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Cyanobionts from the coralloid roots of Cycadales: is there any molecular evidence of cyanotoxins?

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ABSTRACT

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The gymnosperms of the order Cycadales form a symbiosis with cyanobacteria in a special zone of the coralloid roots called the cyanobacterial zone (a green ring between the inner and outer root cortex). Free-living cyanobacteria synthesise secondary metabolites, including cyanotoxic compounds. Given that cyanotoxins were synthesised by certain symbiotic cyanobacteria from lichens and from the gymnosperm *Macrozamia*, this study investigated the presence of genes for four cyanotoxins (microcystin, nodularin, cylindrospermopsin and saxitoxin) in the cyanobionts of *Encephalartos* and *Cycas revoluta* collected in three Portuguese botanical gardens. PCR reactions indicated that the cyanobionts did not have the genes to synthesise microcystin, nodularin and cylindrospermopsin. However, for the saxitoxin gene, three isolates (accessions fEnc1, fEH1, cEH1) showed a positive amplification with the pair of primers *sxt*I683F/*sxt*877R. The BLAST of the nucleotide sequences did indicate a homology to the *sxt*I gene that encodes the enzyme *O*-carbamoyltransferase (OCTASE). Nevertheless, the same samples did not amplify with a second pair of primers for the *sxt*I gene (*sxt*I-F2/*sxt*I-R). Most likely, this result indicates that the OCTASE enzyme is related to the synthesis of other compounds rather than saxitoxin. More research on these cyanobionts should be conducted in the future.

Key words: Cyanobionts, Cycadales, cyanotoxins, saxitoxin, microcystin, cylindrospermopsin, nodularin.

RESUMEN

Cianobiontes en raíces coraloides de Cycadales: ¿hay alguna evidencia molecular de cianotoxinas?

Las gimnospermas del Orden Cycadales forman simbiosis con las cianobacterias en una zona de las raíces coraloides llamada la zona de cianobacterias (un anillo verde entre la corteza interna y externa de la raíz). Las cianobacterias de vida libre sintetizan metabolitos secundarios que incluyen compuestos cianotóxicos. Como algunas cianobacterias simbióticas de líquenes y de la gimnosperma Macrozamia *sintetizan cianotoxinas, se decidió investigar la presencia de los genes de cuatro cianotoxinas (microcistina, nodularina, cilindrospermopsina y saxitoxina) en los cianobiontes aislados de* Encephalartos y Cycas revoluta *recogidas en tres jardines botánicos portugueses. La amplificación de PCR indica que los cianobiontes no tienen los genes para la síntesis de la microcistina, nodularina y cilindrospermopsina. Sin embargo, para el gene de la saxitoxina, tres aislamientos (fEnc1, fEH1, cEH1) mostraron una amplificación positiva con el par de cebadores* sxt*I683F/*sxt*I877R. El BLAST de las secuencias de nucleótidos indicó una homología con el gen* sxt*I que codifica la enzima* O-*carbamoiltransferasa (OCTASA). Sin embargo, las mismas muestras no mostraron amplificación con un segundo par de cebadores para el gen* sxt*I (*sxt*I-F2/*sxt*I-R). Este resultado probablemente apunta a que la enzima OCTASA puede estar relacionada con la síntesis de otros compuestos en lugar de la saxitoxina. Se debería ampliar la investigación con estos cianobiontes en el futuro.*

Palabras clave: *Cianobiontes, Cycadales, cianotoxinas, saxitoxina, microcistina, cilindrospermosina, nodularina.*

INTRODUCTION

Several free-living cyanobacteria of the genera *Anabaena*, *Nodularia*, *Nostoc*, *Aphanizomenon* and others synthesize bioactive secondary metabolites, such as the cyanotoxins (the hepatotoxins microcystin and nodularin, the neurotoxin saxitoxin and the cytotoxin cylindrospermopsin). Those substances, when released in water bodies, have adverse effects on cells or tissues after their ingestion by animals or uptake by phytoplankton, zooplankton and plants. These effects poses a health problem due to not only food web bioconcentration and bioaccumulation but also poisoning through the ingestion of contaminated food (Wiegand & Pflugmacher, 2005; Jungblut & Neilan, 2006; Pearson *et al.*, 2010; Merel *et al.*, 2013).

