

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

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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. (type species *Pantanalinema rosanae* sp. nov.) has sheaths and trichomes with slight gliding motility, which distinguish this genus from *Alkalinema* gen. nov. (type species *Alkalinema pantanalense* sp. nov.), which possesses trichomes arranged in an ornate (interwoven) pattern. 16S rRNA gene sequences of strains of *Pantanalinema* and *Alkalinema* exhibited low identity to each other ( $\leq 91.6\%$ ) and to other sequences from known pseudanabaenacean genera ( $\leq 94.3$  and  $93.7\%$ , respectively). In a phylogenetic reconstruction, six sequences from strains of *Pantanalinema* and four from strains of *Alkalinema* formed two separate and robust clades (99% bootstrap value), with the genera *Oculatella* and *Phormidesmis*, respectively, as the closest related groups. 16S–23S rRNA intergenic spacer sequences and secondary structures of strains of *Pantanalinema* and *Alkalinema* did not correspond to any previous descriptions. The strains of *Pantanalinema* and *Alkalinema* were able to survive and produce biomass at a range of pH (pH 4–11) and were also able to alter the culture medium to pH values ranging from pH 8.4 to 9.9. These data indicate that cyanobacterial communities in underexplored environments, such as the Pantanal wetlands, are promising sources of novel taxa.

The 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, 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 *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; Johansen & Casamata, 2005; Perkerson *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 gene phylogenies have also led to the separation and definition of new genera, such as *Desmonostoc* (Hrouzek *et al.*, 2013), *Oxynema* (Chatchawan *et al.*, 2012),

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S–23S rRNA gene sequences 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.

*Nodosilinea* (Perkerson *et al.*, 2011) and *Oculatella* (Zammit *et al.*, 2012), which emerged from strains of the genera *Nostoc*, *Phormidium* and *Leptolyngbya*.

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 facultatively motile trichomes (Rippka *et al.*, 1979). Komárek & 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, facultatively motile with firm, thin, hyaline sheaths, rarely pseudobranched and with parietal thylakoids. However, the morphology of the genus *Leptolyngbya* is not taxonomically informative, since 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 (Casamatta *et al.*, 2005; Johansen *et al.*, 2011; Silva *et al.*, 2014; Andreote *et al.*, 2014). Furthermore, *Leptolyngbya* is recognised as clearly polyphyletic (Johansen *et al.*, 2011; Perkerson *et al.*, 2011; Zammit *et al.*, 2012), reinforcing the notion that this genus requires a taxonomic re-evaluation that takes into account more distinguishing characters, such as molecular and ecophysiological data. Consequently, the description of new genera is expected (Albertano & Kováčik, 1994; Taton *et al.*, 2003; Casamatta *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). The latter researchers isolated a number of homocytous cyanobacterial strains that are genetically diverse based on 16S rRNA gene phylogeny, and some of these strains are unrelated to the *Leptolyngbya sensu stricto* cluster. These *Leptolyngbya*-like strains were thoroughly analysed in the present study and, from this analysis, two new pseudanabaenacean genera emerged. The descriptions of *Pantalaninema* gen. nov. and *Alkalinema* gen. nov. were based on a polyphasic approach taking into consideration morphological traits, the phylogeny of the 16S rRNA gene, 16S–23S rRNA intergenic spacer (ITS) secondary structures and growth responses to culture pH.

The cyanobacterial strains investigated in this study were recovered from water of saline–alkaline lakes (Pantanal da Nhecolândia, municipality of Aquidauana, Mato Grosso do Sul State, Brazil). Nine strains were isolated previously and identified as members of the family 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 cycle at 25 ± 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 16S–23S rRNA gene sequence from strain CCIBt1046 and the 16S–23S rRNA ITS sequences from the remaining strains were amplified by PCR using the primers 27F1/23S30R (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 (P. Green, University 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 gene nucleotide sequences available for the nine pseudanabaenacean strains (Andreote *et al.*, 2014), the generated sequence of strain CCIBt1046 and related sequences retrieved from GenBank were aligned, refined (16S rRNA matrix with length of 1491 bp) and used for phylogenetic reconstruction. The maximum-likelihood method was used for phylogenetic inference with the MEGA program package, version 5 (Kimura's two-parameter model of sequence evolution) (Tamura *et al.*, 2011). Robustness of the phylogenetic tree was estimated by bootstrap analysis using 1000 replications. The generated 16S–23S rRNA ITS sequences were used for secondary structure folding analysis. The folding of conserved regions (D1–D1', Box B and V3 regions) was analysed using the 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-SEM 1.21 (Schattner *et al.*, 2005).

