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# NON-HETEROCYTOUS CYANOBACTERIA FROM BRAZILIAN SALINE-ALKALINE LAKES

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*Running title:* Homocytous and unicellular cyanobacteria from Pantanal

Abstract

Saline-alkaline lakes are extreme environments that limit the establishment and development of life. The Nhecolândia, a subregion of the Pantanal wetland in Brazil, is characterized by the existence of ~500 saline-alkaline lakes, which support an underexplored and rich diversity of microorganisms. In this study, unicellular and homocytous cyanobacteria from five saline-alkaline lakes were accessed by culture-dependent approaches. Morphological evaluation and analyses of near complete sequences (~1400 nt) of the 16S rRNA genes were applied for phylogenetic and taxonomic placement. This polyphasic approach allowed for the determination of the taxonomic position of the isolated strains into the following genera: *Cyanobacterium*, *Geminocystis*, *Phormidium*, *Leptolyngbya*, *Limnothrix* and *Nodosilinea*. In addition, fourteen Pseudanabaenales and Oscillatoriales representatives of putative novel taxa were found. These sequences fell into five new clades that could correspond to new generic units of the Pseudanabaenaceae and Phormidiaceae families.

Keywords: extreme environment, alkaline environment, saline environment, phylogeny, polyphasic approach, Oscillatoriales, Pseudanabaenales, Chroococcales, Synechococcales, tropical wetlands, Brazil.

## 1. Introduction

Cyanobacteria are an ancient phylum of oxygenic photoautotrophic bacteria with a diverse morphology and physiology. This group's functional plasticity associated with its long evolutionary history has allowed it to disperse around the world, including into extreme environments with high and low temperatures, a range of salinities, pH levels and limited availability of nutrients (Taton et al. 2003, 2006, Turicchia et al. 2009, Bahl et al. 2011, Rigonato et al. 2012).

A number of heterocytous and nonheterocytous genera have been reported from saline and alkaline lakes (Jones and Grant 1999, López-Archilla et al. 2004, Foti et al. 2008, Tsyrenova et al. 2011, Dadheech et al. 2012, 2013). The isolation and characterization of these strains can generate interesting data about cyanobacterial distribution and behavior. This approach can be valuable for solving selected taxonomic questions and also for providing biological material useful for applied and physiological studies (Ward et al. 1998, López-Cortéz et al. 2001, Taton et al. 2006). Several studies have shown that strains from extreme environments are sources of interesting metabolites with biotechnological applications (Grant 1992, Horikoshi 1999, Silva-Stenico et al. 2012, Taton et al. 2012).

The Brazilian Pantanal is the largest continental wetland on the planet, extending over  $2 \times 10^5$  km<sup>2</sup> (Por 1995). It consists of a patchwork of subregions with highly variable hydrological, chemical and pedological features (Rezende-Filho et al. 2012). Nhecolândia is a peculiar 24,000 km<sup>2</sup> subregion of the Pantanal that contains a myriad of shallow saline-alkaline and freshwater lakes coexisting in close proximity. In saline-alkaline lakes, inorganic ion concentrations exceed  $2.5 \text{ g}\cdot\text{L}^{-1}$  and electrical conductivity exceeds  $3 \text{ mS}\cdot\text{cm}^{-1}$ , with pH values above 8.9. Extreme values up to  $50 \text{ g}\cdot\text{L}^{-1}$ ,  $70 \text{ mS}\cdot\text{cm}^{-1}$  and 10.5, respectively, have been recorded during the dry season. The high pH conditions with a  $\text{NaHCO}_3$  chemical profile are positively correlated with the electrical conductivity (Furian et al. 2013) and also associated with high contents of dissolved organic carbon up to  $500 \text{ mgC}\cdot\text{L}^{-1}$  (Mariot et al. 2007). These lakes are hypersaline (athalassohaline) soda lakes with a similar chemical composition to the lakes of the East Africa Rift Valley (Ventosa and Arahal 1989, Duarte et al. 2012).

Blooms of microalgae and cyanobacteria occur usually when the electrical conductivity of the water exceeds about  $3 \text{ mS}\cdot\text{cm}^{-1}$ . These blooms are seasonal or permanent, depending on the intensity of the rainfall during the wet season (De-Lamonica-Freire and Heckman 1996, Santos and Sant'Anna 2010, Santos et al. 2011).

Few studies have focused on the occurrence and distribution of cyanobacteria in the Nhecolândia, and were based on floristic surveys (De-Lamonica-Freire and Heckman 1996, Oliveira and Calheiros 2000, Malone et al. 2007, Santos and Sant'Anna 2010). Cyanobacteria were reported as the dominant group in the saline-alkaline lakes from the Pantanal wetland during the dry season (De-Lamonica-Freire and Heckman 1996). Otherwise, the genetic diversity of cyanobacteria from this environment remains mostly unexplored.

In this study, the cultured diversity of unicellular and homocytous cyanobacteria from the saline-alkaline lakes in Pantanal da Nhecolândia was assessed by morphology and phylogeny of 16S rRNA genes, allowing a robust systematic evaluation of these strains.