Several heterocystic nitrogen-fixing cyanobacteria belonging to a few genera such as *Anabaena* or *Nostoc* establish symbiotic associations with eukaryotic organisms such as sponges, fungi, and plants (bryophytes, pteridophytes, gymnosperms and angiosperms). The symbioses in plants occur in extracellular or intracellular pre-existing or newly formed anatomical structures and are non-everlasting, with a *de novo* infection by the cyanobacterium and formation of the partnership each time a new plant develops; the exceptions are the pteridophytes belonging to the family *Azollaceae*, which form a permanent symbiosis with the cyanobacterium *Anabaena azollae*. In those symbioses, the cyanobacterium fixes the atmospheric nitrogen into ammonia, nitrates or nitrites that can be released and absorbed by the host, and the host, in turn, provides other nutrients such as sugars as well as protection against abiotic and biotic factors (Rai *et al.*, 2000; Usher *et al.*, 2007).

The order Cycadales is an ancient group that occurs in tropical and subtropical regions. The order is divided into two families, Cycadaceae (only containing the genus *Cycas*) and Zamiaceae (with the remaining 10 genera, in which are included the genus *Encephalartos*) (Zonneveld, 2012). All the members of the order Cycadales form, in addition to the main root, finger-like lateral roots (called coralloid roots) that protrude from the ground or remain at a shallow depth in the soil and in which infection or colonization by the cyanobacterium occurs (Rai *et al.*, 2000). In the coralloid roots, in a zone called the cyanobacterial ring lying between the inner and the outer root cortex (Fig. 1), inhabit filamentous heterocystous cyanobacteria. These cyanobacteria belong to several genera: *Nostoc* (Costa *et al.*, 1999; Gehringer *et al.*, 2010; Thajuddin *et al.*, 2010; Yamada *et al.*, 2012), *Calothrix* (Gehringer *et al.*, 2010; Thajuddin *et al.*, 2010) or *Anabaena* (Gehringer *et al.*, 2010). As mentioned above, certain free-living cyanobacteria synthesise cyanotoxins, and as the majority of the symbioses are *de novo* infections with cyanobacteria from the surrounding environment, it can be assumed that if the cyanobacteria that form the symbiosis have the genes to synthesise cyanotoxins, those compounds could be produced during the symbiosis. However, research on cyanotoxins in cyanobionts is very recent, and current information is still scarce. Nevertheless, the microcystin (MC) variants MC-LR and MC-XR have been found in the cyanobionts of the lichens *Pannaria* (Oksanen *et al.*, 2004), *Peltigera*, *Sticta*, *Lobaria* and *Nephroma* (Kaasalainen *et al.*, 2012, 2013), and nodularin (Nod) has been detected in the cyanobionts of the lichens *Sticta* and *Peltigera* (Kaasalainen *et al.*, 2012). In the gymnosperms, nodularin has been detected in the *Nostoc* symbiont of *Macrozamia riedlei* and *M. serpentina* (Gehringer *et al.*, 2012), but the presence of these cyanotoxins in other cyanobionts associated with the Cycadales has not yet been demonstrated.

The aim of this study was to evaluate the possible presence of four cyanotoxic genes (microcystin, nodularin, cylindrospermopsin and saxitoxin) in the cyanobionts of the coralloid roots of Cycadales collected in three Portuguese botanical gardens.

MATERIALS AND METHODS

Plant material

Coralloid roots located at or a few centimetres below the soil surface were collected from the

living collections of three Portuguese botanical gardens in northern and central mainland Portugal (Table 1). The gymnosperms from which the coralloid roots were collected belong to the order Cycadales and to the families Cycadaceae and Zamiaceae. The roots were washed and scrubbed in tap water to remove soil and stored at 4 °C.

Isolation and culture of the cyanobionts

Two sets of cyanobionts from the coralloid roots were used in this research –fresh isolated cyanobionts and cultured cyanobionts in culture medium. The coralloid roots were disinfected with hydrogen peroxide (2%) and washed in sterile water. For the fresh isolated cyanobionts, 1 cm long sections of the coralloid roots were cut, the root cap peeled off and the cyanobacterial zone transferred to Eppendorf tubes, homogenised in a vortex to detach the cyanobionts and stored at −20 °C until DNA extraction.

