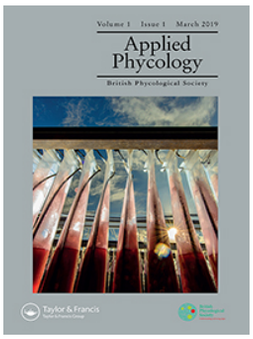

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Novel marine cyanobacteria from the Atlantic coast of Brazil

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ABSTRACT

Marine cyanobacteria have emerged as a source of promising bioactive compounds. The isolation of strains from tropical environments seems to be an important step in the search for molecules displaying bioactivity, besides being valuable for biotechnological purposes. Thus, the objective of the present study was to describe marine cyanobacterial strains, isolated and characterized in terms of morphology and molecular phylogeny, as well as screened for biologically active compounds. Five strains were isolated and morphologically characterized as *Leptolyngbya* (CENA553, CENA554 and CENA555) and *Geitlerinema* (CENA552 and CENA556). Considering the phylogenetic results, these strains did not match the clusters of the type-species or reference strains, indicating the possible emergence of new generic entities morphologically related to *Leptolyngbya* and *Geitlerinema*. Among the isolated strains, CENA552 and CENA556 were selected for chemical analyses. Five known nucleosides with biological activities and two amino acids were recognized and isolated from the CENA556 strain. The GC-MS analyses of strains CENA552 and CENA556 revealed distinct classes of non-polar compounds, with a predominance of octadecane (CENA556), 2-hexyl-1-decanol (CENA552) and neophytadiene (CENA552 and CENA556) hydrocarbons. All nucleosides and amino acids were highly abundant, indicating these morphotypes as promising sources of these bioactive compounds. This is the first report considering taxonomy/molecular phylogeny as well as chemistry prospection of morphotypes resembling *Geitlerinema*-like isolated from the Brazilian coastal area, highlighting the potential of these taxa for biotechnological applications.

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GC-MS analysis; marine environment; non-heterocytous strains; phylogeny; nucleosides; natural products

Introduction

Marine microorganisms have attracted attention as a promising source of natural products (Gerwick & Fenner, 2013). Among them, Cyanobacteria have been extensively investigated due to their wide metabolic and genetic/genomic diversity (Calteau et al., 2013), which in turn might be useful for the elaboration of natural products. Cyanobacteria have proven to be an important source of bioactive natural products with toxic activities, such as harmful toxins (Rinehart, Namikoshi, & Choi, 1994; Schuurmans et al., 2018) as well as molecules with potential beneficial bioactivities, such as cytotoxic, anti-tumour, antiviral, algaecide and immunosuppressive properties (Panjiar, Mishra, Yadav, & Verma, 2017). Some reports also emphasize the importance of cyanobacterial bioproducts because of their anti-tumour and anti-inflammatory activities (Gerwick & Moore, 2012; Silva-Stenico et al., 2013, 2012; Villa, Lieske, & Gerwick, 2010; Wrasidlo et al., 2008).

Although the Brazilian seashore extends for about 7,400 km and has a continental shelf area of about 3.4 million km^2, few studies have been conducted investigating the marine cyanobacterial diversity and its potential for natural product biosynthesis (Baeta-Neves & Tribuzi, 1992; Branco, Moura, da Silva, & Bittencourt-Oliveira, 2003; Branco, Sant'Anna, Azevedo, & Sormus, 1996; Branco, Sant'Anna, Paiva Azevedo, & De, Sormus, 1997; Caires et al., 2018a, 2018b; Caires, Sant'Anna, & de Castro Nunes, 2013; Crispino & Sant'Anna, 2006; Nogueira & Ferreira-Correia, 2001; Silva, Genuário, Vaz, & Fiore, 2014). In recent years, the isolation of marine cyanobacteria from Brazilian coastal areas, followed by their polyphasic evaluation, has led to the characterization of new strains (Silva et al., 2014) as well as the description of novel genera, for example, *Halotia* (Genuário, Vieira Vaz, Hentschke, Sant'Anna, & Fiore, 2015), *Aliterella* (Rigonato et al., 2016), *Neolyngbya* (Caires et al., 2018b) and *Capillus* (Caires et al., 2018c). Some of these isolated

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Note: Nucleotide sequence data reported are available in the GenBank database under the accession numbers: MF084980-MF084984.

strains have been already investigated regarding the production of bioactive secondary metabolites of biomedical and biotechnological interest (Caires et al., 2018a; Silva et al., 2014). In addition, recently erected taxa such as *Moorea* (Engene et al., 2012), *Caldora* (Engene et al., 2015), *Toxifilum* (Zimba, Huang, Foley, Linton, & Vis, 2017) and *Dapis* (Engene, Tronholm, Paul, & De Clerck, 2018) have also been reported to be prolific producers of bioactive compounds.

Natural compounds isolated from marine sources include nucleosides and amino acids, which show a broad range of bioactivities and biotechnological applications (Huang et al., 2014; Niu & Tan, 2015; Ollivaux, Soyez, & Toullec, 2014; Yang, Zheng, Peng, Qiang, & Yuan, 2003). The nucleoside spongosine and the modified nucleosides spongothymidine and spongouridine, for example, were initially isolated from the marine sponge *Cryptotethya crypta* (Bergmann & Burke, 1955; Bergmann & Feeney, 1950; Bergmann & Swift, 1951). Spongothymidine and spongouridine later led to the development of cytarabine (ara-C) and vidarabine (ara-A), both with anticancer action (Rangel & Falkenberg, 2015). Since then, many marine organisms have revealed the ability to produce nucleosides with unusual structures and significant bioactivities (Huang et al., 2014; Niu & Tan, 2015). Additionally, it is known that microorganisms produce and metabolize *D*-amino acids (Hopkins, O'Dowd, & Shiel, 1997) and that certain antibiotics produced by bacteria and fungi also contain *D*-amino acids (Friedman, 1999), reinforcing their biotechnological importance.