## 2. Materials and Methods

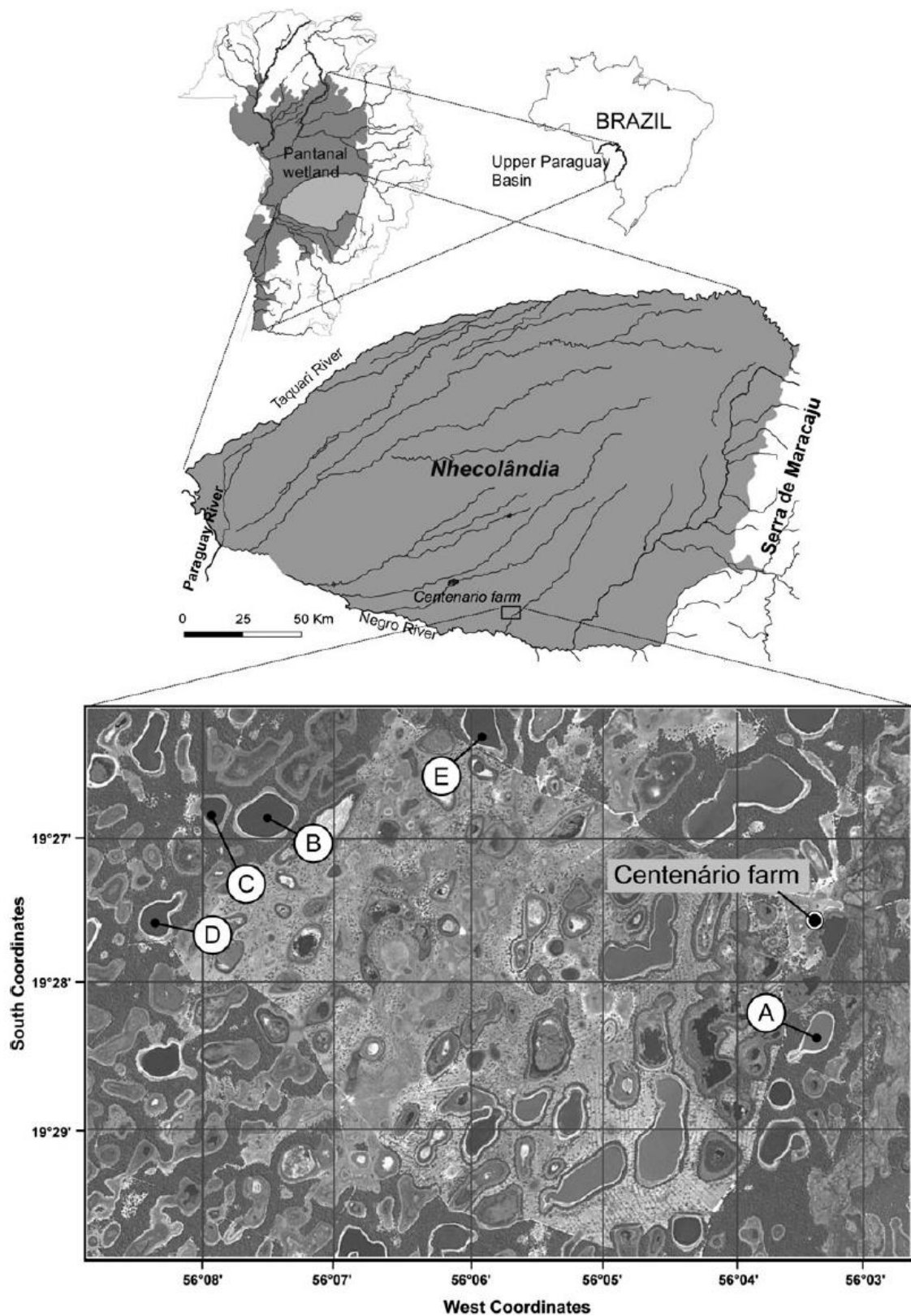
### 2.1 Site and Sampling

The Nhecolândia is located between the Taquari River (North) and the Negro River (South). In this region, the number of lakes has been estimated from 12,000 to 17,500, including 500 to 600 saline-alkaline lakes (Oliveira et al. 2011). Despite great differences in chemical composition between freshwater and saline-alkaline lakes, all surface waters in the region belong to the same chemical family, corresponding to several concentration stages of the Taquari River water that supplies the region (Rezende-Filho et al. 2012). The concentration stage depends on the hydrological functioning of each lake, which is itself controlled by the relative importance of low-permeability soils as a barrier to movement of water into or out of the lakes through the subsurface (Barbiero et al. 2008). Furian et al. (2013) have concluded that the high pH salinity observed in some lakes results from an ongoing

process of accumulation and evaporation under relatively humid climatic conditions and poor drainage.

The sampling was carried out in Southern Nhecolândia at Centenário farm located in the north of the city of Aquidauana, Mato Grosso do Sul State (Fig. 1). The region has a tropical humid climate with a short dry season. The mean annual air temperature is about 25°C, ranging from 21°C in winter to 32°C in summer. Mean annual precipitation (P) is about 1,100 mm, and the annual evapotranspiration (ETP) is approximately 1,400 mm, giving a hydrological deficit of about 300 mm. Precipitation and ETP show a pronounced seasonal cycle, with most rainfall occurring from October to April and with excesses from November to March (Tarifa 1986).