All strains were tested for their ability to survive and/or grow under different pH conditions. The medium pH was adjusted using 1 M HCl or 2 M NaOH. Initially, each strain was grown for 15 days in 250 ml glass flasks containing 50 ml BG-11 medium under the temperature and light conditions described above. 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 liquid BG-11 at pH 4, 5, 7, 9 and 11. Each strain was grown for 14 days, and the final pH was measured at the end of the experiment. The effects of pH were analysed by determining dry weight after cultivation. To determine dry weight, flasks were dried for 7 days or more to obtain

**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 rosanaeae</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.

a constant mass. All experiments were conducted in duplicate. Two flasks containing 5 ml liquid BG-11 (pH 7.2) without inoculum were used as controls. The dry weight 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 (R Core Team, 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 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. Six strains (CENA516, CENA517, CENA521, CENA537, CENA539 and CCIBt1046) had a rounded to slightly conical apical cell, motile trichomes with sheaths and hormogonium formation via cell disintegration (Fig. 1, Table 1). The remaining strains (CENA528, CENA529, CENA 530 and CENA 531) exhibited a narrowed or rounded-conical apical cell, no sheath and were non-motile (Fig. 2, Table 1). In addition, the trichomes of the second group of strains exhibited an ornate mat (interwoven) pattern and hormogonium formation via trichome fragmentation (Fig. 2).

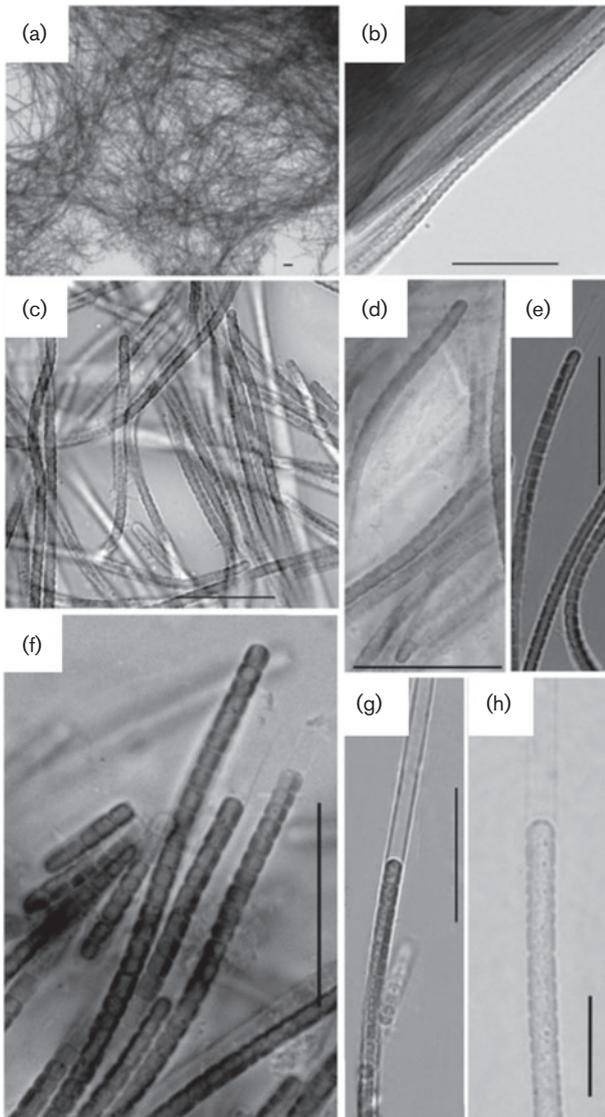
Nearly complete 16S rRNA gene sequences (1412–1415 bp) for nine strains were obtained by Andreote *et al.* (2014), and one was generated in this study (strain CCIBt1046), which was 1415 bp. Additionally, the 16S–23S rRNA ITS regions of all ten strains were sequenced (474–570 bp), characterized and folded (Table 2).