Considering that marine tropical cyanobacteria have been characterized as prolific sources of bioactive secondary metabolites (Armstrong, Vaz, Genuário, Fiore, & Debonsi, 2019; Caires et al., 2018a; Engene et al., 2013, 2012, 2015; Silva et al., 2014), we hypothesize that the strains isolated from the Brazilian Atlantic coast can be a potential source of natural products. For this purpose, we isolated marine cyanobacterial strains, which were characterized in terms of morphology and molecular phylogeny, as well as subjected to nucleoside and amino acid prospection. These investigations allowed insights into the production of nucleosides from marine cyanobacterial morphotypes resembling *Geitlerinema*, highlighting that genera other than *Lyngbya* can be a promising source of natural products in this environment.

Material and methods

Sampling sites and cyanobacterial isolation

Periphytic samples were collected along the shoreline of three different beaches (Corsário Natural Refuge - 23° 31′ 53.88" S/45° 9′ 51.63" W; Fortaleza Beach - 23° 31′ 23,62" S/45°10′ 0,68" W; and Dura Beach - 23° 30′ 6,30" S/45° 10′ 21,50" W) in the municipality of Ubatuba, northern coast of São Paulo state, Brazil (Fig 1). After sampling, the environmental samples were kept in cold SWBG-11 medium (Castenholz, 1988) during transportation to the laboratory. Three culture media, specific for marine cyanobacterial strains, were used for isolation: ASN-III (Rippka et al., 1979), SWBG-11 and MN (Castenholz, 1988) supplemented with cycloheximide (70 mg·L^{-1}). The collected material was fractionated mechanically and the sections were inoculated into the above mentioned liquid media. After growth, sub-samples were streaked onto solid media (1.2% of agar w/v). Repeated streaking and microscopic observation were performed until monocyanobacterial cultures were established. Cultured cells were grown under a 14:10 h light:dark cycle with white fluorescent irradiation (40 μmol·m^{-2}·s^{-1}) at 24 ± 1°C, during all isolation steps.

Characterization of cyanobacterial strains

Morphological evaluation

Cyanobacterial strains were identified using the diacritical morphological traits for genera (Komárek & Anagnostidis, 2005, 1989, 1986), taking into account the classification systems proposed by Hoffmann, Komárek, and Kaštovský (2005) and Komárek, Kaštovský, Mareš, and Johansen (2014). In addition, updated literature describing novel taxa and strains was consulted (Obuekwe et al., 2019; Strunecký et al., 2017; Tinpranee, Worananthakij, Incharoensakdi, & Phunpruch, 2018). Microscopic inspections were conducted with a Zeiss Axioskop 40 light microscope equipped with an Axio Vision LE 4.6 digital imaging system (Carl Zeiss Jena, Germany).

Molecular and phylogenetic analyses using 16S rRNA gene sequences

Total genomic DNA was extracted from cyanobacterial strains using a modified CTAB-based extraction method adapted for cyanobacteria (Fiore, Moon, Tsai, Lee, & Trevors, 2000). The 16S + 16S-23S ITS rRNA gene region was PCR-amplified using the primer set 27F1 (Neilan et al., 1997) and 23S30R (Taton et al., 2003), as described previously (Genuário, Corrêa, Komárek, Fiore, 2013). The amplicons were cloned into a pGEM-T Easy Vector System (Promega, Madison), according to the manufacturer's manual. Ultra-competent *Escherichia coli* DH5a cells were transformed and recombinant plasmids were purified from white colonies using the UltraClean Standard Mini Plasmid Prep Kit (MoBio). Plasmids containing the fragments were sequenced using the M13F/R primer

