GENETIC POTENTIAL FOR TOXICITY: BLOOMS OF CYANOBACTERIA IN THE ITAIPU RESERVOIR, BRAZIL

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Abstract. Cyanobacteria are algae of great importance to environmental and public health because they produce toxins, such as microcystins. The same species of cyanobacteria may show microcystin-producing and non-microcystin-producing genotypes, which are impossible to differentiate based on morphological characteristics. The presence of the mcyA gene has been used as an indirect diagnosis for the identification of microcystin-producing cyanobacteria. The aim of this work was to evaluate if the blooms of cyanobacteria in the Itaipu reservoir present a microcystin-producing genotype. To that end, the phycocyanin intergenic spacer (PC-IGS) and partial mcyA gene were amplified and sequenced. The presence of these genes was observed in all the samples, indicating the toxicological potential of the blooms of cyanobacteria in the Itaipu reservoir. The phylogenetic analyses of sequences from cyanobacteria blooms isolated from the Itaipu reservoir and sequences available in GenBank demonstrated the genetic similarity between samples from Itaipu and species from the *Microcystis* genus.
Key words: *Microcystin, PC-IGS, mcyA.*

Introduction

Cyanobacteria are ubiquitous organisms that can be found in many diversified aquatic environments. Their presence is important because they are known to produce cyanotoxins (Carmichael, 1994) that are capable of affecting the aquatic biota, which results in poisonous effects even in terrestrial mammals (Sivonsen and Jones, 1999). Cyanobacteria produce several different types of toxins, including microcystins. Microcystins are a hepatotoxin produced by a cluster of 10 genes consisting of 55,000 base pairs (bp) arranged in two probable operons, mcyA-C and mcyD–J, which encode for a complex enzymatic system involved in microcystin biosynthesis (Tillett *et al*., 2000), that acts by covalently binding to phosphatase proteins, which disrupts cellular signaling and can lead to hepatic tumors, as described by (Falconer *et al*., 1994; Ito *et al*., 1997). As such, the presence of microcystin-producing cyanobacteria in bodies of water has obvious relevance to public health.

In 1995, Neilan *et al*. described a pair of primers that are derived from the coding region of the α and β subunits of the phycocyanin (cpcA and cpcB) and the intergenic spacer between these two subunits (PC-IGS). These primers can be used to amplify DNA by PCR (polymerase chain reaction) and, with the use of restriction enzymes (RFLP), can aid in the identification of many cyanobacteria genera. But the primers selectively amplify both toxic and non-toxic variants of DNA of cyanobacteria that can be present in the complex microbial communities of aquatic environments.

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Another way to study the taxonomy and phylogeny of cyanobacteria is to sequence the PC-IGS and compare its sequence with those of several species of cyanobacteria that are available in GenBank using BLAST (Basic Local Alignment Search Tool) (Baker *et al*., 2002). The PG-IGS of cyanobacteria is appropriate for phylogenic studies because the two phycocyanin subunits α and β that flank the intergenic spacer are extremely conserved, which allows the annealing of primers across many species. The intergenic spacer, on the other hand, is very variable and allows the distinction of species of cyanobacteria (Neilan *et al*., 1995).

The toxicity of cyanobacteria cannot be evaluated by morphological characteristics. The difficulty in the differentiation among toxic and non-toxic strains has been overcome by the development of quantitative analyses of cyanotoxins in water. Recently, amplification of a fragment of the mcyA gene by PCR allowed the identification of cyanobacteria with a microcystin-producing genotype in blooms of water reservoirs. (Tillett *et al*., 2001) synthesized a pair of specific primers to amplify the mcyA gene of *Microcystis* species; however, the primers did not effectively amplify the mcyA gene of other cyanobacteria genera. (Hisbergues *et al*., 2003) synthesized a different pair of primers that correspond to another fragment of the mcyA gene, which allowed the selective distinction of microcystin and non-microcystin-producing cyanobacteria from several genera.