To culture the cyanobionts in liquid medium and under controlled conditions, the cyanobionts from coralloid roots were isolated using the method described by Gehringer *et al.* (2010) with some modifications. To isolate the cyanobionts, 1-mm transverse sections of sterile coralloid roots were placed on agar plates containing BG_0-11 medium at pH 7.4 (Rippka *et al.*, 1979), 2% agar and 100 μg/ml cycloheximide. The plates were incubated in a culture chamber at 24 °C, with a photoperiod of 16 h light/ 8 h dark and a light intensity of 40 μmol m^{-2} s^{-1}. After 2 months, the colonies of each section were cultured in liquid BG_0-11 medium containing 100 μg/ml cycloheximide in the same conditions described above. After the observation of the cyanobiont filaments of each isolate under a light microscope (Olympus BX41, Olympus, Lisbon, Portugal), all isolates of each gymnosperm were placed in a single culture with liquid BG_0-11 without cycloheximide. Every month, the biomass was collected by centrifugation at 4600 rpm (10 min, 4 °C), and stored at −20 °C until DNA extraction.

DNA extraction, PCR amplification and sequencing

The DNA of both cyanobionts (freshly isolated and cultured) were extracted with the PureLink® Genomic DNA MiniKit (Invitrogen, Lisbon, Portugal) according to the manufacturer's instructions. The DNA was quantified using a Qubit®fluorometer (Invitrogen) with the Quant-iT® dsDNA HS assay following the manufacturer's instructions. The DNA was stored at −20 °C.

Table 1. List of accession designations of cyanobionts isolated from several Cycadales collected in three Portuguese botanical gardens and used in this study. *Lista de códigos de los cianobiontes aislados de varios ejemplares de Cycadales recogidos en tres jardines botánicos portugueses utilizados en este estudio.*

Accession[a]	Species	Origin and year of collection	Collector
Order Cycadales-Family Cycadaceae			
CR1	*Cycas revoluta*	Coimbra Botanical Garden, 2012	A. L. Pereira
CR2	*Cycas revoluta*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
Order Cycadales-Family Zamiaceae			
Enc1	*Encephalartos sp.*	Porto Botanical Garden, Lake Garden, 2012	A. L. Pereira
EH1	*Encephalartos horridus*	Coimbra Botanical Garden, 2012	A. L. Pereira
EH2	*Encephalartos horridus*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
EP1	*Encephalartos paucidentatus*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
EV1	*Encephalartos villosus*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
EA1	*Encephalartos altensteinii*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
EL1	*Encephalartos lebomboensis*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
ELeh1[b]	*Encephalartos lehmannii*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira
ELeh2	*Encephalartos lehmannii*	Tropical Botanical Garden, Lisbon, 2012	A. L. Pereira

[a] The accession designation of the cyanobionts was given by the manuscript authors.

[b] It was only possible to use the cultured cyanobiont.

To perform the PCR reactions, the thermocycler Biometra TProfessional (Biometra, Göttingen, Germany) was used. The amplification products were separated in 1.5% agarose gel electrophoresis running in TAE 1× at 150 V, 25-30 min and stained with 0.2 μg/ml ethidium bromide (BioRad, Hercules, CA, USA). The 1 Kb Plus DNA ladder (Invitrogen) was used as a molecular size marker.

Four cyanotoxic genes were assessed with specific primers: 1) *mcy*A-cd1F/*mcy*A-cd1R for microcystin A synthetase (*mcy*A) (Hisbergues *et al.*, 2003); 2) *hep*F/*hep*R for the microcystin/nodularin aminotransferase (AMT) domain (*mcy*E/*nda*F) (Jungblut & Neilan, 2006); 3) K18/M4 and M13/M14 multiplex PCR for cylindrospermopsin (*cyn*) (Schembri *et al.*, 2001; Fergusson & Saint, 2003); and 4) *sxt*I683F/*sxt*I877R (*sxt*1) (V. Ramos, unpublished) and *sxt*I-F2/*sxt*I-R (*sxt*2) (Kellmann *et al.*, 2008a; Brito *et al.*, 2012) for the saxitoxin (*sxt*I). Each 20 μl reaction contained 1 μl of 0.5 μM of each primer (Invitrogen), 2 μl of 0.1 μg/μl DNA, 9 μl Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal) and 7 μl of ultrapure sterile water. A negative control (with sterile ultrapure water) and a positive control (*M. aeruginosa* LEGE91094 for the microcystin and microcystin/nodularin genes, *A. ovalisporum* for the cylindrospermopsin gene and *A. gracillaris* LMECYA 40 from INSA for the saxitoxin gene) were included in each reaction. The DNA of the negative cyanotoxic strain *Anabaena azollae* from the pteridophyte *Azolla filiculoides* (obtained in the Botanical Garden of Lisbon University) was also included. For all the amplification reactions, an initial denaturation at 95 °C for 5 min, a final extension at 72 °C for 7 min and a hold at 4 °C were included. The protocols were as follows: 1) 35 cycles of 95 °C 90 s, 56 °C 30 s, 72 °C 50 s for *mcy*A; 2) 35 cycles of 92 °C 20 s, 56 °C 30 s, 72 °C 1 min for *mcy*E/*nda*F; 3) 30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 7 min for *cyn*; and 4) 35 or 30 cycles of 94 °C 10 s, 52 °C 20 s, 72 °C 1 min for *sxt*1 and *sxt*2, respectively.