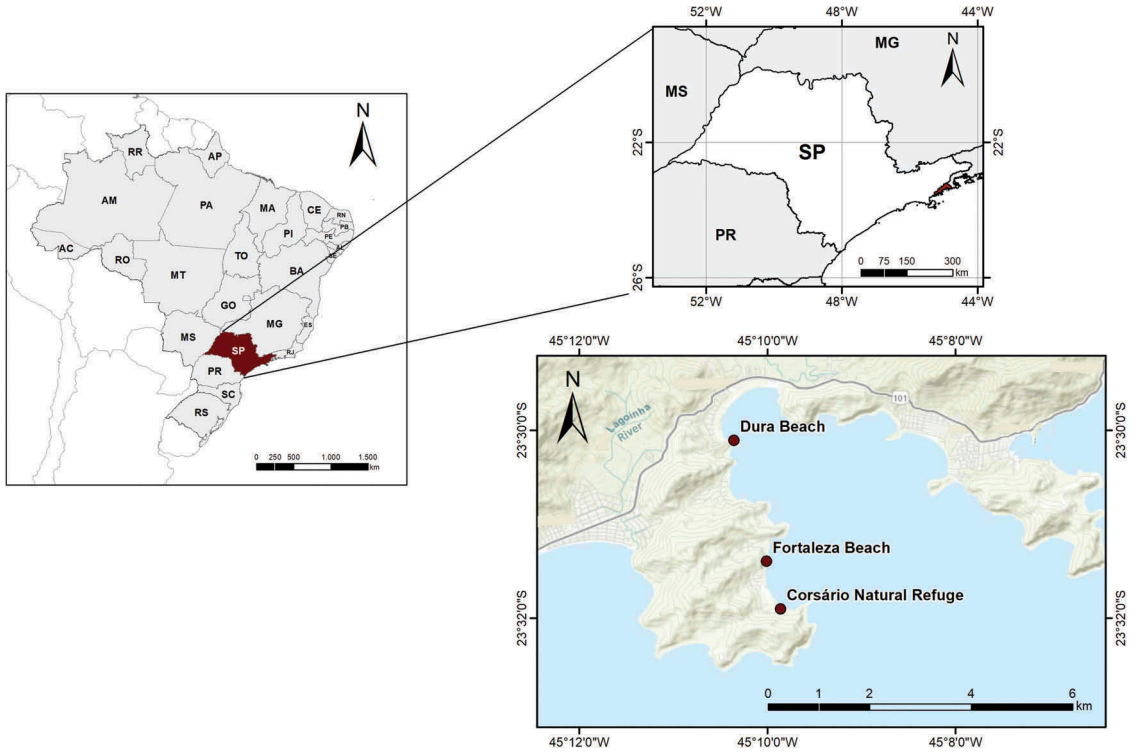

Figure 1. Sampling sites located at Ubatuba municipality, coast of São Paulo state, Brazil. Left side: Map of Brazil (São Paulo state is highlighted in red). Right side (top): Map of São Paulo state. Right side (bottom): The sampling sites for cyanobacterial isolation (Dura beach, Fortaleza beach and Corsário Natural Refuge).

set and the 16S rRNA internal primers 341–357F, 357–341R, 685–704F, 704–685R, 1099–1114F, and 1114–1099R (Lane, 1991), as described by Genuário et al. (2013). The sequenced fragments were assembled using the Phred/Phrap/Consed software (Philip Green, University of Washington, Seattle, USA) and only bases with >20 quality were considered.

The 16S rRNA gene sequences obtained in this study and related ones retrieved from GenBank were aligned using *CLUSTAL W* from the MEGA version 5 software package (Tamura et al., 2011) and trimmed (16S rRNA gene matrix with a 1,470 bp length). A total of 148 sequences were considered and used to infer their phylogenetic position on the basis of the maximum likelihood (ML) method included in the MEGA version 5.0. The Kimura two-parameter model of sequence evolution with gamma-distributed evolutionary rates and with an estimated proportion of invariable sites (K2+G+I) was selected as the best fitting model, applying the model-testing function of MEGA version 5. Statistical confidence of the inferred evolutionary relationships was assessed by bootstrapping (1,000 replicates). The novel nucleotide sequences were deposited in the NCBI GenBank database under the accession numbers MF084980-MF084984.

Biomass production, extraction and chromatographic analyses of natural products

Among the isolated cyanobacterial strains, CENA552 and CENA556 were selected for natural product prospection due to their yield in terms of biomass production. The two strains were respectively grown in SWBG-11 and ASN-III media, supplemented with cyanocobalamin (10 $\mu g \cdot L^{-1}$) for 180 days at 25°C, under a 14:10 h light:dark cycle with white fluorescent irradiation of 40 $\mu mole \cdot m^{-2} \cdot s^{-1}$. Every four weeks, the biomass was centrifuged and re-inoculated into a fresh medium. At the end of the cultivation period, about 30 L of culture medium was filtered and the biomass obtained was used for the next steps.

The filtered biomasses were extracted twice with dichloromethane/methanol–CH_2Cl_2/MeOH (2:1), and once with pure ethyl acetate (EtOAc). The three crude extracts were combined and dried under vacuum, yielding extracts of 10.5 and 13.4 g for the CENA552 and CENA556 strains, respectively. The extracts were subjected to liquid-liquid partition using 100 mL of a methanol/water – MeOH/H_2O (95:5) mixture – which was then extracted three times with hexane (50 mL each time). In this way, hexane fractions of 6.6 and 10.1 g as well as MeOH/H_2O (95:5) fractions of 3.2 and 3.3 g were generated for the CENA552 and CENA556 strains, respectively. The hydro-methanolic part was analysed

for the isolation of more polar compounds and the hexane partition was injected into a GC-MS instrument for the identification of non-polar compounds.

The chemical profile of the MeOH/H_2O (95:5) fractions was determined by HPLC-DAD using a C8 (250 × 4.6 mm – Shimadzu Shim-pack) analytical column at room temperature, according to the acetonitrile (CH_3CN) gradient in water: 0 min – 2% CH_3CN; 40 min – 100% CH_3CN; 50 min – 100% CH_3CN; 55 min – 2% CH_3CN; and 60 min – 2% CH_3CN, at a flow of 1 mL·min^{-1}.

Chemical isolation and characterization of compounds

In order to isolate its major constituents, the polar fraction (MeOH/H_2O 95:5) of CENA556 was selected due to the yield in terms of biomass for this strain. The fraction was subjected to reversed-phase HPLC-PDA (LC-6AD, SPD-M10A, Shimadzu, Kyoto, Japan) using a semi-preparative C8 column (250 × 20 mm, Shimadzu Shim-pack). Gradient step elution of acetonitrile in water from 2 to 8% for 45 minutes at a flow rate of 10 mL·min^{-1} was implemented and resulted in seven major compounds, including five nucleosides and two amino acids. Proposed natural products (NPs) were characterized by NMR (^{1}H, COSY^{1}H^{1}H, HSQC and HMBC) and high-resolution mass (ESI-TOF) analyses. NMR experiments were conducted using a Bruker Avance 500 MHz spectrophotometer for ^{1}H NMR and 125 MHz for ^{13}C NMR. All NMR data were recorded in CD_3OD. Mass spectral data were determined using a ZQ 2000 mass spectrometer (Waters, Milford, Massachusetts, USA).