These alternative approaches for the detection of toxic cyanobacteria by amplification of the mcy gene has been applied to several genera, such as: *Microcystis* (Davis *et al*., 2009; Kurmayer *et al*., 2003; Via-Ordorika *et al*., 2004; Tillett *et al*., 2001), *Planktothrix* (Kurmayer *et al*., 2004), *Leptolyngbya* and *Geitlerinema* (Richardson *et al*., 2007), *Anabaena* (Kaebernick *et al*., 2002), *Nostoc* (Hisbergues *et al*., 2003), and others.

The evaluation of toxicity by PCR techniques is consistent with results obtained by the quantitative analyses of toxins in water by methods such as HPLC (High-pressure liquid chromatography), MALDI-TOF MS (Matrix-assisted laser desorption/ionization Team-Of-Light mass spectrometry) and ELISA (Enzyme-linked immunosorbent assay). Several authors have demonstrated a correlation between the presence of mcy genes and the detection of microcystin in water (Baker *et al*., 2002; Boaru *et al*., 2006; Davis *et al*., 2009; Kurmayer *et al*., 2004; Hisbergues *et al*., 2003; Mankiewicz-Boczek, *et al*., 2006; Oberholster *et al*., 2009; Via-Ordorika *et al*., 2004).

One of the most important reservoirs of South America, the Itaipu reservoir was built in 1982 and is located in the southwest of Paraná State (24° 15' – 25° 33' S-latitude; 54° 00' – 54° 37' W-longitude), and it demarcates part of the border between Brazil and Paraguay. The reservoir presents a flooded area of 1.350 km^2 and a residence time of 40 days. In general, the annual oscillations of the water level are less than one meter (Bini, 2001). Ecological or even taxonomy work on the algae community in the Itaipu reservoir is very rare. As such, the aim of this work was evaluate the toxicity of natural blooms of cyanobacteria in the Itaipu reservoir by the investigation of the presence of the mcyA gene.

Materials and methods

Two of the eight tributaries on the Brazilian margin of the Itaipu reservoir were chosen to collect the biological samples. Specifically, the tributaries that are formed by

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the São Francisco Verdadeiro (SV) and São Francisco Falso (SF) rivers were selected for this study (*Fig. 1*).