Water samples were collected from five shallow, non-stratified, saline-alkaline lakes (Fig. 1). Additionally, a sediment sample was collected from one lake (Salina Verde) that had a heavy cyanobacterial bloom. The geographic coordinates and physico-chemical parameters of these lakes are reported in Table 1.



**Figure 1** - Location of the studied sites in the Nhecolândia, Pantanal wetland. (A) Salina Verde; (B) Salina Grande; (C) Salina Preta; (D) Salina 67 mil; and (E) Salina Centenário.

## 2.2 Cyanobacterial isolation and morphological evaluation

In the laboratory, cyanobacterial growth was achieved by inoculating one milliliter of each water sample into flasks containing 75 mL of BG-11 medium (Allen 1968). After visible growth, the cultures were subjected to repeat streaking onto BG-11 solid medium and examined by microscopic observation until completely purified. Mono-specific cyanobacterial cultures were grown under white

fluorescent light ( $40 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) with a 14:10 h light:dark (L:D) cycle at  $25 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$ . The isolated strains were maintained in the Molecular Ecology of Cyanobacteria Laboratory collection (CENA/USP), Piracicaba, São Paulo State, Brazil. The morphological characterization was performed according to the systematic scheme proposed by Komárek and Anagnostidis (1986, 1999), revised by Hoffmann et al. (2005) and Komárek (2010), as well as recent revisions for new genera.

### 2.3 DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the method described by Fiore et al. (2000). PCR amplification and sequencing of the 16S rRNA gene (average length, 1,400 nt) were performed as previously described (Fiore et al. 2007). The quality of the DNA and specificity of the PCR were analyzed by agarose gels (1 % w/v) using the Low DNA mass ladder (Life Technologies/Invitrogen, Carlsbad, CA, USA). The gels were stained with the SYBR Safe DNA gel stain (Life Technologies/Invitrogen) and visualized under UV light. The PCR products were cloned using the pGEM-T Easy vector system (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. Competent *E. coli* DH5 $\alpha$  cells were transformed, and plasmids containing the cloned gene were extracted by an alkaline lysis method (Birnboim and Doly, 1979). The plasmids containing the gene fragments were sequenced with the M13 forward and reverse primer set and the 16S rRNA internal primer sets, 357F/357R, 704F/704R and 1114F/1114R (Lane 1991) to cover the entire fragment (1,400 nt), using the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies/Applied Biosystems, Foster City, CA, USA). The cycle sequencing reaction was performed starting at  $95 \text{ }^{\circ}\text{C}$  for 1 min followed by 35 cycles of the following:  $95 \text{ }^{\circ}\text{C}$  for 15 s,  $50 \text{ }^{\circ}\text{C}$  for 15 s and  $60 \text{ }^{\circ}\text{C}$  for 2 min. After completing the sequencing reaction, the DNA was precipitated using a sodium acetate buffer (1.5 M sodium acetate, pH 9.0 and 250 mM EDTA) and washed with 100 % and 70 % ethanol. The purified DNA was re-suspended in Hi-Di formamide (Life Technologies/Applied Biosystems), and the sample was placed in an ABI PRISM 3500 genetic analyzer (Life Technologies/Applied Biosystems).