Gas chromatography-mass spectrometry conditions

The fractions obtained from the CENA552 and CENA556 strains were analysed in order to compare their volatile compound profile. Each extract was re-suspended to a final concentration of 1.0 mg·mL^{-1} for further analysis. The samples were evaluated using a gas chromatograph coupled to a mass spectrometer (GC-MS; Shimadzu), with an EN5MS (30 m × 0.25 mm × 0.25 µm, SGE Analytical Science) column, equipped with a split injector heated to 270°C. The carrier gas used was helium (99.999%) at a flow rate of 1.20 mL·min^{-1}; the injection volume was 1 µL and injector split ratio was 1:40. The oven temperature program was initiated at 100°C and held for 3.0 min before being increased to 220°C and then to 310°C at time points 10.0 and 20.0 min, respectively. The mass spectrometer was set to observe *m/z* 40–600 in positive EI mode, with electron impact at 70 eV and the ion source temperature of 280°C. For compound identification, only EIMS similarity scores of ≥90% library matches (Wiley 7 lib, FFNSC1.3 lib and Nist 08 lib) were considered. Additionally, KI values were calculated by evaluating an external standard set of *n*-alkanes (C_8-C_{36}) under the same conditions and with the same column, and compared to literature values for the identified molecules.

Results

Characterization of cyanobacterial strains

Five cyanobacterial strains were isolated from periphyton attached to rocks collected along the seashore of Corsário Natural Refuge, Ubatuba municipality, São Paulo state, Brazil (Fig 1). According to morphological examination, CENA552 and CENA556 belonged to the order Oscillatoriales, family Coleofasciculaceae *sensu* Komárek et al. (2014) and genus *Geitlerinema;* while CENA553, CENA554 and CENA555 were assigned to the order Synechococcales, family Leptolyngbyaceae *sensu* Komárek et al. (2014) and genus *Leptolyngbya* (Figs 2–10).

Almost complete 16S rRNA gene sequences (1,411–1,412 bp) were obtained for all novel marine cyanobacterial strains. BLAST analysis (Table 1) showed identities ranging from 93.2% to 99.7% with sequences retrieved from uncultured and cultured cyanobacterial strains belonging to the genera *Geitlerinema, Amazoninema* and *Leptolyngbya*. According to the phylogenetic tree (Fig 11), the novel sequences from Brazilian marine strains fell into two different clusters (C-I and C-II). Two of these new sequences, from CENA552 and CENA556, grouped together (64% bootstrap support) and were included in a cluster containing sequences of uncultured bacteria and *Geitlerinema* morphotypes with 96% of bootstrap support (cluster C-I). Cluster C-I was sister group (92% bootstrap support) of two clades formed mainly by strains identified as *Geitlerinema* spp. The remaining sequences, obtained from CENA553, CENA554 and CENA555, grouped together with 88% of bootstrap support (cluster C-II) and were placed in a part of the tree including uncultured cyanobacteria, *Geitlerinema, Halomicronema* and *Amazoninema* sequences, even if without any statistical support (Fig 11).

Chemical isolation and characterization of compounds

A total of seven major compounds including five nucleosides and two amino acids were isolated and/or identified from the polar fraction (MeOH/H_2O 95:5) of the organic extract obtained for the CENA556 strain (Fig 12). The nucleosides were identified as uridine (Fig 12, **1**) (13.0 mg) (Duan et al., 2011; Ishikawa, Kondo, & Kitajima, 2003; Li, Deng, Li, Fu, & Lin, 2004; Yalçin et al., 2003); 2′-deoxyuridine (**2**) (14.0 mg) (Abou-Hussein, Badr, & Youssef, 2007; Li et al., 2004; Youssef, Badr, Shaala,