The 16S rRNA gene sequences of the six strains that had motile trichomes and sheaths exhibited low identities (<94.3%) 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 (Fig. 3), the studied strains grouped together in a robust clade (99% bootstrap value), 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. PCC8501 (Fig. 3). The 16S–23S rRNA ITS sequences from these six strains ranged in length from 474 to 478 bp, and tRNA genes were absent. Four different secondary structures were observed for D1–D1', three for Box-B and two for the V3 region (Table 2, Fig. 4).

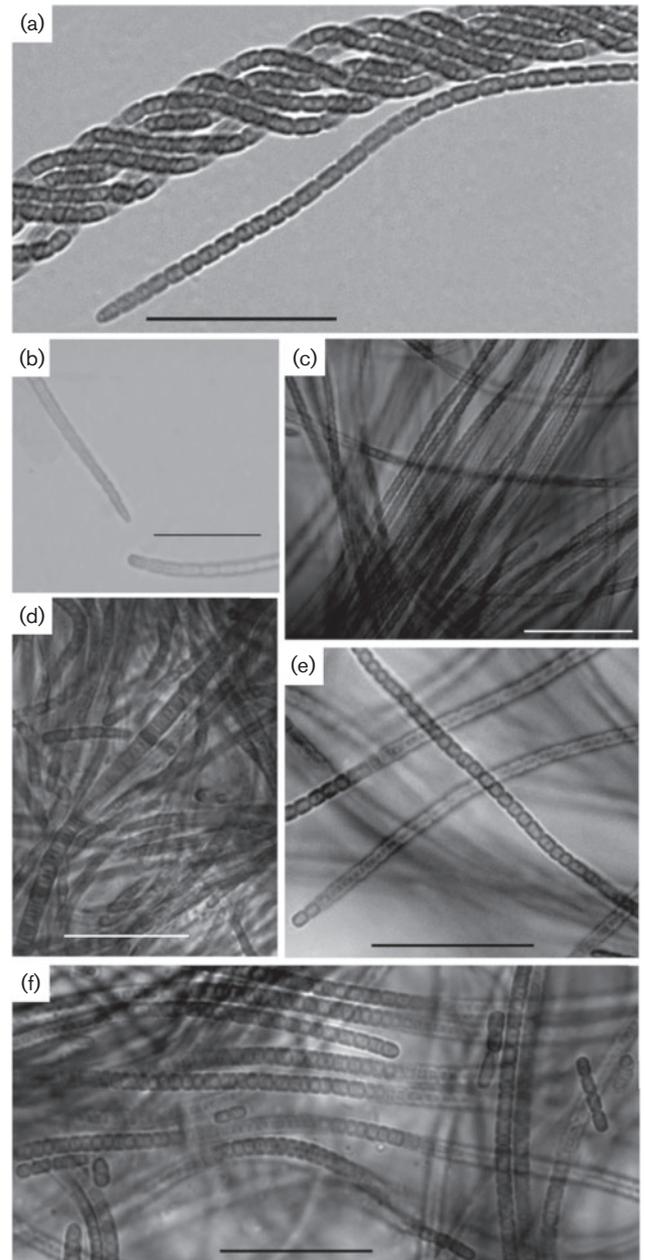
The remaining four strains shared 16S rRNA gene sequence identities lower than 93.5% with those retrieved from GenBank. These four sequences shared identities  $\geq 99.6\%$  and formed a stable clade (99% bootstrap value). This clade had an external branch consisting of an orphan sequence of cf. *Leptolyngbya* sp. Greenland-9, which was isolated from alkaline soil (Roeslers *et al.*, 2007). In addition, this clade was related to *Phormidesmis*, *Leptolyngbya frigida* ANT.L53B.2 and *Phormidium* sp. SAG 37.90 (Fig. 3). The ITS sequences of these four strains were 570 bp long, and both tRNA genes (Ile and Ala) were identified (Table 2, Fig. 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 novel 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* gen. nov. and *Alkalinema* gen. nov., as well as the description of the type species for each genus, *Pantanalinema rosanaeae* sp. nov. and *Alkalinema pantanalense* sp. nov., respectively. No morphological or molecular differences were observed among the strains of each genus and they were therefore named at the same species level; the diagnoses are presented below.