### 2.4 Sequence processing and phylogenetic analysis

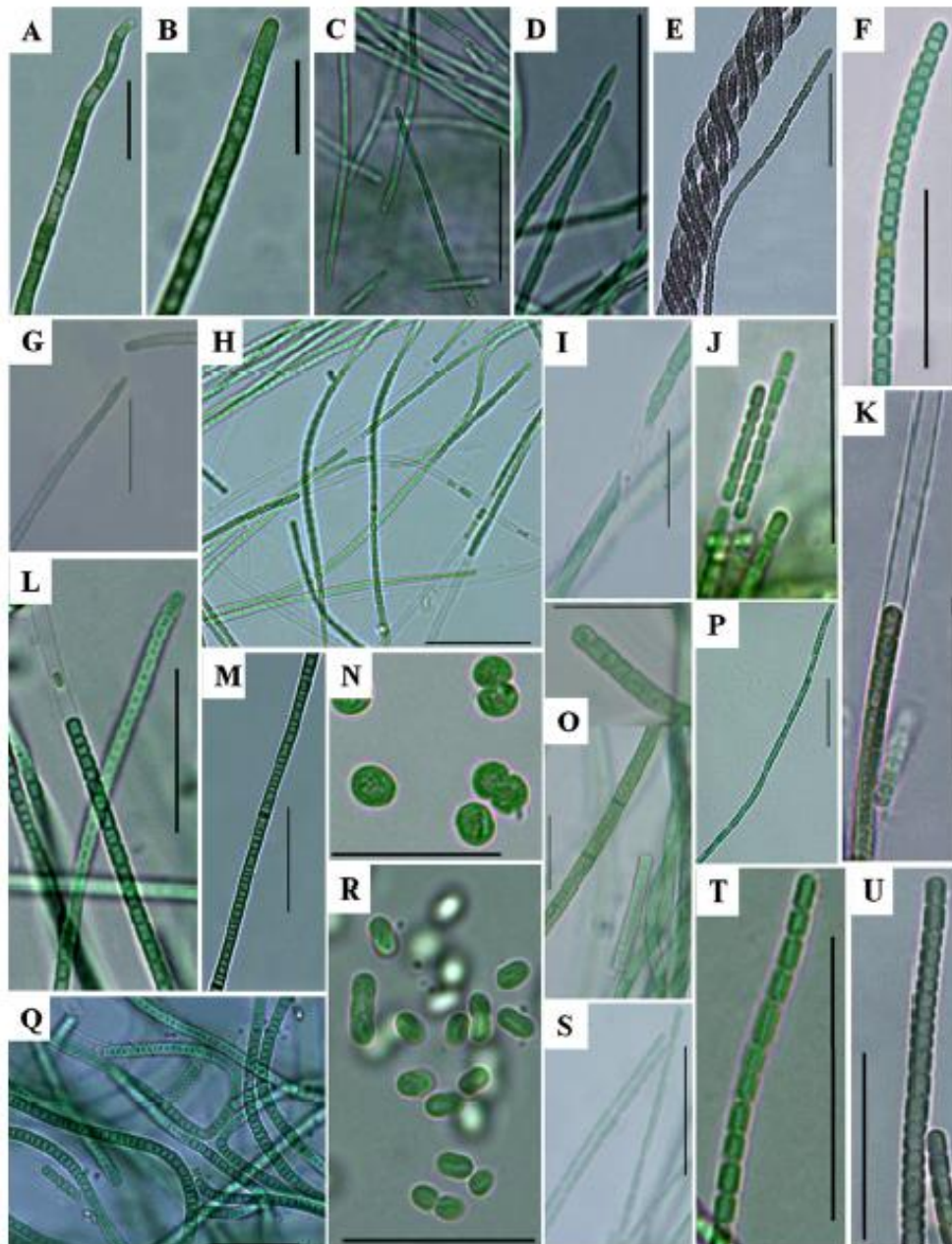
The 16S rRNA sequenced fragments were assembled into contigs using the Phred/Phrap/Consed software (Philip Green, Univ. of Washington, Seattle, USA). The sequences were trimmed by considering only bases with a quality score above 20. For accurate phylogenetic analyses of 16S rRNA, the best-matched sequences for each sequenced strain and the reference sequences were retrieved from GenBank. Phylogenetic analysis was performed using Mega 5.1 software (Tamura et al. 2011). All sequences were aligned using Muscle. Maximum likelihood was performed by applying the Gamma evolutionary model distributed with Invariant sites (G+I) and the Kimura-2 parameter as defined by the BIC (Bayesian Information Criterion). Gaps were treated as missing data, and bootstrap resampling was performed using 1,000 replications. The 16S rRNA gene sequences were deposited in the NCBI GenBank (Table 2).

## 3. Results and Discussion

Twenty-eight non-heterocytous cyanobacterial strains were isolated from the studied saline-alkaline lakes. The evaluation of morphological features organized these strains within the orders Chroococcales, Synechococcales, Pseudanabaenales and Oscillatoriales (Fig. 2, Table 2). The two unicellular isolated strains were affiliated to Cyanobacteriaceae and Merismopediaceae and the 26 homocytous to Pseudanabaenaceae (24) and Phormidiaceae (2).

The 16S rRNA gene sequences of unicellular and homocytous morphotypes showed a mixed distribution in the phylogenetic reconstruction (Fig. 3), corroborating with other studies showing that the orders Chroococcales, Synechococcales, Pseudanabaenales and Oscillatoriales are polyphyletic (Hoffmann et al. 2005, Furtado et al. 2009, Shih et al. 2013). Although Cyanobacteria is a monophyletic phylum, a consensus was not yet reached regarding the origin and evolutionary history of its multicellularity or concerning which morphotypes emerged earliest (Sánchez-Baracaldo et al.,