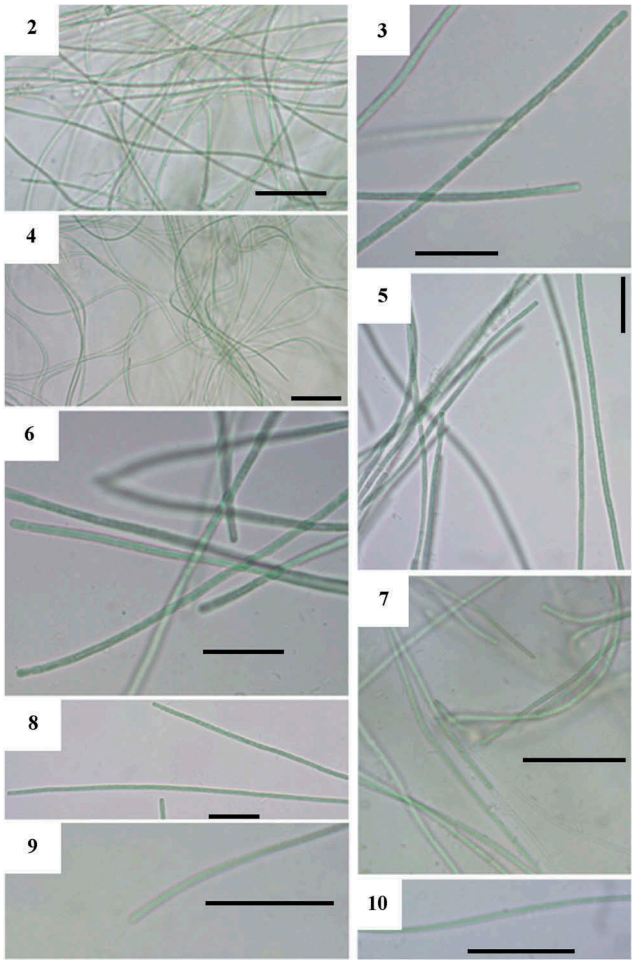

Figure 2–10. Photomicrographs of the isolated cyanobacterial strains. *Leptolyngbya*-like: CENA553 (2 and 9); CENA555 (4 and 10); CENA554 (7). *Geitlerinema*-like: CENA556 (3 and 6); CENA552 (5 and 8). Scale Bars: 2, 3, 6, 9 and 10 = 20 µm; 4, 5, 7 and 8 = 10 µm.

Mohamed, & Bamanie, 2015) in a mix with *L*-phenylalanine; thymidine (**3**) (6.0 mg) (Liu, Ye, Qiang, Liao, & Zhao, 2008; Sgarrella et al., 2007; Youssef et al., 2015); adenosine (**4**) (9.0 mg) (Ciuffreda, Casati, & Manzocohi, 2007; Ishikawa et al., 2003; Jamison, Boddy, & Molinski, 2014; Wang et al., 2009); and 2′-deoxyadenosine (**5**) (8.0 mg) (Ciuffreda et al., 2007; Sgarrella et al., 2007). The amino acids were identified as *L*-phenylalanine (**6**) (7.0 mg) (Mitchell, Bao, Benz, & Zhang, 2009; Tian, Yin, Sun, & Luo, 2002; Treweeke, Hitchcock, Pardoe, & Caddick, 2005), and *D*-leucine (**7**) (22.0 mg) (Menahem & Mastai, 2006), instead of *L*-leucine, the more usual isomer.

GC-MS analyses

The extracts obtained from the CENA552 and CENA556 strains were evaluated by applying a GC-MS-based approach. The majority of the compounds found belong to the classes of alcohol, aldehyde, ester and aliphatic hydrocarbons. The complete identification of the compounds is listed in Table 2.

Discussion

Cyanobacterial characterization

Initially, based solely on morphological features, the novel cyanobacterial strains were identified as belonging to the genera *Geitlerinema* (CENA552 and CENA 556) and *Leptolyngbya* (CENA553, CENA554 and CENA555) (Figs 2–10). Morphologically, members of *Geitlerinema* differ from those of *Leptolyngbya* by very active gliding motility and lack of sheath envelopes (Anagnostidis, 1989; Castenholz, 2001).

However, according to the phylogeny based on 16S rRNA gene (Fig 11), the novel sequences did not group into clusters including the type species of the above mentioned genera (Fig 11 – *Geitlerinema sensu stricto* and *Leptolyngbya sensu stricto*). The sequences retrieved from the novel marine *Leptolyngbya*-like strains (CENA553, CENA554 and CENA555 – Cluster C-II) also clustered apart from *Nodosilinea* (Perkerson et al., 2011), *Alkalinema* and *Pantanalinema* (Vaz et al., 2015), *Oculatella* (Zammit, Billi, & Albertano, 2012), *Plectolyngbya* (Taton, Wilmotte, Šmarda, Elster, & Komárek, 2011) and *Amazoninema* (Genuário, De Souza, Monteiro, Sant´Anna, & Melo, 2018), genera erected for morphotypes resembling *Leptolyngbya*. In addition, these novel sequences did not exhibit any phylogenetic relationship with *Leptolyngbya*-like sequences recovered from Brazilian Atlantic coastal areas (Silva et al., 2014). The same pattern was observed for the sequences taken from the novel *Geitlerinema*-like strains (CENA552 and CENA556 – Cluster C-I), which were not placed within the typical *Geitlerinema* cluster or within the *Anagnostidinema* group.

Nevertheless, considering the Bergey Manual of Systematic Bacteriology (Castenholz, 2001), the novel 16S RNA sequences retrieved from *Geitlerinema*-like strains (cluster C-I) were associated with the sequence of the reference strain *Geitlerinema* sp. PCC7105 (Fig 11), which has been recognized as the reference strain/sequence for the "marine *Geitlerinema*" lineage, and not for the entire genus *Geitlerinema*. Therefore, the CENA552 and CENA556 strains must be considered novel members within this phylogenetic lineage (marine lineage of *Geitlerinema*-like strains), taking into account both the high similarity (>99.6%) with *Geitlerinema* sp. PCC7105 (Table 1) and the strong phylogenetic support of cluster C-I (96% bootstrap)

Table 1. Sequence identity (%) of 16S rRNA gene among the isolated strains and other cyanobacterial strains available in GenBank.

