.M.V. Vaz et al.** sp. nov. (Figure 1) [ro.sa´ne.ae. N.L. gen. n. *rosanae*, of Rosane; named after the late Rosane Aguiar, a Brazilian phycologist and cyanobacterial physiologist, *in memoriam*]

In nature, *Pantanalinema rosanae* strains grow in saline-alkaline water in the Brazilian Pantanal wetland. The trichomes are microscopic, and in liquid media, they grow as olive-green

colonies attached to the tube walls. Filaments are entangled and flexuous. The cells are slightly constricted, and the cross wall is quite translucent. The sheath is hyaline, firm, attached to the trichome and always present. Cells are isodiametric or wider than longer, 1.2–3.1  $\mu\text{m}$  (length), 1.5–3.1  $\mu\text{m}$  (width). The apical cell is cylindrical with a rounded apex. Cell content is homogeneous and brownish-green or olive-green. Hormogonia formation occurs via trichome disintegration.

Holotype: BRAZIL, Mato Grosso do Sul State, Pantanal wetlands, 05/27/2010, *Laurent Barbiero* (herbarium preparation of cultured material CENA516), Herbarium of Institute of Botany (SP428.663).

Reference strain: CENA516

Type location: Grows in water samples collected from “Salilna Verde” in the Pantanal wetlands, Mato Grosso do Sul State, Brazil.

Studied material: BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.664); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.665); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.666); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.667); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 04/22/2008 *Camila F. S. Malone* (SP400.858).

Habitat: Grows in water samples collected from “Salilna Verde” the in Pantanal wetlands.

Etymology: Named in honour of Dr. Rosane Aguiar (*in memoriam*), a Brazilian phycologist and cyanobacterial physiologist from the Federal University of Viçosa, Minas Gerais state, Brazil.

#### **Diagnosis for *Alkalinema* gen. nov.** (Figure 2)

***Alkalinema* (M.G.M.V. Vaz et al.) gen. nov.** (Al.ka.li.ne´ma. N.L. n. *alkali* (from Arabic article *al*, the; Arabic n. *qaliy*, ashes of saltwort), *alkali*; Gr. neut. n. *nema* thread, filament; N. L. neut. n. *Alkalinema*: a filament from alkaline lakes).

In nature, *Alkalinema* gen. nov. strains grow in saline-alkaline lakes in the Brazilian Pantanal wetland. The trichomes are microscopic, and in liquid media, they grow as free-floating trichomes that are distributed in the culture flask. The trichomes are frequently organised in ornated (interwoven) mats. Macroscopically, the culture has a reddish to brownish colour. Trichomes do not have sheaths but do have a diffluent mucilage and are non-motile. Cells are isodiametric or longer than wider, 1.5–4.1  $\mu\text{m}$  (length), 1.1–2.2  $\mu\text{m}$  (width) with a narrowed or rounded-conical apical cell. Cell content is homogeneous and reddish to brownish. Hormogonia formation occurs via trichome disintegration.

Type species: *Alkalinema pantanalense* (type strain: CENA528)

***Alkalinema pantanalense* M.G.M.V. Vaz et al. sp. nov.** (Figure 2) [pan.ta.nal.en´se. N.L. neut. adj. *pantanalense*, pertaining to Pantanal, a Brazilian wetland].

The trichomes are microscopic, and in liquid media, they grow as free-floating trichomes that are evenly distributed in the culture flask. Macroscopically, the culture has a reddish to brownish colour. The trichomes do not have sheaths but do have a diffluent mucilage and are non-motile. They are organised in ornated (interwoven) mats. Cells are isodiametric or longer than wide, 2.0–4.1  $\mu\text{m}$  (length), 1.7–2.2  $\mu\text{m}$  (width) with a narrowed apical cell. Cell content is homogeneous and reddish to brownish. Hormogonia formation occurs via trichome disintegration.

Holotype: BRAZIL, Mato Grosso do Sul State, Pantanal wetlands, 27/05/2010, *Laurent Barbiero* (SP428.668) (herbarium preparation of cultured material CENA528), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CENA528

Type location: Grows in water samples collected from “Salina Preta” in the Pantanal wetlands, Mato Grosso do Sul State, Brazil.

Studied material: BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.669); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero*

(SP428.670); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 05/27/2010, *Laurent Barbiero* (SP428.671).

Habitat: Grows in water samples collected from “Salina Preta” in the Pantanal wetlands.

Etymology: Named in reference to the sampling site, Pantanal.