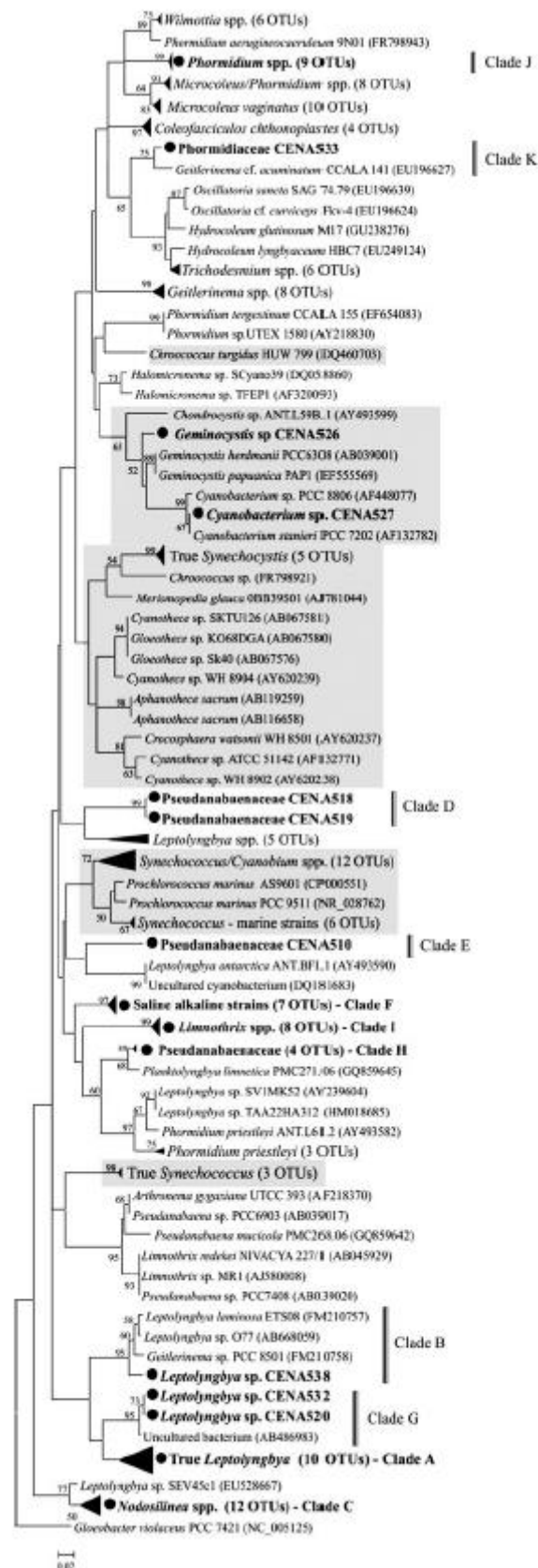
2005; Schirrmeister et al., 2011). Furthermore, cellular division genes determine cyanobacterial multicellularity and filamentous phenotypes may result from modifications of the gene regulatory network controlling cell division (Mori and Johnson 2001, Miyagishima et al. 2005, Dagan et al. 2012). Whereas morphological evaluation is still needed due to the traditional morphological classification of Cyanobacteria, it is not always in agreement with molecular phylogeny. Therefore, the systematics of Cyanobacteria has been repeatedly reviewed in order to arrive at a consensus (Komárek and Anagnostidis 1986, 1999, Hoffmann et al. 2005, Komárek 2010).



**Figure 2** – Microphotographs from selected cyanobacterial strains related to each phylogenetic cluster. **A** and **B**: *Phormidium* sp. CENA 525; **C** and **D**: *Leptolyngbya* sp. CENA 538; **E**: Pseudanabaenaceae CENA 530; **F**: *Leptolyngbya* sp. CENA 532; **G**: Pseudanabaenaceae CENA 528; **H**: *Nodosilinea* sp. CENA 522; **I** and **O**: Phormidiaceae CENA 533; **J**: *Nodosilinea* sp. CENA 512; **K**: Pseudanabaenaceae CENA537; **L** : *Leptolyngbya* sp. CENA 540; **M**: *Leptolyngbya* sp. CENA 542; **N**: *Geminocystis* sp. CENA 526; **P**: *Limnothrix* sp. CENA 545; **Q**: *Leptolyngbya* sp. CENA 520; **R**: *Cyanobacterium* sp. CENA 527; **S**: Pseudanabaenaceae CENA 519; **T**: Pseudanabaenaceae CENA 510; **U**: *Leptolyngbya* sp. CENA 517.



### 3.1 Synechococcales and Chroococcales



**Figure 3** – Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains of Chroococcales, Synechococcales (on grey boxes), Oscillatoriales and Pseudanabaenales. Bootstrap values equal to or greater than 50 % are indicated at the nodes. The studied strains are in the clades indicated by a filled and bold circle.



Morphologically, the strain CENA526 has solitary rounded or slightly oval cells and hemispherical cells after cell division; the cells did not form colonies, lacked gelatinous envelopes and had cell diameters of 4.08-7.15  $\mu\text{m}$ . The CENA527 strain contained both solitary cells or cells in groups of two after division, and the cells were more or less cylindrical, without gelatinous envelopes, and had cell lengths of 2.30- 3.50  $\mu\text{m}$  and cell widths of 2.10-2.33  $\mu\text{m}$ . These morphological features are characteristic of *Synechocystis aquatilis* and *Synechococcus* cf. *nidulans*, respectively, identified previously in this environment (Santos and Sant'Anna 2010). However, the CENA526 strain had a larger cell diameter than the type-species *Synechocystis aquatilis* strain PCC 6803 (Korelusová et al. 2009), and the *S. aquatilis* strain observed previously in the Pantanal wetlands (Santos and Sant'Anna 2010). Likewise, the CENA527 strain had a cell width larger than the typically observed *Synechococcus nidulans* strains (Komárek and Anagnostidis 1999).

Based on phylogeny of the 16S rRNA gene sequences, the two unicellular strains and their most similar morphotypes mentioned above were not related (Fig. 3, Fig. S1). The sequence of CENA526 belonged to a clade formed by strains belonging to the recently described genus *Geminocystis* (Korelusová et al. 2009) and *Cyanobacterium*. The 16S rRNA sequence of CENA526 exhibited 96.3 % identity to *G. herdmanni* PCC 6308, described as the type-species for the *Geminocystis* genus, which was previously identified as *Synechocystis* sp. PCC 6308. According to Korelusová et al. (2009), the main morphological differences between *Synechocystis* and *Geminocystis* are the cell diameter, the ultra-structural position of the thylakoids and the phylogenetic position. The cell size of CENA526 strain (4.08-7.15  $\mu\text{m}$  diameter) is bigger than the reference strain, *Synechocystis* sp. PCC 6803 (1-5  $\mu\text{m}$  diameter), and the *Geminocystis* cluster is not related with the typical *Synechocystis* cluster (Fig. 3). Currently, only two species in the *Geminocystis* genus have been described; one was isolated from a freshwater lake in the USA, and the other was isolated from the soil in Papua New Guinea. Furthermore, considering the cut-off value of 95 % of 16S rRNA sequence identity for genus definition (Wayne et al. 1987, Stackebrandt and Goebel 1994, Komárek 2010), CENA526 belonged to the *Geminocystis* genus and could represent a novel species from tropical saline-alkaline lakes.