Strain	Length (bp)	Closest match in GenBank (Accession number)	C* (%)	I** (%)
Geitlerinema sp. CENA552	1412	Uncultured bacterium Pb-1y-7 (FN794239)	100	99.7
		Uncultured bacterium CpH7-1 (FN794262)	100	99.6
		Uncultured bacterium Pb1y-46 (FN794241)	100	99.6
		Geitlerinema sp. PCC7105 (AF132780)	99	99.7
		Uncultured bacterium CpH7-9 (FN794263)	100	99.5
		Uncultured bacterium CpH7-49 (FN794267)	100	99.5
		Uncultured bacterium CpH4-3 (FN794257)	100	99.4
		Uncultured bacterium Pb1y-86 (FN794247)	100	99.4
		Geitlerinema sp. A28DM (FJ410907)	98	99.8
		Geitlerinema sp. BBD_P2b-1	100	97.5
Leptolyngbya sp. CENA553	1411	Uncultured Cyanobacterium Alchichica AL33_1_1B_32 (JN825331)	100	95.9
		Uncultured cyanobacterium Alchichica AL52_2_1B_11 (JN825330)	95	95.7
		Uncultured bacterium Dstr_B02 (GU118152)	99	93.9
		Uncultured bacterium SGUS386 (FJ202307)	100	93.6
		Geitlerinema sp. PCC7407 (CP003591)	100	93.6
		Amazoninema brasiliense CMAA1609 (MF002133)	100	93.5
		Amazoninema brasiliense CMAA1608 (MF002132)	100	93.5
		Amazoninema brasiliense CMAA1602 (MF002130)	100	93.5
		Uncultured bacterium SGUS1412 (FJ202607)	100	93.5
		Uncultured Cyanobacterium Alchichica AQ1_1_1B_17 (JN825332)	100	93.3
Leptolyngbya sp. CENA554	1412	Uncultured Cyanobacterium Alchichica AL33_1_1B_32 (JN825331)	100	95.7
		Uncultured cyanobacterium Alchichica AL52_2_1B_11 (JN825330)	95	95.4
		Uncultured bacterium Dstr_B02 (GU118152)	99	93.6
		Geitlerinema sp. PCC7407 (CP003591)	100	93.4
		Uncultured bacterium SGUS386 (FJ202307)	100	93.3
		Amazoninema brasiliense CMAA1609 (MF002133)	100	93.3
		Amazoninema brasiliense CMAA1608 (MF002132)	100	93.2
		Uncultured bacterium SGUS1412 (FJ202607)	100	93.2
		Amazoninema brasiliense CMAA1602 (MF002130)	100	93.2
		Uncultured Cyanobacterium Alchichica AQ1_1_1B_17 (JN825332)	100	93.2
Leptolyngbya sp. CENA555	1412	Uncultured Cyanobacterium Alchichica AL33_1_1B_32 (JN825331)	100	95.8
		Uncultured cyanobacterium Alchichica AL52_2_1B_11 (JN825330)	95	95.5
		Uncultured bacterium Dstr_B02 (GU118152)	99	93.7
		Geitlerinema sp. PCC7407 (CP003591)	100	93.4
		Amazoninema brasiliense CMAA1609 (MF002133)	100	93.3
		Amazoninema brasiliense CMAA1608 (MF002132)	100	93.2
		Uncultured bacterium SGUS1412 (FJ202607)	100	93.2
		Amazoninema brasiliense CMAA1602 (MF002130)	100	93.3
		Amazoninema brasiliense CMAA1603 (MF002131)	100	93.2
		Leptolyngbya frigida ANT.L53B.2 (AY493576)	100	93.2
Geitlerinema sp. CENA556	1412	Uncultured bacterium Pb-1y-7 (FN794239)	100	99.6
		Uncultured bacterium CpH7-1 (FN794262)	100	99.5
		Uncultured bacterium Pb1y-46 (FN794241)	100	99.5
		Geitlerinema sp. PCC7105 (AF132780)	99	99.6
		Uncultured bacterium CpH7-9 (FN794263)	100	99.5
		Uncultured bacterium CpH7-49 (FN794267)	100	99.4
		Uncultured bacterium CpH4-3 (FN794257)	100	99.3
		Uncultured bacterium Pb1y-86 (FN794247)	100	99.3
		Geitlerinema sp. A28DM (FJ410907)	98	99.7
		Geitlerinema sp. BBD_P2b-1	100	99.2

[a]Published sequences; *Coverage, ** Identity

(Fig 11). Yet, the origin from marine environments is another ecological feature shared by the novel strains and *Geitlerinema* sp. PCC7105. It is also important to mention that the emergence of a "marine *Geitlerinema* group" has been previously discussed, suggesting the possible erection of a new generic unit apart from *Geitlerinema sensu stricto* (Strunecký et al., 2017; Tinpranee et al., 2018).

The CENA553, CENA554 and CENA555 strains may represent a new generic unit considering the low similarity observed with *Geitlerinema* sp. PCC7407 (<93.6%) and *Amazoninema* spp. (<93.5%) (Table 1), and the weak association with them in the phylogenetic reconstruction (Fig 11). Likewise, their clustering with uncultured cyanobacterial clones from the Mexican Alchichica alkaline lake reinforces their novelty to science as an isolated culture. Therefore, these novel strains could be a source of undiscovered natural products since the genetic variability regarding their 16S rRNA sequences may also reflect dissimilarities in their genome content.