To identify the cyanobiont, a PCR reaction was performed with the pair of primers 27F (Neilan *et al.*, 1997) and CYA781R (Nübel *et al.*, 1997) in 20 μl containing 1 μl of 0.5 μM of each primer (Invitrogen), 2 μl of 0.1 μg/μl DNA, 9 μl Supreme NZYTaq 2x Green Master Mix (NZYTech) and 7 μl of ultrapure sterile water. The amplification protocol included an initial denaturation at 95 °C 5 min, 35 cycles of denaturation at 92 °C, 20 s, annealing at 50 °C, 30 s, extension at 72 °C, 1 min, with a final extension at 72 °C 5 min and a hold at 4 °C. Negative (with sterile ultrapure water) and positive (*M. aeruginosa* LEGE91094) controls were included.

The PCR products were cut from the agarose gel and purified with the kit Cut&Spin Gel Ex-

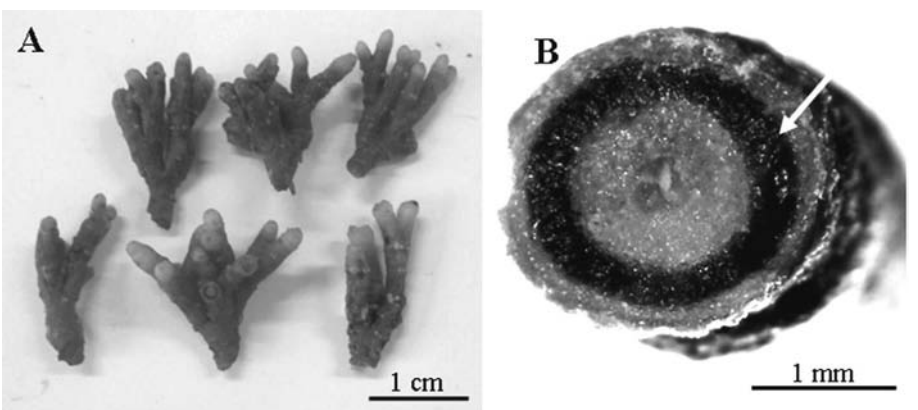

Figure 1. Coralloid roots of *Cycas revoluta*. (A) Coralloid roots. (B) Transverse section of a coralloid root with a green ring of cyanobacteria (arrow).). *Raíces coraloides de* Cycas revoluta*. (A) Raíces coraloides. (B) Sección transversal de una raíz coraloide con un anillo verde de cianobacterias (flecha).*

traction Columns (GRiSP, Porto, Portugal) according to the manufacturer's instructions. The positive PCR reactions for cyanotoxins were re-amplified and purified as described above. The PCR products were sequenced by Macrogen® (Amsterdam, The Netherlands). To correct mismatches and gaps, the forward and reverse sequences were aligned with the free software *multialin* 5.4.1 (Corpet, 1988) and matched using BLASTN 2.2.28 (Zhang *et al.*, 2000; Morgulis *et al.*, 2008) on the NCBI database.

RESULTS AND DISCUSSION

This paper is the first report on the possible presence of cyanobacterial genes involved in the synthesis of toxins by cyanobionts isolated from Cycadales collected in three Portuguese botanical gardens.