The investigation of several pseudanabaenacean strains isolated from saline-alkaline lakes of the Brazilian Pantanal wetlands led to the description of two novel cyanobacterial genera, *Pantanalinema* and *Alkalinema*. Morphologically, these two novel genera are similar to strains of *Leptolyngbya*, a genus that exhibits poor morphological diacritic traits. Furthermore, the genetic diversity within this genus exceeds its morphological diversity, clearly supporting its polyphyletic status (Casamata *et al.*, 2005; Johansen *et al.*, 2011; Perkerson III *et al.*, 2011; Zammit *et al.*, 2012). Therefore, the genus *Leptolyngbya* requires a taxonomic re-evaluation and definition of diacritical features (Komárek and Hauer, 2013). Data emerging from a phylogeny based on 16S rRNA gene sequences from *Leptolyngbya sensu lato* has led to the separation, correction and definition of the new genera *Nodosilinea*, *Haloleptolyngbya* and *Oculatella* (Perkerson III *et al.*, 2011; Dadheech *et al.*, 2012; Zammit *et al.*, 2012). The phylogenetic tree based on 16S rRNA gene sequences generated in this study corroborates reports on the polyphyl status of the genus *Leptolyngbya*. The *Pantanalinema* and *Alkalinema* clades are not related to each other or to the true *Leptolyngbya* clade. In addition, the 16-23S rRNA ITS sequences and secondary structures of *Pantanalinema* and *Alkalinema* are dissimilar and do not correspond to the related clusters *Leptolyngbya* and *Oculatella*. These data support the separation of *Pantanalinema* and *Alkalinema* from other genera.

The *Pantanalinema* clade consists of sequences of six strains isolated from three saline-alkaline lakes in the Brazilian Pantanal wetlands and two sequences of uncultivated bacteria from alkaline soil in Mexico (Valenzuela-Encinas *et al.*, 2009). In the constructed phylogenetic tree, this clade was related to the *Oculatella* and *Leptolyngbya sensu lato* clusters. However, the sequences included in these two clades exhibited low identity (<93.1 %) compared to *Pantanalinema*. Likewise, the morphological traits of *Pantanalinema* also differed from those of *Oculatella*. The *Pantanalinema* genus is characterised by homogeneous cell content with brownish-green or olive-green colouration, without any spots or granulation. In contrast, the autapomorphic characteristics of *Oculatella* are the purple-red cell colour and the presence of an orange spot at the tip of the trichome, which contains a rhodopsin-like pigment (Zammit *et al.*, 2012). Furthermore, *Oculatella* and *Leptolyngbya* can, albeit rarely, possess false branches, which has not been observed for *Pantanalinema*. Comparing the sequences of the 16S-23S ITS of the *Pantanalinema* strains, stable lengths (474–478 bp) and the absence of both tRNA genes were noted. The D1-D1', Box-B and V3 regions of the *Pantanalinema* strains were longer (81–82, 43–44 and 57–58 bp, respectively) than those of *Leptolyngbya sensu stricto* and other *Leptolyngbya* morphotypes (51–65, 11–41 and 23–76 bp, respectively) (Johansen *et al.*, 2011). The D1-D1', Box-B and V3 secondary structures of the *Pantanalinema* strains demonstrated large differences in terms of the numbers and shapes of these bulges when compared with the respective structures of the *Leptolyngbya sensu lato* and *Oculatella* strains. However, the basal portions of these structures were conserved in terms of sequence and nucleotide number. The differences observed in the bulges of all regions highlighted the novelty of the *Pantanalinema* strains when compared with the most related morphotypes, corroborating the results from the phylogenetic reconstruction.

The *Alkalinema* clade consisted of the sequences of four strains isolated from a saline-alkaline lake in the Brazilian Pantanal wetlands. The most related sequences (Cf. *Leptolyngbya* sp. Greenland\_9 and *Phormidesmis* spp.) were from strains that were also isolated from alkaline environments in Greenland (Roeselers *et al.*, 2007) and Belize (Turicchia *et al.*, 2009), respectively. The *Alkalinema* clade was distantly related (identities <93.7 %) to the *Leptolyngbya sensu stricto* cluster and to

*Leptolyngbya*, *Phormidium* and *Plectolyngbya*. Morphologically, *Alkalinema* strains possessed a particular arrangement of trichomes, designated here as ornate (interwoven) mats, which differed from the trichome arrangement of strains of the genera *Leptolyngbya*, *Phormidesmis* and *Plectolyngbya*. The absence of sheaths is another diacritical feature of this new genus, while their presence is almost obligatory for *Leptolyngbya* (Rippka *et al.*, 1979; Komárek and Anagnostidis 2005) and obligatory for the genera *Plectolyngbya* and *Phormidesmis* (Taton *et al.*, 2011; Komárek *et al.*, 2009). Furthermore, cell size and shape are clearly different among these genera. The *Leptolyngbya* genus comprises cells that are 0.5 to 3.5  $\mu\text{m}$  wide and trichomes that are rarely solitary and rarely pseudobranched (Komárek and Anagnostidis 2005). The *Plectolyngbya* genus possesses trichomes that are morphologically similar to those of *Leptolyngbya*, but the false branching is obligatory, and it occasionally forms multiple trichomes in a single sheath (Taton *et al.*, 2011). The *Phormidesmis* genus is characterised by the moliniform shape of its trichomes and the presence of one or rarely two or several trichomes in a sheath (Komárek *et al.*, 2009). The four *Alkalinema* strains had 16-23 ITS sequences of the same length (570 bp), and they exhibited both tRNAs. The D1-D1' and V3 regions of these strains were shorter (64 and 56 bp, respectively) than those of *Leptolyngbya sensu stricto* and other *Leptolyngbya* strains (51–65 and 23–76 bp, respectively), while the Box-B region was longer (48 bp) than those of related genera (11–41 bp) (Johansen *et al.*, 2011). The *Alkalinema* secondary structures of the D1-D1', Box-B and V3 regions were also dissimilar compared with those from *Leptolyngbya sensu stricto* (Johansen *et al.*, 2011). As no data regarding the lengths and secondary structures of the 16-23S ITS regions of *Phormidesmis* and *Plectolyngbya* exist, no comparisons were possible.