**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).

The ten strains tolerated the pH values tested (4, 5, 7, 9 and 11) and were able to grow under these conditions. Significant differences were not observed in terms of biomass production for the different treatments (Kruskal–Wallis rank sum test: Kruskal–Wallis  $\chi^2=1.3848$ , d.f.=4,  $P=0.8468$ ). After 14 days, the pH of the BG-11 culture medium was 8.4–9.9, demonstrating that these strains were able to induce alkalization of the culture medium. The pH of the BG-11 culture medium used as a control did not change significantly, ranging from 7.2 to 7.4 (mean values).



**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  $\mu\text{m}$ .

**Diagnosis for *Pantanalinema* M. G. M. V. Vaz et al. gen. nov. (Fig. 1)**

*Pantanalinema* (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 the Pantanal).

In nature, known strains grow in saline–alkaline lakes in the Brazilian Pantanal wetland. The trichomes are microscopic

**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 rosaneae</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

and in liquid medium grow attached to the tube bottom and walls. Macroscopically, the colonies are olive green. Filaments are entangled and flexuous. The motile trichomes are characterized 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 they are long, 1.2–3.1 µm long by 1.5–3.1 µm wide. The apical cell is cylindrical with a rounded to slightly conical apex. Cell content is homogeneous and brownish green or olive green. Hormogonium formation occurs via cell disintegration (false necridial cells) or trichome fragmentation.

Type species: *Pantanalinema rosaneae* (type strain: CENA516).

#### **Diagnosis for *Pantanalinema rosaneae* M. G. M. V. Vaz et al. sp. nov. (Fig. 1)**

*Pantanalinema rosaneae* (ro.sa'ne.ae. N.L. gen. n. *rosaneae* of Rosane; named after the late Rosane Aguiar, a Brazilian phycologist and cyanobacterial physiologist, *in memoriam*).

In nature, known strains grow in saline–alkaline water in the Brazilian Pantanal wetland. The trichomes are microscopic and, in liquid medium, 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 they are long, 1.2–3.1 µm long by 1.5–3.1 µm wide. The apical cell is cylindrical with a rounded apex. Cell content is homogeneous and brownish green or olive green. Hormogonium formation occurs via trichome disintegration.

Holotype: BRAZIL, Mato Grosso do Sul State, Pantanal wetlands, 27 May 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 Salina Verde in the Pantanal wetlands, Mato Grosso do Sul State, Brazil.