The search for cyanotoxic genes in cyanobionts is recent and has primarily been performed in samples of lichens and Cycadales collected in the wild. In cyanolichens belonging to several species collected from all around the world, two hepatotoxins, i.e., nodularin and microcystin, have been detected (Oksanen *et al.*, 2004; Kaasalainen *et al.*, 2012, 2013). In addition, in *Nostoc* isolated from the coralloid roots of *M. riedlei* and *M. serpentine* (collected in Australia), the hepatotoxin nodularin was detected (Gehringer *et al.*, 2012). However, in the cyanobionts isolated from the coralloid roots of several *Encephalartos* species and *C. revoluta* collected in Portuguese botanical gardens and used in the present research, the genes for microcystin and nodularin were not detected (Fig. 2A). In addition, the gene for cylindrospermopsin (a cytotoxin that inhibits protein synthesis) was also not detected for any of the cyanobionts analysed. These results indicate that the cyanobionts do not have the genes to synthesise the hepatoxins or the cytotoxins. As is also the case in the free-living cyanobacteria, the cyanobiont strains of different Cycadales can be toxic and/or non-toxic, or the cyanobiont strain that synthesises those cyanotoxins can depend on the collection site (wild or botanical gardens). The cyanotoxins (microcystins and nodularin) found in cyanolichens and *Macrozamia* sp. were identified as products of *Nostoc*

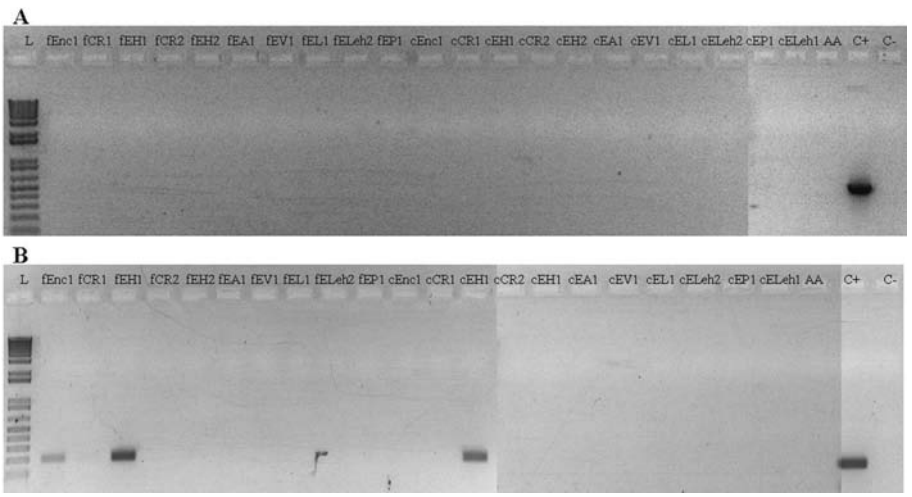

Figure 2. Agarose gel of the amplification for both fresh and cultured symbiotic cyanobacteria isolated from several Cycadales collected at three botanical gardens. (A) Aminotrasferase (AMT) domain of microcystin and nodularin. (B) Saxitoxin gene using the primers *sxt*I683F/*sxt*I877R. L, ladder; AA, *A. azollae*; C+, positive control; C–, negative control. See Table 1 for cyanobiont accession designation. The letter f or c before the accession designation refers to fresh isolated and cultured cyanobionts, respectively. *Gel de agarosa de la amplificación para cianobacterias aisladas (frescas y cultivadas) de varias Cycadales cultivadas en tres jardines botánicos. (A) Dominio de aminotransferasa (AMT) de microcistina y nodularina. (B) Gen de la saxitoxina con los cebadores* sxt*I683F/*sxt*I877R. L, gradiente; AA,* A. azollae*; C +, control positivo; C–, control negativo. Véase la Tabla 1 para la designación de código del cianobionte. La letra f o c antes del código se refiere a cianobiontes aisladas frescas y cultivadas, respectivamente.*

(Oksanen *et al.*, 2004; Gehringer *et al.*, 2012; Kaasalainen *et al.*, 2012, 2013), a genus that has both toxic and non-toxic strains. In our research, the cyanobionts isolated from all the coralloid roots of Cyacadales were identified as *Nostoc* by the BLASTN of the 16S gene (unpublished results).