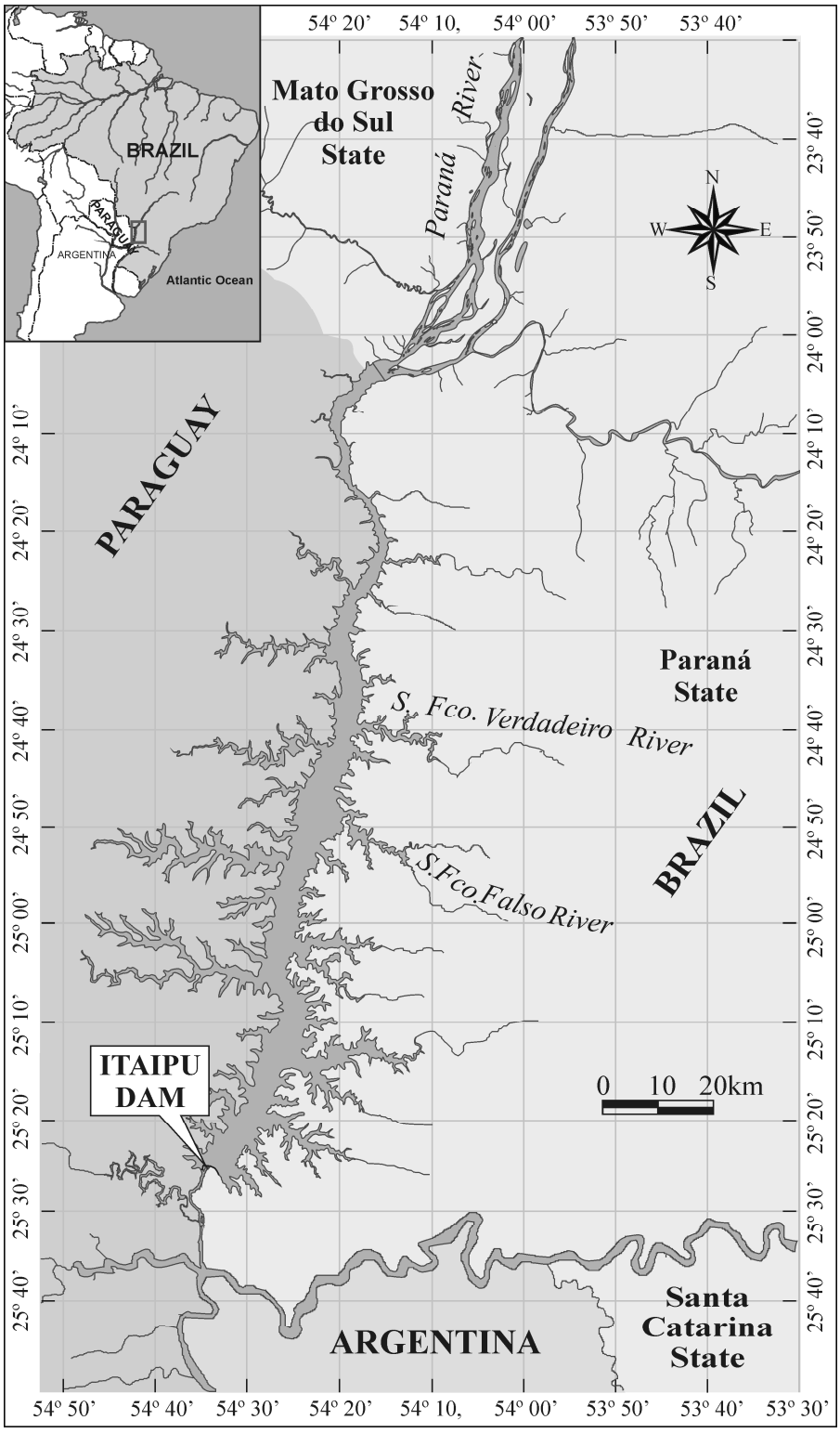

Figure 1. *Sampling sites in the two tributaries of the Itaipu reservoir (São Francisco Verdadeiro and São Francisco Falso rivers)*

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Approximately 150 ml of water from the subsurface that contains the cyanobacteria bloom was collected in October 2007. The collections were performed at four locations (SV1, SV2, SV3 and SV4) at the São Francisco Verdadeiro tributary and in one location (SF1) at the São Francisco Falso tributary. An aliquot of each sample was stored at 4°C for DNA extraction.

For DNA extraction, 2.0 ml of each sample was put into an Eppendorf tubes and centrifuged at 10,000 rpm for 10 min to pellet the cells. To each tube, 500 µl of extraction buffer was added (1 M Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, 140 mM β-mercaptoethanol, 5 M NaCl, 5% CTAB, and Sarcosyl 10%), in the presence of lysozyme (1 mg/ml) and incubated in a water bath at 37°C for 30 min. Proteinase K (50 µg/ml) was added and the cells were incubated for 2 h in a water bath at 60°C. Cellular debris was isolated from DNA by a phenol/chloroform extraction. The purified DNA was precipitated with a mixture of saline solution and ethanol overnight. The DNA was washed with ethanol to remove excess salt and treated with RNAse in a 37°C water bath for 2 h. Extracted DNA was quantified by agarose (1%) gel electrophoresis, using as a reference DNA from the bacteriophage λ at concentrations of 25, 50 and 100 ng/l.

The primer pair PCβF (5'-GGCTGCTTGTTTACGCGACA-3') and PCαR (5'-CCAGTACCA-CCAGCAACTAA-3') (Neilan *et al*., 1995) was used for amplification of the phycocyanin intergenic spacer (PC-IGS), which is a positive control for the presence of cyanobacteria DNA in the sample. The primer pair mcyA-Cd1R (5'-AAAAGTGTTTTATTAGCGGCTCAT-3') and mcyA-Cd1F (5'-AAAATTAAAAGCCGTATCAAA-3') (Hisbergues *et al*., 2003) was used for the partial amplification of the mcyA gene.