The 16S rRNA sequence of CENA527 was included in a well-supported cluster (bootstrap value of 99 %) with two sequences from *Cyanobacterium* and had 98.7 % identity to *C. stanieri* PCC 7202, the type-species for this genus. This fact along with morphological similarity of CENA527 to *Cyanobacterium* (cell dimensions and a visible lengthwise striation) and the occurrence of at least three *Cyanobacterium* species (*C. synechococcoides*, *C. minervae* and *C. diachloros*) in saline-alkaline environments, allowed the identification of this strain as *Cyanobacterium* sp.

### 3.2 Oscillatoriales

In the filamentous oscillatorialean group, the two Phormidiaceae strains (CENA525 and CENA533) were preliminarily identified as *Phormidium* spp. based on morphological features. The *Phormidium* genus is characterized by quadratic or sub-quadratic cells that are cylindrical and isopolar, have non-branched and homocytous filaments, and form irregular aggregates with more or less parallel-oriented trichomes (Komárek and Anagnostidis 2005). Despite the fact that these two strains showed similar cell size and trichome morphology, CENA533 had necridic cells and sheaths, while CENA525 possessed motile trichomes (Fig. 2). The occurrence of *Phormidium* morphotypes in the Brazilian Pantanal wetlands has been documented (Santos and Sant'Anna 2010).

The 16S rRNA sequence of *Phormidium* sp. CENA525 shared 99.0 % identity with *Phormidium* sp. LEGE 06072 and *Phormidium acuminatum* PCC 6304 (synonymous with *Oscillatoria acuminata* PCC 6304) (Komárek and Anagnostidis 2005). The CENA525 sequence grouped in a highly supported clade (99 % bootstrap value) containing several sequences from Phormidiaceae (Fig. 3, Clade J; Fig. S2). These strains had no coherent morphological traits (such as apical cell morphology) but were obtained from extreme environments, such as the poles, hypersaline environments and hot springs. According to Chatchawan et al. (2012), the typical *Phormidium* phylogenetic cluster comprises *Phormidium* sp. UTEX 1580 and *Phormidium tergestinum* CCALA 155, but this cluster

had little relationship with the cluster containing the CENA525 sequence. In addition, the CENA525 sequence shared 89.9 % identity with the typical *Phormidium* representatives.

The genus *Phormidium* is a polyphyletic group with few diacritic morphological characters (Strunecký et al. 2011, Chatchawan et al. 2012). Traditionally, this genus is divided into eight groups that differ in the morphology of the apical end cells (Komárek and Anagnostidis 2005). However, recent studies have demonstrated that this traditional division is not supported by the *Phormidium* phylogeny. Polyphasic approaches have been applied in an attempt to clarify the taxonomic position of the strains that have been assigned as *Phormidium* spp, and new genera, such as *Coleofasciculus* (Siegesmund et al. 2008), *Phormidesmis* (Komárek et al. 2009, Turicchia et al. 2009), *Wilmottia* (Strunecký et al. 2011) and *Oxynema* (Chatchawan et al. 2012) have been described.

The CENA533 sequence had an identity of 92.2 % with sequences of the genera, *Coleofasciculus* (synonymous with *Microcoleus chthonoplastes*), *Arthrospira*, *Spirulina*, *Lyngbya*, *Hydrocoleum* and *Oscillatoria*, below the cut-off value used to delimit genera (Wayne et al. 1987, Stackebrandt and Goebel 1994, Komárek 2010). Notwithstanding, this sequence grouped in a well-supported clade (bootstrap value of 75 %) with the short sequence of *Geitlerinema* cf. *acuminatum* CCALA 141 (1,165 nt) (Fig. 3, Clade K). The latter strain was isolated from the soil of Romania and was first identified as *Microcoleus vaginatus* (Vaucher) Gomont ex Gomont, renamed as *Phormidium animale* (C. Agardh ex Gomont) Anagnostidis et Komárek and recently reclassified as *Geitlerinema* by Lokmer (2007). However, considering the morphological features (*Geitlerinema* obligately has motile trichomes and an absence of sheaths - Komárek and Anagnostidis 2005), CENA533 must be excluded from this genus (Fig. 2). Therefore, CENA533 was identified only at the family level.