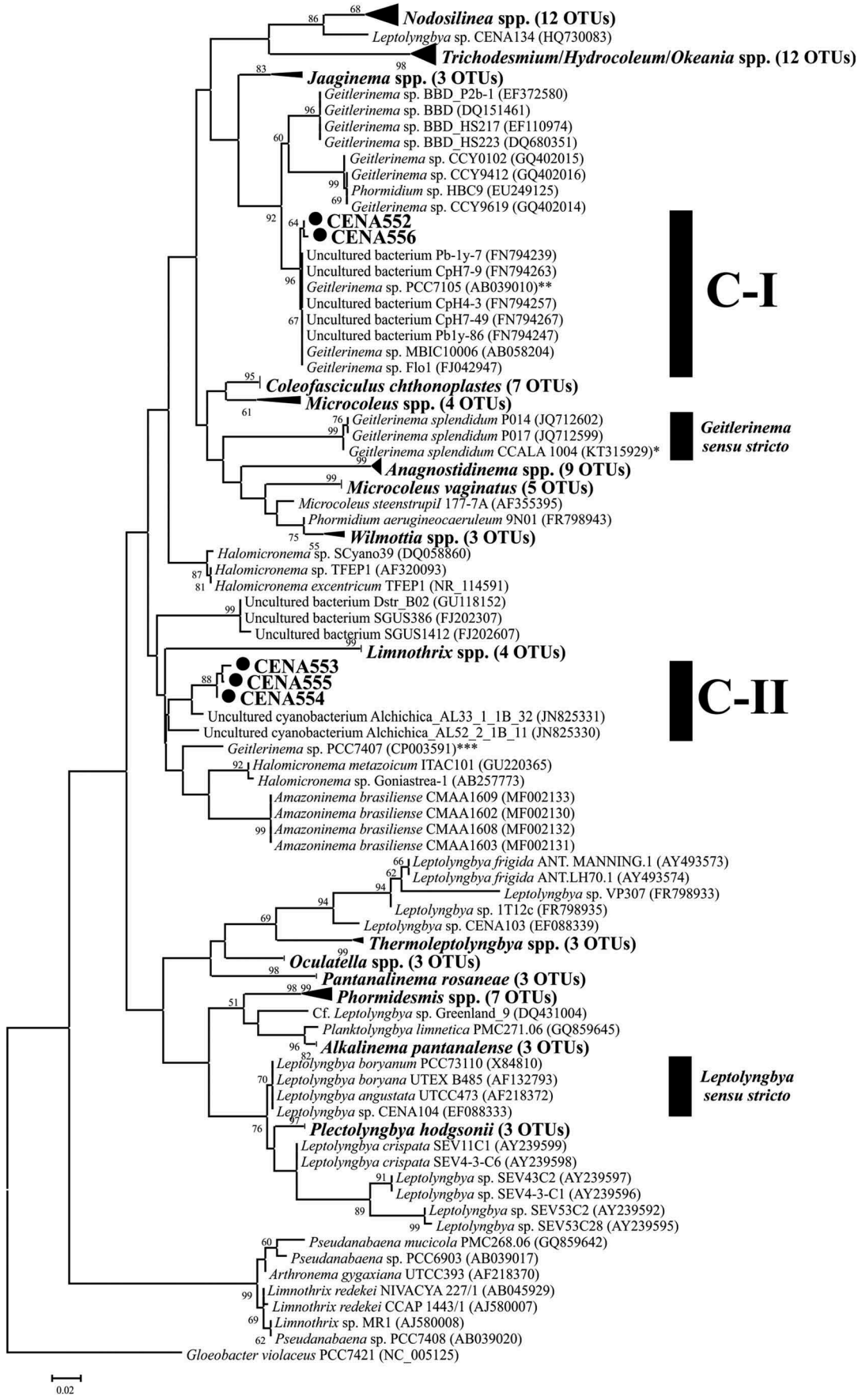

Figure 11. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences of filamentous homocytous strains. The sequences retrieved from the novel strains isolated in this study are shown in bold with a black circle. A bootstrap test involving 1,000 resamplings was performed and bootstrap values greater than 50% are given in front of the relevant nodes. (*) Epitype and reference strain/sequence (*Geitlerinema*) according to Strunecký et al., 2017; (**) and (***) Reference strains according to Bergey's Manual of Systematic Bacteriology. The strain PCC7105 (**) corresponds to the "marine *Geitlerinema*". OTU: Operational taxonomic unit(s).