The PCR conditions were as described by (Prioli *et al*., 2002) and consisted of the following steps: 95°C for 10 min, 35 cycles of 95°C for 90 s, 56°C for 30 s, 72°C for 50 s and a final step of 72°C for 7 min. PCR fragments were submitted to electrophoresis in a 1,5% agarose gel with a standard DNA ladder 100 bp (Gibco BRL). The gels were stained with ethidium bromide.

Approximately 50 ng of DNA from each reaction was used in the sequencing reactions using the MegaBase automatic sequencer (Amersham), according to the manufacturer's instructions. Fifty-one sequences of the PC-IGS and 15 sequences of the partial mcyA gene from species of cyanobacteria of the orders Chroococcales, Nostocales and Oscillatoriales were selected from GenBank for the phylogeny analysis. Only sequences published in scientific journals were selected.

The sequences were aligned using the Clustal W (Thompson *et al*. 1994) computer program and edited with the Bioedit program (Hall, 1999). The choice of evolutionary model, using the Akaike Information Corrected Criterion (AICc) and Bayseian Information Criterion (BIC) procedures, was performed using the Paup 4.0b4 (Swofford, 2002) and Modeltest 3.0 (Posed and Crandall, 1998) programs. The nucleotide diversity matrix and the Neighbor-Joining dendrogram were built with the Mega 4.0.1 program (Tamura *et al*., 2007). The principal coordinate scatter plot was built using the eigenvectors after Lingoes correction criterion with the Statistica 7.1 program (StatSoft, Inc., 2005).

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Results

Phycocyanin Intergenic Spacer (PC-IGS)

The results of the PCR using primers to amplify the PC-IGS produced fragments of approximately 650 bp (*Fig. 2*), which confirmed the presence of cyanobacteria DNA in the samples.

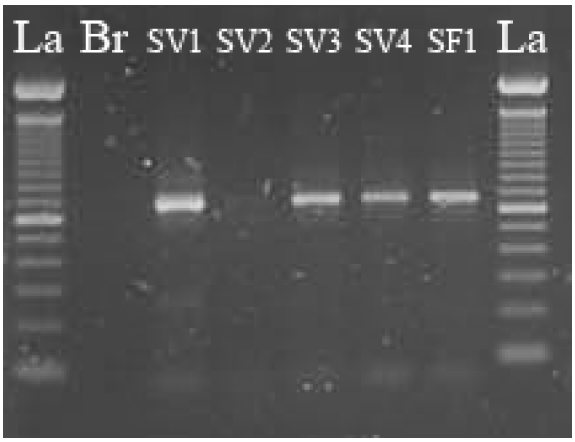

Figure 2. *A 1,5 % agarose gel including a 100 bp ladder (La) and the negative control (Br). Fragments of 650 bp correspond to the amplification product of the PC-IGS from samples of cyanobacteria blooms from São Francisco Verdadeiro (SV1, SV2, SV3 and SV4) and São Francisco Falso (SF1) tributaries, using the PCβF and PCαR primers*

The PCR products for the samples from the São Francisco Verdadeiro (SV1, SV2, SV3 and SV4) tributary were sequenced. After the alignment, a 320 bp sequence was obtained that encodes for part of the phycocyanin β subunit, the complete intergenic spacer and part of the α subunit. The Tamura-Nei evolutionary model was selected for phylogenetic analysis. The sequence indicated that there were six nucleotide substitutions points among the four samples. No substitution was found between the samples SV2 and SV3. The transition/transversion bias was 1.7 and the distribution of nucleotide bases was A=0.247, T=0.241, C=0.263 and G=0.248.

The results of the Neighbor-Joining dendrogram and the scatter plot, that were built with the Itaipu reservoir samples, in combination with sequences available from GenBank, made it was possible to characterize the three orders of cyanobacteria that were analyzed. The sequences of the cyanobacteria samples from the Itaipu reservoir aligned with species from the *Microcystis* genus in the Chroococcales cluster (*Fig. 3* and *Fig. 4*).