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

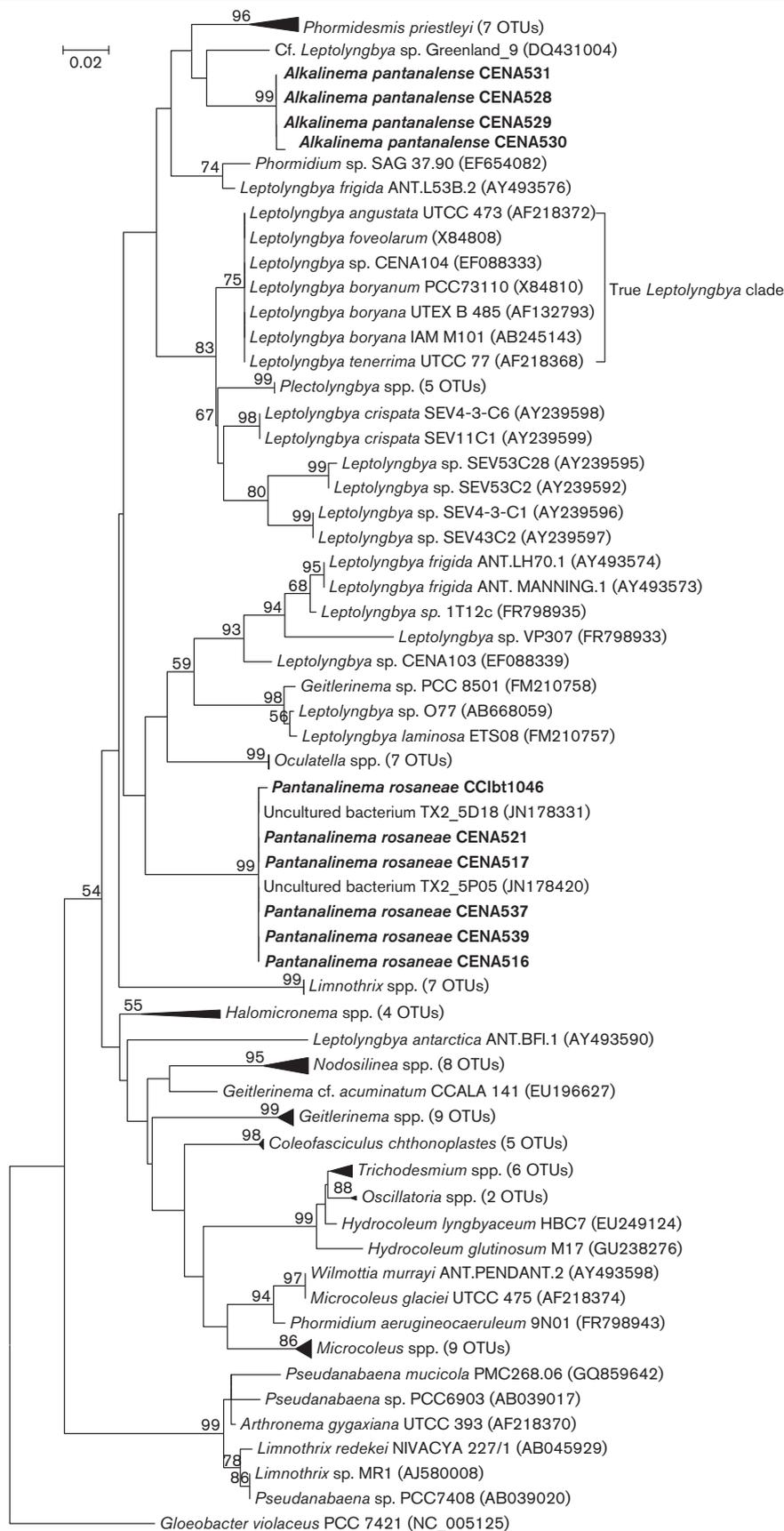
Habitat: Grows in water samples collected from Salina Verde in the Pantanal wetlands.

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.