The *Pantanalinema* and *Alkalinema* strains survived and produced biomass at the different tested pH values, which permitted them to be classified as acid and alkali tolerant. In addition, these strains were able to alter the final pH values to 8.40–9.9, characterising them as alkalophilic. Given that *Pantanalinema* and *Alkalinema* strains were not axenic, the heterotrophic bacteria community associated to the cyanobacterial filaments may be involved on the pH alteration or both together (cyanobacteria and heterotrophic bacteria) may coordinate this pH modification. In a general way, cyanobacterial strains are known as the most alkaliphilic microorganisms and frequently dominate alkaline environments, such as soda lakes or saline-alkaline lakes (Summerfield & Sherman, 2008; Mogelhoj *et al.*, 2006; Pikuta *et al.*, 2007; Dadheech *et al.*, 2012; Santos *et al.*, 2011; Santos & Sant'Anna 2010). Cyanobacterial bloom formation is usually accompanied by an elevation in pH as a consequence of the removal of CO<sub>2</sub> favoured for photosynthesis (Summerfield & Sherman, 2008). However, cyanobacteria also can survive under acidic conditions (Almer *et al.*, 1974; Kwiatkowski & Roff, 1976; Steinberg *et al.*, 1998). Despite these reports, there are relatively few studies showing how cyanobacteria tolerate acid stress and how they neutralise high-pH culture medium. It has been shown that acid-tolerant cyanobacteria maintain a neutral cytoplasmic pH, although how they maintain a strong transmembrane gradient is unknown, as is how they increase the external pH via photosynthesis (Steinberg *et al.* 1998). It was reported that the cyanobacterium *Geminocystis* sp. PCC6308 (formerly *Synechocystis* sp. PCC6308) responds to acid stress via mechanisms that involve cell concentration-dependent neutralisation of the external medium, maintenance of a transmembrane pH gradient, and maintenance of photosystem II efficiency (Huang *et al.*, 2002). Similar mechanisms might be involved in the alkalinisation of culture medium by *Pantanalinema* and *Alkalinema* strains, but these findings deserve further and more detailed investigation.

The two new genera *Pantanalinema* and *Alkalinema* were described under the provisions of the International Code of Nomenclature for algae, fungi, and plants, based on a polyphasic approach merging molecular, morphological, ecological and physiological data. Until now, *Pantanalinema* strains were found in three saline-alkaline lakes from the Brazilian Pantanal wetlands. Additionally, two sequences of uncultivated bacteria from alkaline soil in Mexico that were grouped in the same phylogenetic clade demonstrated a more widespread distribution. In contrast, *Alkalinema* was found only in one saline-alkaline lake from the Brazilian Pantanal wetlands. However, its close phylogenetic

relationship to cf. *Leptolyngbya* sp. Greenland\_9 and *Phormidesmis*, which were isolated from alkaline environments, demonstrates an ecological relationship. Phylogeny of the 16S rRNA gene sequence has been successfully used to support the separation of novel cyanobacterial genera, such as *Haloleptolyngbya*, *Coleofasciculus*, *Halomiconema*, *Plectolyngbya*, *Wilmottia*, *Phormidesmis*, *Nodosilinea*, *Oxynema*, *Oculatella*, *Mojavia*, *Chakia*, *Calochaete*, *Toxopsis*, *Iphinoe*, *Loriellpsis*, *Desmonostoc* and *Halotia* (Abed *et al.*, 2002; Řeháková *et al.*, 2007; Siegesmund *et al.*, 2008; Turicchia *et al.*, 2009; Lamprinou *et al.*, 2011; Perkerson *et al.*, 2011; Strunecký *et al.*, 2011; Taton *et al.*, 2011; Chatchawan *et al.*, 2012; Dadheech *et al.*, 2012; Lamprinou *et al.*, 2012; Zammit *et al.*, 2012; Hauer *et al.*, 2013; Hrouzek *et al.*, 2013; Komárková *et al.*, 2013; Genuário *et al.*, 2014). In addition, morphology, physiological and ecological data are complementary markers for genus delimitation and should be used to improve the descriptions of new taxa. The ITS sequences and secondary structures of *Pantanalinema* and *Alkalinema* did not correspond to previous descriptions, demonstrating that ITS can be used as an auxiliary tool for the description of new genera. Additionally, investigations of the cyanobacterial communities in different and underexplored environments, such the Pantanal wetlands, identify novel taxa, even via simple and inexpensive methods such as isolation techniques.

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