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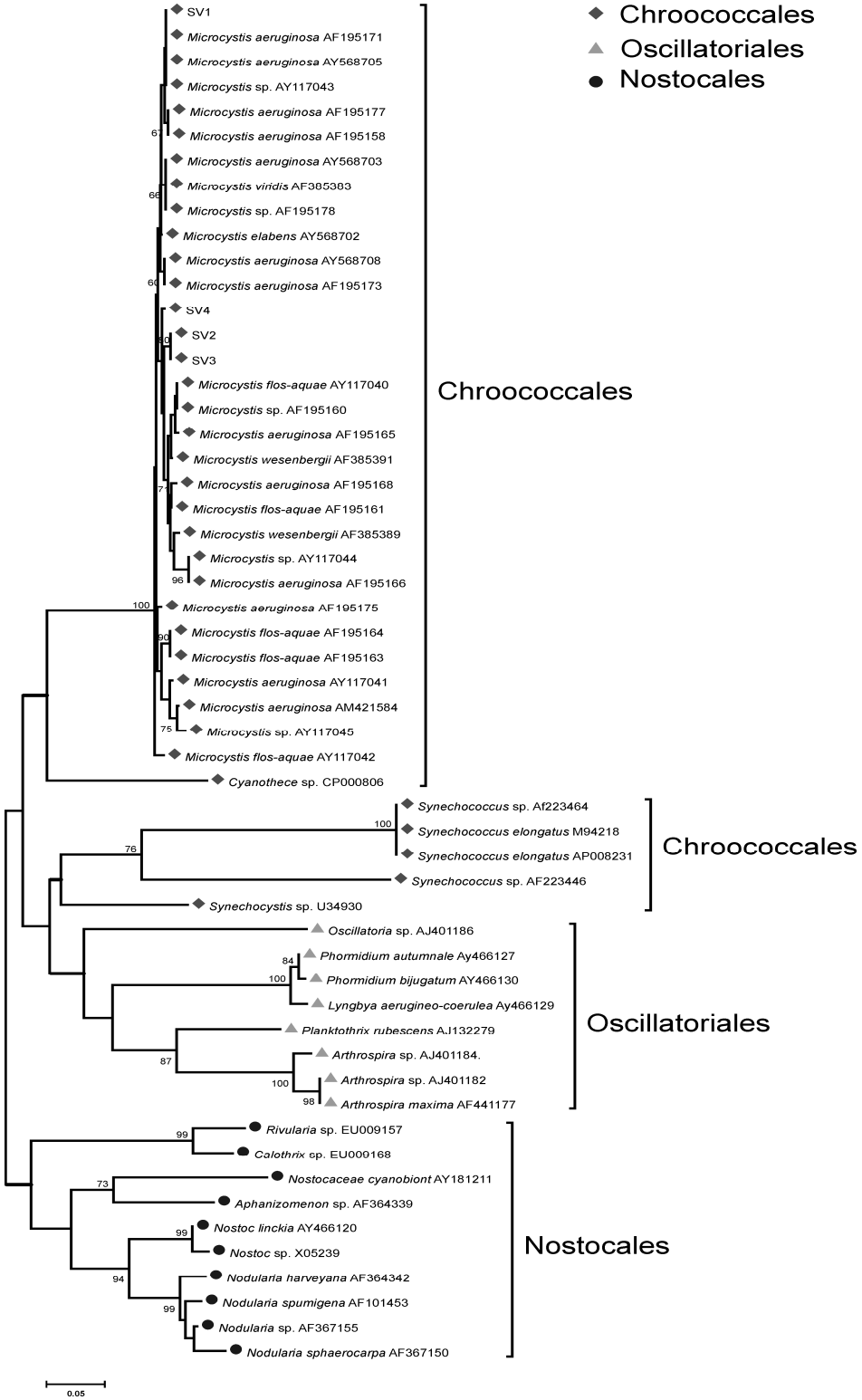