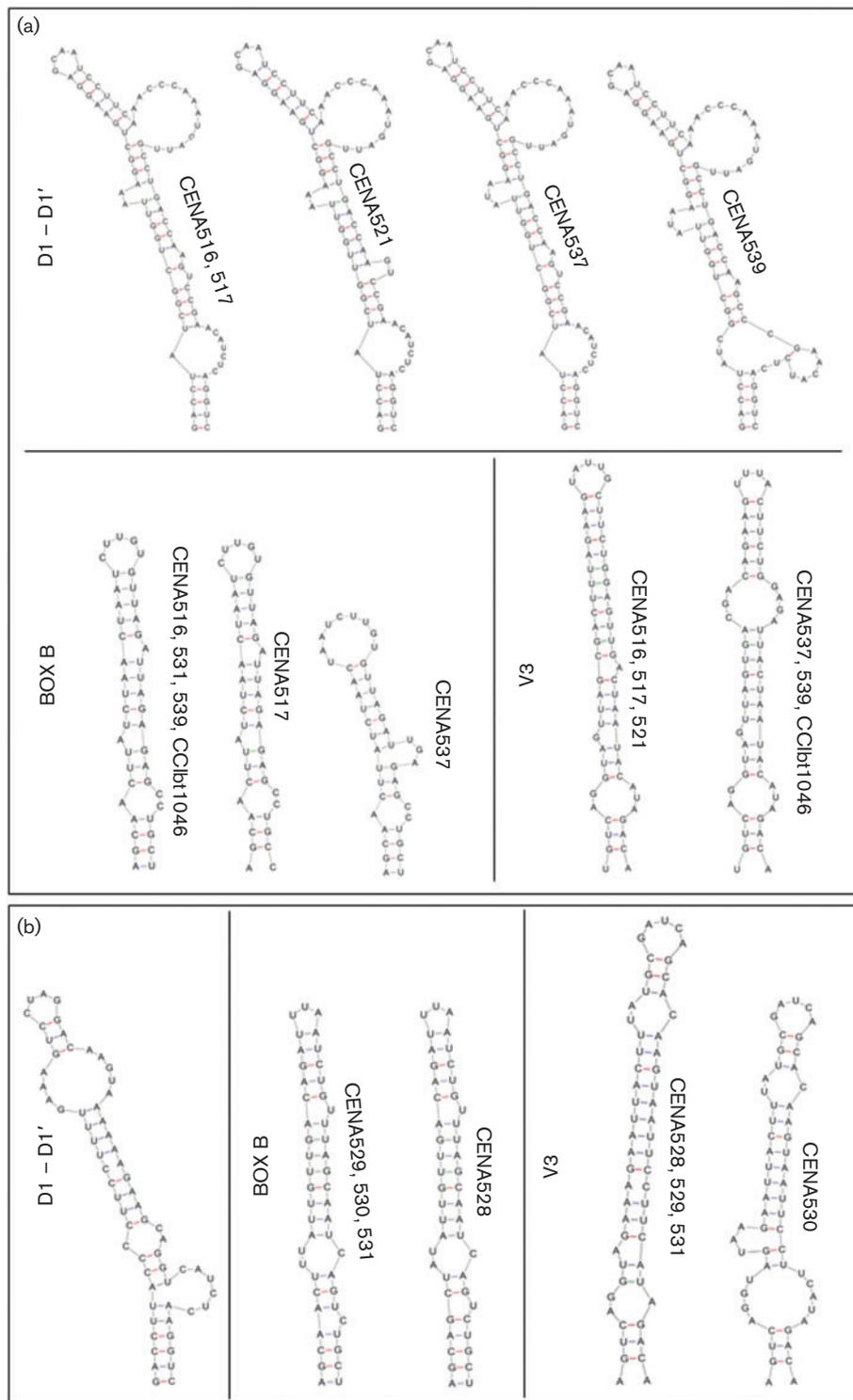
#### **Diagnosis for *Alkalinema* M. G. M. V. Vaz et al. gen. nov. (Fig. 2)**

*Alkalinema* (Al.ka.li.ne'ma. N.L. n. *alkali* alkali, from Arabic n. *al qaliy* the ashes of saltwort; Gr. neut. n. *nema* thread, filament; N.L. neut. n. *Alkalinema* a filament from alkaline lakes).

In nature, known strains grow in saline–alkaline lakes in the Brazilian Pantanal wetland. The trichomes are microscopic and, in liquid medium, they grow as free-floating trichomes that are distributed in the culture flask. The trichomes are frequently organised in ornate (interwoven)



**Fig. 3.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains of the Pseudanabaenales and Oscillatoriales. The studied strains of *Pantanalinema* 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.



**Fig. 4.** Folded 16S–23S rRNA ITS secondary structures of strains of *Pantanalinema* (a) and *Alkalinema* (b).

mats. Macroscopically, the culture has a reddish to brownish colour. Trichomes do not have sheaths but do have a diffuent mucilage and are non-motile. Cells are isodiametric or longer than they are wide, 1.5–4.1 µm long

by 1.1–2.2 µm wide, with a narrowed or rounded-conical apical cell. Cell content is homogeneous and reddish to brownish. Hormogonium formation occurs via trichome disintegration.

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

**Diagnosis for *Alkalinema pantanalense* M. G. M. V. Vaz et al. sp. nov. (Fig. 2)**

*Alkalinema pantanalense* (pan.ta.na.len'se. N.L. neut. adj. *pantanalense* pertaining to the Pantanal, a Brazilian wetland).