The saxitoxins are neurotoxins produced by a few marine and freshwater cyanobacteria. Only recently has it been possible to sequence the complete saxitoxin gene cluster. One of the genes –*sxt*I– encodes the enzyme *O*-carbamoyltransferase (OCTASE), which transfers a carbamoyl group for a compound having a hydroxyl group, such as the saxitoxin precursor (Kellmann *et al.*, 2008a, 2008b; Moustafa *et al.*, 2009; Al-Tebrineh *et al.*, 2010; Murray *et al.*, 2011). In the present research, three cyanobionts –a fresh isolate from *Encephalartos* sp. (fEnc1) collected in the Porto botanical garden and fresh and cultured isolates from *E. horridus* (fEH1 and cEH1) collected in the Coimbra botanical garden– showed positive amplification for the *sxt*I gene with the pair of primers *sxt*I862F/*sxt*I877R (Fig. 2B). The BLASTN of the nucleotide sequences in the NCBI database, indicate the presence of the *sxt*I gene due to their homology with the *sxt*I gene of the free-living cyanobacteria *Aphanizomenon* and *Cylindrospermosis raciborskii* (Table 2). However, the DNA of the same samples did not amplify with the pair of primers *sxt*I-F2/*sxt*I-R, which amplifies a larger fragment

Table 2. The homologies for the saxitoxin gene with the BLASTN of the three cyanobionts isolated from three Cycadales. *Las homologías para el gen de la saxitoxina con el BLASTN de los tres cyanobiontes aislados a partir de tres Cycadales.*

Accession[a]	BLASTN isolate match and gene	NCBI reference	Homology	E-value
fENC1	*Aphanizomenon flos-aquae* NH-5, *O*-carbamoyltransferase (sxt1)	EU439559	81%	2e-31
	Aphanizomenon sp. NH-5, PSP[b]	EU603710	81%	2e-31
	Cylindrospermosis raciborskii CENA305, *O*-carbamoyltransferase (sxt1)	KC894589	77%	4e-18
	Cylindrospermosis raciborskii CENA302, *O*-carbamoyltransferase (sxt1)	JX175232	77%	4e-18
	Cylindrospermosis raciborskii T3, *O*-carbamoyltransferase (sxt1)	EU439556	77%	4e-18
fEH1	*Aphanizomenon flos-aquae* NH-5, *O*-carbamoyltransferase (sxt1)	EU439559	81%	2e-30
	Aphanizomenon sp. NH-5, PSP[b]	EU603710	81%	2e-30
	Cylindrospermosis raciborskii CENA305, *O*-carbamoyltransferase (sxt1)	KC894589	77%	1e-17
	Cylindrospermosis raciborskii CENA302, *O*-carbamoyltransferase (sxt1)	JX175232	77%	1e-17
	Cylindrospermosis raciborskii T3, *O*-carbamoyltransferase (sxt1)	EU439556	77%	1e-17
cEH1	*Aphanizomenon flos-aquae* NH-5, *O*-carbamoyltransferase (sxt1)	EU439559	81%	1e-33
	Aphanizomenon sp. NH-5, PSP[b]	EU603710	81%	1e-33
	Cylindrospermosis raciborskii CENA305, *O*-carbamoyltransferase (sxt1)	KC894589	77%	6e-21
	Cylindrospermosis raciborskii CENA302, *O*-carbamoyltransferase (sxt1)	JX175232	77%	6e-21
	Cylindrospermosis raciborskii T3, *O*-carbamoyltransferase (sxt1)	EU439556	77%	6e-21

[a] fENC1, cyanobiont fresh isolate from the coralloid roots of *Encephalartos* sp.; fEH1, cyanobiont fresh isolate from the coralloid roots of *Encephalartos horridus*; cEH1, cyanobiont cultured from the coralloid roots of *Encephalartos horridus*.

[b] PSP, paralytic shellfish poisoning.

of the *sxt*I gene. In addition, the saxitoxin was not detected in any strain of free-living *Nostoc*. The enzyme *O*-carbamoyltransferase (OCTASE), besides being identified in the gene cluster of the saxitoxin is also related to the biosynthesis of nodulation factors and of other secondary metabolites, such as antibiotics (Kellmann *et al.*, 2008a; Parthier *et al.*, 2012). Perhaps, in the case of the cyanobionts fEnc1, fEH1 and cEH1, the homology with the OCTASE gene may indicate that the cyanobionts synthesise other secondary compounds in which the OCTASE enzyme is required.

In conclusion, the cyanobionts from Cycadales grown in three Portuguese Botanical Gardens do not have the genes to synthesize the cyanotoxins nodularin, microcystin and cylindrospermopsin. However, one cyanobiont isolated from the coralloid roots of *Encephalartos* sp. (fEnc1) and two from *E. horridus* (fEH1 and cEH1) showed amplification for the saxitoxin gene. As the saxitoxin was not identified in free-living *Nostoc* strains, it is necessary to conduct more research on the DNA samples isolated in the present study that were positive for saxitoxin.

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