Figure 3. *Neighbor-Joining dendrogram built with the Tamura-Nei model, with 10,000 Bootstraps, from sequences of the PC-IGS from samples of cyanobacteria blooms from São Francisco Verdadeiro (SV1, SV2, SV3 and SV4) tributary and sequences available in GenBank*

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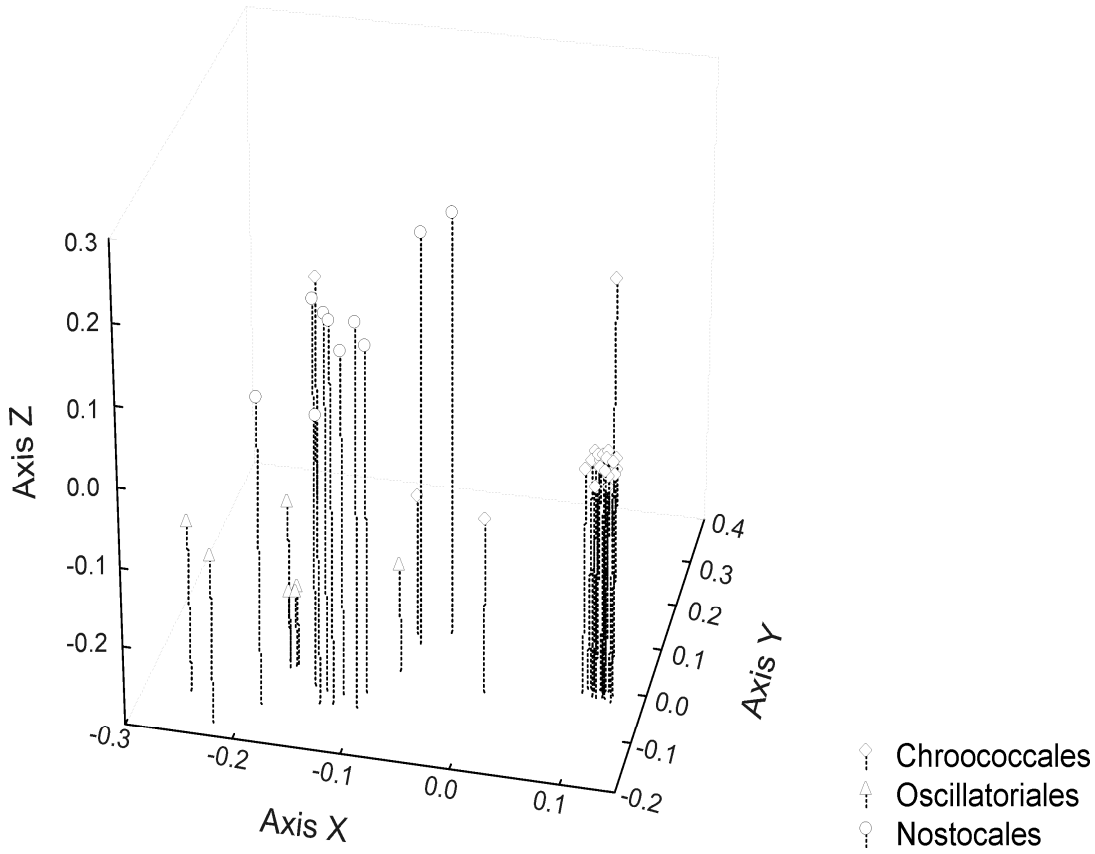

Figure 4. *Scatter plot built with the eigenvectors obtained from the Tamura-Nei genetic distance matrix from PC-IGS sequences from samples of cyanobacteria blooms from São Francisco Verdadeiro (SV1, SV2, SV3 and SV4) tributary and sequences available in the GenBank*

Toxicity

Partial amplification of the mcyA gene produced a DNA fragment of approximately 300 bp for all analyzed samples (*Fig. 5*). The presence of the mcyA gene in all samples indicates that the cyanobacteria blooms present in the Itaipu reservoir are from a microcystin-producing genotype strain.