The trichomes are microscopic and, in liquid medium, 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 diffuent mucilage and are non-motile. They are organized in ornate (interwoven) mats. Cells are isodiametric or longer than they are wide, 2.0–4.1 µm long by 1.7–2.2 µm wide, with a narrowed apical cell. Cell content is homogeneous and reddish to brownish. Hormogonium formation occurs via trichome disintegration.

Holotype: BRAZIL, Mato Grosso do Sul State, Pantanal wetlands, 27 May 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, 27 May 2010, *Laurent Barbiero* (SP428.669); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 27 May 2010, *Laurent Barbiero* (SP428.670); BRAZIL, Mato Grosso do Sul State, Pantanal wetland, 27 May 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 (Casamatta et al., 2005; Johansen et al., 2011; Perkinson et al., 2011; Zammit et al., 2012). Therefore, the genus *Leptolyngbya* requires taxonomic re-evaluation and the definition of diacritical features (Komárek & Hauer, 2013). Data emerging from a phylogeny based on 16S rRNA gene sequences from *Leptolyngbya sensu lato* have led to the separation, correction and definition of the new genera *Nodosilinea*,

*Haloleptolyngbya* and *Oculatella* (Perkinson 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 of the polyphyletic 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 16S–23S rRNA gene 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 reconstructed 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%) to sequences of *Pantanalinema*. Likewise, the morphological traits of *Pantanalinema* differed from those of *Oculatella*. The genus *Pantanalinema* is characterized 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, strains of *Oculatella* and *Leptolyngbya* can, albeit rarely, possess false branches, which has not been observed for *Pantanalinema*. Comparing the sequences of the 16S–23S rRNA 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 strains 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 strains of *Leptolyngbya sensu lato* and *Oculatella*. 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 (from 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 & 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 genus *Leptolyngbya* comprises strains with cells that are 0.5–3.5 µm wide and trichomes that are rarely solitary and rarely pseudobranched (Komárek & Anagnostidis, 2005). Members of the genus *Plectolyngbya* possess 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 genus *Phormidesmis* is characterized 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 16S–23S rRNA 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 strains of *Leptolyngbya sensu stricto* and other strains of *Leptolyngbya* (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 secondary structures of the D1–D1', Box-B and V3 regions in *Alkalinema* 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 16S–23S rRNA ITS regions of *Phormidesmis* and *Plectolyngbya* exist, no comparisons were possible.

The strains of *Pantanalinema* and *Alkalinema* survived and produced biomass at the different tested pH, which permitted them to be classified as acid- and alkali-tolerant. In addition, these strains were able to alter the final pH to 8.4–9.9, characterising them as alkaliphilic. Given that the strains of *Pantanalinema* and *Alkalinema* were not axenic, the heterotrophic bacterial community associated with the cyanobacterial filaments may be involved in the alteration of pH, or both organisms together (cyanobacteria and heterotrophic bacteria) may coordinate this pH modification. Cyanobacterial strains are generally known as the most alkaliphilic micro-organisms and frequently dominate alkaline environments, such as soda lakes or saline–alkaline lakes (Summerfield & Sherman, 2008; Møgelhøj *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 can also survive under acidic conditions (Almer *et al.*, 1974; Kwiatkowski &

Roff, 1976; Steinberg *et al.*, 1998). Despite these reports, relatively few studies have shown how cyanobacteria tolerate acid stress and how they neutralize 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 neutralization 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 alkalization of the culture medium by strains of *Pantanalinema* and *Alkalinema*; 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. To date, strains of *Pantanalinema* have been 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 demonstrate 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 used successfully to support the separation of novel cyanobacterial genera, such as *Haloleptolyngbya*, *Coleofasciculus*, *Halomicronema*, *Plectolyngbya*, *Wilmottia*, *Phormidesmis*, *Nodosilinea*, *Oxynema*, *Oculatella*, *Mojavia*, *Chakia*, *Calochaete*, *Toxopsis*, *Iphinoe*, *Loriellopsis* and *Desmonostoc* (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). 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 16S–23S rRNA ITS sequences and secondary structures of *Pantanalinema* and *Alkalinema* did not correspond to previous descriptions, demonstrating that the ITS sequence 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 as the Pantanal wetlands, can lead to the identification of novel taxa, even via simple and inexpensive methods such as isolation techniques.

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