### 3.3 *Pseudanabaenales*

A total of 24 pseudanabaenacean strains were isolated. The only representative of the genus *Limnothrix* was the CENA545 strain. *Limnothrix* is defined as those morphospecies containing isopolar and slightly bent trichomes, absence of false branching, and cylindrical, elongated and generally unconstricted cells (1-6 µm in width) with apical or central aerotopes (Komárek and Anagnostidis 2005). Blast analysis showed that the sequence of *Limnothrix* sp. CENA545 shared 99.7 % identity with sequences of *Limnothrix redekei* 2LT25S01, *Limnothrix* sp. CENA109, *Limnothrix* sp. CENA110 and *Limnothrix* sp. CENA111. The same comparison between our sequence and the sequence of the reference strain for the genus, *Limnothrix redekei* NIVA-CYA227/1 (Suda et al. 2002), revealed an 88.3 % identity. This dissimilarity was reflected in the phylogenetic reconstruction because our strain did not group with the reference strain (Fig. 3; Fig. S2). Studies on the phylogeny of *Limnothrix* strains have demonstrated that their sequences are distributed mainly into two well-supported clades; one clade contains the reference sequence and other sequences from different pseudanabaenacean genera, and the other clade is comprised of sequences exclusively assigned as *Limnothrix* spp. (Bernard et al. 2011, Perkerson et al. 2010, Zhu et al. 2012). These findings indicate that the reference strain for *Limnothrix* should be re-evaluated. Alternatively, one of the sequences included in Clade I could be used as a reference sequence because the whole group has coherent morphology and is stable in a well-supported phylogenetic cluster (Fig. 3, Clade I).

All of the other isolated pseudanabaenacean strains had morphological features similar to the genus *Leptolyngbya*, which is defined as consisting of strains with very thin trichomes (less than 3.5 µm in width) and cells that are isodiametric or have longer lengths than widths (Komárek and Anagnostidis 2005). *Leptolyngbya* is a polyphyletic assemblage. *Leptolyngbya sensu stricto* contains the type-species *L. boryana* in a monophyletic and well-supported clade, and *Leptolyngbya sensu lato* comprises several morphologically and phylogenetically related lineages (Taton et al. 2006, Johansen et al. 2011).

The isolated strains *Leptolyngbya* sp. CENA534, *Leptolyngbya* sp. CENA540 and *Leptolyngbya* sp. CENA542 showed 99.7 % sequence identity with *Leptolyngbya* spp. and grouped into the *Leptolyngbya sensu stricto* clade (Fig. 3 – Clade A). *Leptolyngbya* sp. CENA538 shared 96.7 % sequence identity with *Leptolyngbya* sp. O-77 and grouped in a separate and highly supported clade with *Leptolyngbya laminosa* ETS-08 and *Geitlerinema* sp. PCC 8501. Interestingly, these strains

were isolated from different thermophilic environments (Fig. 3 – Clade B). *Leptolyngbya* sp. CENA520 and *Leptolyngbya* sp. CENA532 grouped together in a separate clade (Fig. 3 – Clade G). *Leptolyngbya* possesses few morphological features that are useful for its identification; thus, misidentifications are common and have hampered the definition of new taxa. Nevertheless, it is difficult to propose new taxonomic arrangements inside *Leptolyngbya sensu lato* because several studies have reported that the genetic diversity exceeds the morphological diversity (Casamatta et al. 2005, Komárek and Anagnostidis 2005, Johansen et al. 2008).

Recently, the new monophyletic genus *Nodosilinea* was described from strains previously identified as *Leptolyngbya* spp. Morphologically, this new genus comprises species with isodiametric or longer than wide cells, that possess immotile trichomes with a sheath and that have the ability to form nodules when exposed to limited light stress (Perkerson et al. 2011). Five strains isolated in this study were assigned to *Nodosilinea*: CENA512, CENA515, CENA522, CENA523 and CENA546. All of these strains showed morphological features consistent with *Nodosilinea* spp., except for the nodulation under low light, which was not yet assessed in the isolated strains. Additionally, these strains shared > 97 % sequence identity with other *Nodosilinea* spp., and in the phylogenetic analysis fell into the *Nodosilinea* cluster (Fig. 3, Clade C).

Thirteen isolates with *Leptolyngbya*-like morphological traits corresponded to novel Pseudanabaenaceae strains because they showed < 95 % identities in Blast analyses. These sequences grouped into four exclusive clades in the phylogenetic reconstruction (Fig. 3, Clades D, E, F and H), all demonstrating some morphological coherence (Fig. 2). Likewise, within these clades, the 16S rRNA sequences had the same length and shared at least 99 % sequence identity. Clade F (97 % bootstrap value) was formed by five sequences generated in this study and two uncultured sequences also retrieved from an extreme environment (saline-alkaline soil - Valenzuela-Encinas et al. 2009). Interestingly, all of these strains showed slight gliding motility. Clade H (89 % bootstrap value) exclusively comprises four sequences and has a sister taxon, *Planktolyngbya limnetica* PMC271.06 (99 % bootstrap value). The strains within this clade had morphological characteristics similar to those of *Planktolyngbya* spp. (Komárek and Anagnostidis 2005), but differed in their trichome organization into ornate mats (Fig. 2). Taking into account these well-defined clades and the origin of the strains, Clades H and F may represent two novel generic units that must be better explored in a future study.

Clades D and E are also formed by new Pseudanabaenales strains (< 95 % identity in the Blast analysis) but with weak support (bootstrap value  $\leq$  51 %). To correctly define these strains, more data on related strains are needed.