The partial microcystin synthetase gene (mcyA) of three samples from Itaipu reservoir was sequenced. After alignment, a 236 bp sequence was obtained. Only one nucleotide substitution point was observed, which was in the sample from the São Francisco Falso (SF1) tributary. The transition/transversion bias was 1.7, with nucleotide base distribution of A=0.265, T=0.328, C=0.155 and G=0.253.

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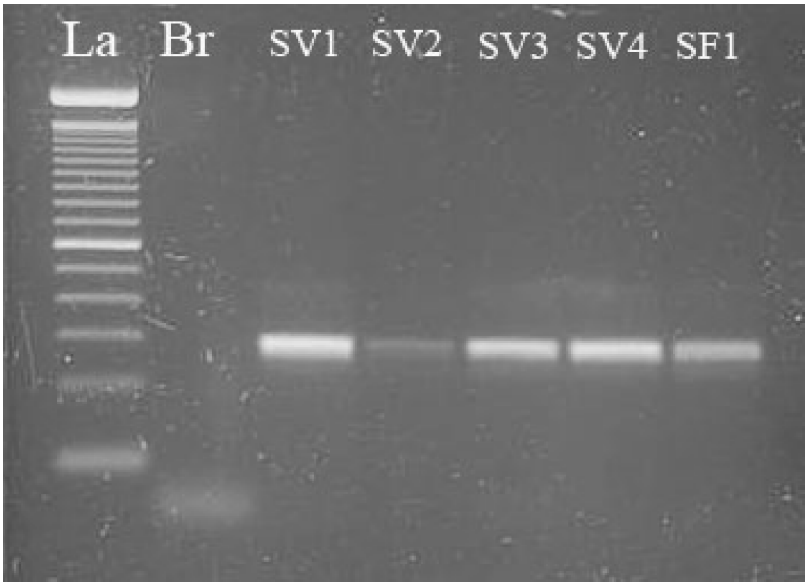

Figure 5. *A 1,5% agarose gel including a 100 bp ladder (La), and the negative control (Br) Fragments of 300 bp correspond to the amplification product of the partial mcyA gene from samples from the São Francisco Verdadeiro (SV1, SV2, SV3 and SV4) and São Francisco Falso (SF1) tributaries, using the mcyA-Cd1R and mcyA-Cd1F primers*

The Neighbor-Joining dendrogram separated the samples from the Itaipu reservoir into a single cluster, which was very close to the cluster of the species from the *Microcystis* genus (*Fig. 6*). This results were consistent with the results obtained with the PC-IGS sequences, The genetic analyses of the partial sequence of the mcyA gene were also useful in the characterization of the three orders of cyanobacteria.

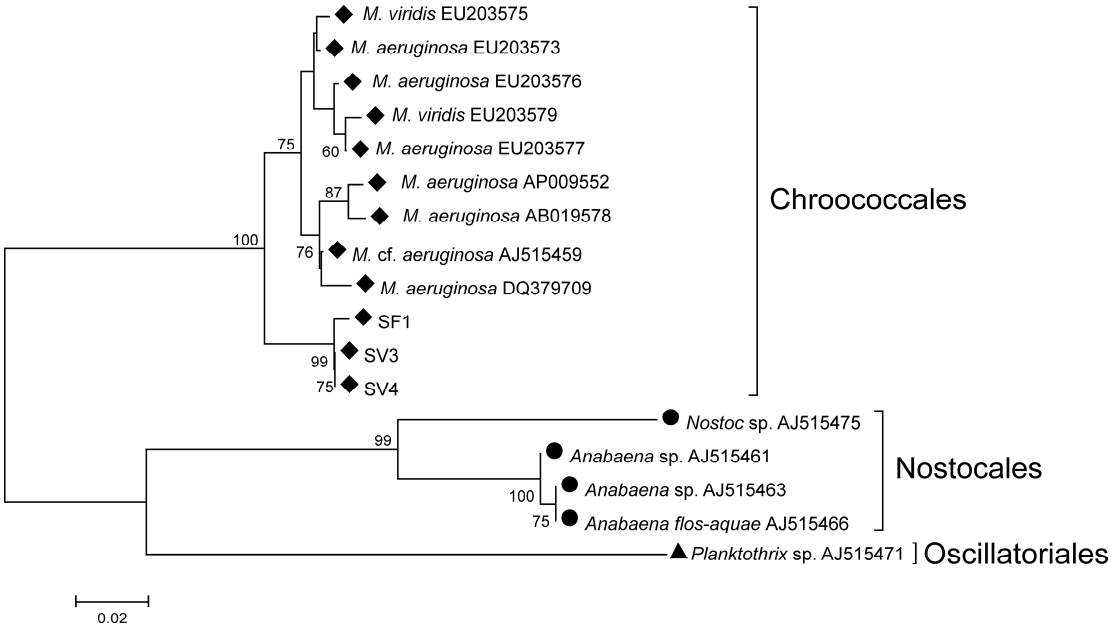

Figure 6. *Neighbor-Joining tree built with the p distance, with 10,000 bootstrap, using sequences of the partial mcyA gene from samples of cyanobacteria blooms from the São Francisco Verdadeiro (SV3 and SV4) and São Francisco Falso (SF1) tributaries and sequences available in GenBank*

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Discussion

Amplification of the PC-IGS sequence is a positive control for the presence of cyanobacteria DNA in the samples. (Neilan *et al*., 1995) used primers of the PC-IGS for the selective PCR amplification of genetic material of cyanobacteria of different samples from aquatic environments. Fragments from toxic genera (*Microcystis* and *Anabaena*) had between 500 and 740 bp. All of the DNA samples studied from the Itaipu reservoir tested positive for the PC-IGS, which confirms the presence of cyanobacteria in the algae blooms that were studied.

The results of the PC-IGS sequencing demonstrate the genetic relationship among samples from the Itaipu reservoir and species from the *Microcystis* genus, such as *Microcystis aeruginosa*. The exact result, with simple nucleotide peaks in the sequencing, confirms that the extracted DNA of the samples were from one single species since the presence of more than one species (in significant abundance) would show up as multiple nucleotide peaks (Baker *et al*., 2002).

The presence of the mcyA gene in the samples collected from the Itaipu reservoir demonstrates the toxicological power of these cyanobacteria blooms. Environmental conditions, such as light intensity, nutrient concentration, temperature, age and size of the colony, appear to influence microcystin production. However, the environmental factors that control the expression of the microcystin synthetase genes are not yet completely understood (Nishizawa *et al*., 1999).

In fact, the presence of the mcyA gene alone is not enough to infer that the blooms are producing cyanotoxin. In 2009, Ostermaier and Kurmayer demonstrated that the mcyA gene could be inactivated in natural populations, due to a mutation in the gene. However, the presence of the mcyA gene has been correlated with the ability of cyanobacteria to produce microcystin (Tillet *et al*. 2000), and several studies have correlated the presence of mcy genes, included mcyA, with the presence of microcystin in water (Baker *et al*., 2002; Boaru *et al*., 2006; Kurmayer *et al*., 2004; Hisbergues *et al*., 2003; Mankiewicz-Boczek, *et al*., 2006; Via-Ordorika *et al*., 2004). Thus, the results obtained in this work indicated that the species of cyanobacteria that is present in samples from two tributaries of the Itaipu reservoir is from a microcystin-producing strain.

The present study emphasizes the importance of using molecular markers for the identification of potentially toxic cyanobacteria in environmental monitoring programs because this method allows the evaluation of algae before the toxins are released into the water. The presence of a species that contains a genotype for microcystin production is of particular concern for public health in water reservoirs, such at the Itaipu reservoir, because it is used for recreation by the riverine population and tourism

The sequencing of the PC-IGS and the partial mcyA gene is shown to be useful in the molecular analyses of cyanobacteria and must be utilized together for the identification of potentially toxin-producing cyanobacteria.

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