#### 4. Conclusions

This is the first report to describe the diversity of cultured cyanobacteria from the Pantanal saline-alkaline lakes using a polyphasic approach. Few genera were identified by morphological evaluation. However, our phylogenetic analysis indicated a larger number of taxa dispersed into 13 clades, of which five represent novel groups, indicating that morphological diversity underestimates genetic diversity. Our phylogenetic affiliations underscored the relevance of considering the environment in defining the taxonomy of homocytous cyanobacteria because lineages from extreme habitats tended to group together independently of the extreme environmental conditions. These data highlight the importance of polyphasic approaches to determine cyanobacterial taxonomy.

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**Table 1** - Main characteristics and abiotic variables of the studied saline-alkaline lakes from Pantanal da Nhecolândia, MS, Brazil. Measurements were made during the dry and wet seasons, from May 2009 to November 2011.

	Saline-alkaline lakes				
	1 - Verde	2 - Grande	3 - Preta	4 - 67mil	5 - Centenário
Geographic Coordinates	19°28'13"S	19°26'56"S	19°26'56"S	19°27'42"S	19°26'24"S
	56° 3'22"W	56° 7'45"W	56° 7'55"W	56° 8'21"W	56°5'58"W
Area (km <sup>2</sup> )	0.15	0.32	0.09	0.14	0.28
Water level (m)	0.1 to 1.5	Dry to 0.6	1.2 to 2.5	Dry to 0.6	Dry to 0.7
pH <sup>a e</sup>	8.4 to 9.7	9 to 9.8	8.5 to 8.8	8.4 to 10.3	10.02 <sup>b</sup>
EC (mS.cm <sup>-1</sup> ) <sup>c e</sup>	1.98 to 15	2.61 to 18	1.1 to 4.7	0.6 to 67	2.54 <sup>b</sup>
Bloom occurrence	almost permanent	almost permanent	never	seasonal <sup>d</sup>	seasonal <sup>d</sup>

<sup>a</sup> the pH value was measured at 0.3 meters below surface; <sup>b</sup> only one value was determined; <sup>c</sup> EC: Electrical Conductivity measured at 0.3 meters below surface. The higher values were obtained during dry season and the lower ones during the wet season; <sup>d</sup> bloom occurrence was observed only during the dry season; <sup>e</sup> electrical conductivity and pH did not show any significant variability laterally or vertically within a given lake.

**Table 2** – List of isolated strains with taxonomic affiliation, saline-alkaline lake of origin and GenBank access number of 16S rRNA sequences.

Order	Taxonomic assignment	Lake ( <i>Salina</i> )	GenBank
Synechococcales	<i>Geminocystis</i> sp. CENA526	Centenário	KF246492
Chroococcales	<i>Cyanobacterium</i> sp. CENA527	Grande	KF246493
	<i>Leptolyngbya</i> sp. CENA520	Verde	KF246487
	<i>Leptolyngbya</i> sp. CENA532	Preta	KF246498
	<i>Leptolyngbya</i> sp. CENA534	Verde <sup>a</sup>	KF246500
	<i>Leptolyngbya</i> sp. CENA538	67 Mil	KF246502
	<i>Leptolyngbya</i> sp. CENA540	Verde	KF246504
	<i>Leptolyngbya</i> sp. CENA542	Verde	KF246505
	<i>Limnothrix</i> sp. CENA545	67 Mil	KF246506
	<i>Nodosilinea</i> sp. CENA512	Verde	KF246481
	<i>Nodosilinea</i> sp. CENA515	Verde	KF246482
	<i>Nodosilinea</i> sp. CENA522	Verde	KF246489
	<i>Nodosilinea</i> sp. CENA523	Verde	KF246490
	<i>Nodosilinea</i> sp. CENA546	67 Mil	KF246507
Pseudanabaenales	Pseudanabaenaceae CENA510	Verde	KF246480
	Pseudanabaenaceae CENA528	Preta	KF246494
	Pseudanabaenaceae CENA529	Preta	KF246495
	Pseudanabaenaceae CENA530	Preta	KF246496
	Pseudanabaenaceae CENA531	Preta	KF246497
	Pseudanabaenaceae sp. CENA516	Verde	KF246495
	Pseudanabaenaceae sp. CENA517	Verde	KF246496
	Pseudanabaenaceae sp. CENA518	Verde	KF246497
	Pseudanabaenaceae sp. CENA519	Verde	KF246498
	Pseudanabaenaceae sp. CENA521	Verde	KF246488
	Pseudanabaenaceae sp. CENA537	Grande	KF246501
	Pseudanabaenaceae sp. CENA539	Verde	KF246503
Oscillatoriales	<i>Phormidium</i> sp. CENA525	Centenário	KF246491
	Phormidiaceae CENA533	Preta	KF246499

<sup>a</sup> isolated from sediment sample.

**Figure S1** – Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains of Chroococcales and Synechococcales. Bootstrap values equal to or greater than 50 % are indicated at the nodes. The studied strains are shown with a filled and bold circle.

**Figure S2** – Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strains of Oscillatoriales and Pseudanabaenales. Bootstrap values equal to or greater than 50 % are indicated at the nodes. The studied strains are shown with a filled and bold circle. The clades indicated as A-K correspond to those of Figure 3.