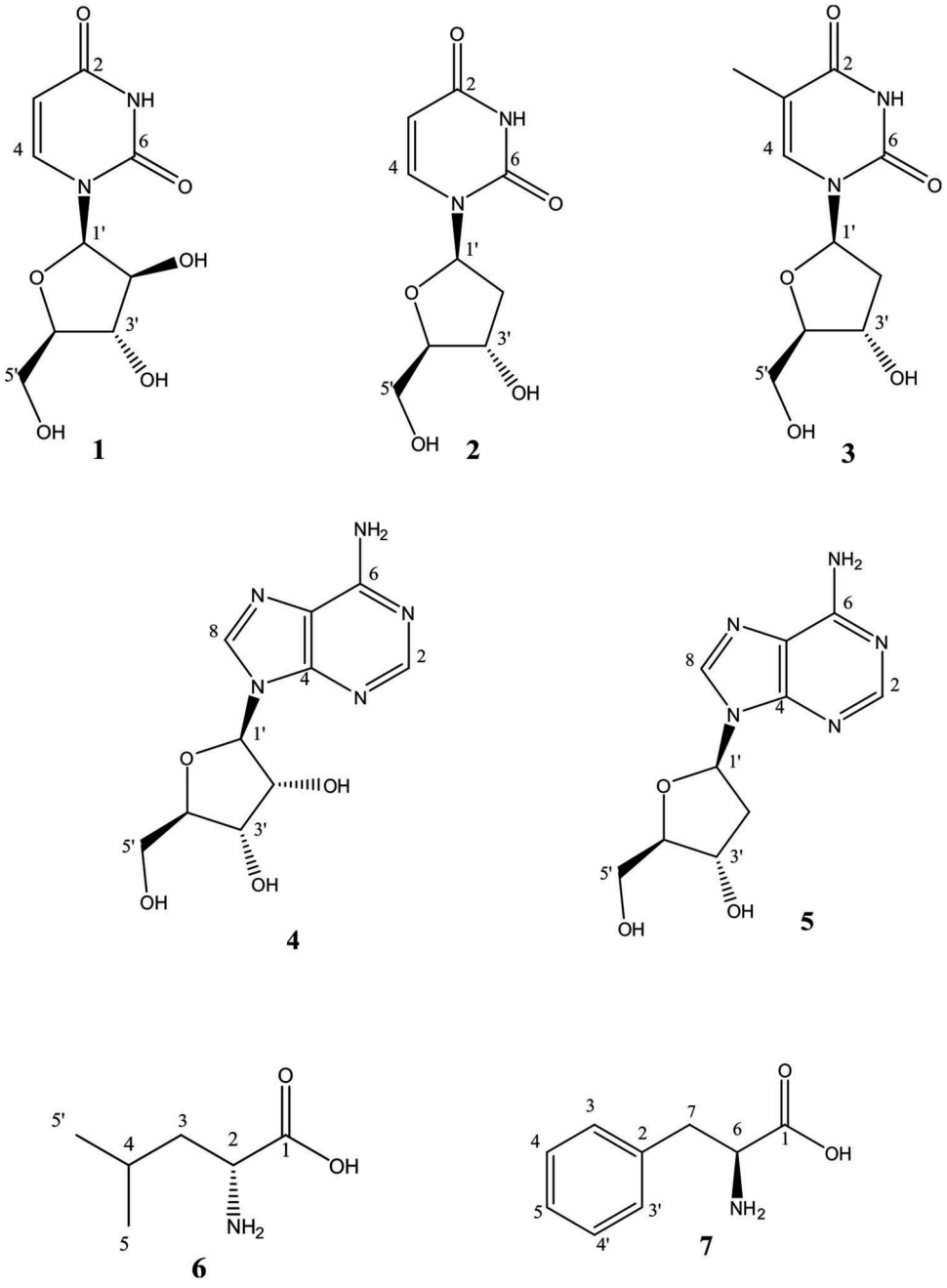

Figure 12. Nucleosides and amino acids isolated from the cyanobacterial strain CENA556 (*Geitlerinema*-like). The nucleosides are: uridine (1), 2′-deoxyuridine (2), thymidine (3), adenosine (4), and 2′-deoxyadenosine (5). The amino acids are: *D*-leucine (6) and *L*-phenylalanine (7).

Over the last few years, phylogenetic studies on tropical marine *Lyngbya* morphotypes have demonstrated their polyphyletic condition based on 16S rRNA gene sequences. Consequently, the genera *Moorea* and *Okenia* were described, comprising species able to produce unique and structurally different secondary metabolites (Engene, Cameron Coates, & Gerwick, 2010; Engene et al., 2013, 2012). Similarly, a revision of the marine cyanobacteria *Phormidium penicillatum* (*Symploca hydnoides*) resulted in the description of the genus *Caldora* (Engene et al., 2015). These authors showed that the production of dolastatin 10 and/or the related compound symplostatin 1 is a common feature among *C. penicillata* strains, and useful as a chemotaxonomic marker (Engene et al., 2015).

Accordingly, Myers, Sekar, and Richardson (2007), examining the cyanobacterial diversity present in the black band disease (BBD) of corals, isolated *Geitlerinema* and *Leptolyngbya* morphotypes and detected their 16S rRNA sequences in DGGE. This study, conducted using culture and uncultured techniques, speculated that toxin

Table 2. Compounds identified by GC-MS in fractions generated from the studied cyanobacterial strains.

Compound	Class	RI[a] (exp.)	RI[a] (lit.)	S[#] (%)	Peak Area (%) HG552[c]	Peak Area (%) HG556[d]
2-hexyl-1-decanol	Alcohol	1702	-	96	15.7	-
Dodecanal	Aldehyde	1409[d]	1409[e]	97[d]	-	2.8
Hexadecanal (palmitaldehyde)		1800	1808[f]	91	-	3.8
Methyl (*Z*)-9-hexadecenoate (methyl palmitolate)	Ester	1897 [d]	1901[e]	96[d]	-	2.2
Methyl hexadecanoate (methyl palmitate)		1925[c] 1921[d]	1925[L§]	97	4.3	2.0
Ethyl hexadecanoate		1986	1993[e]	95	-	3.7
Pentadecane	Aliphatic hydrocarbons	1512	1512[L§]	98	-	4.5
Octadecane		1810	1804[g]	98	-	24.5
3-Octadecene		1807	1818[h]	96	2.5	-
2,6,10-Trimethyl, 14-ethylene-14-pentadecene (Neophyta diene)		1838[c] 1839[d]	1839[e]	95[c,d]	7.5	21.2

[a] RI: Retention Index; S[#]: Similarity with databank; [L§]: Identified by MS spectra and comparison with a computer databank (Library: Wiley 7 lib, FFNSC 1.3 lib and Nist 08 lib); HG552[c]: hexane fraction of *Geitlerinema*-like CENA552; HG556[d]: hexane fraction of *Geitlerinema-like* CENA556; [e]: (de Oliveira et al., 2009); [f]: (de Felício et al., 2010);[g]: (Sun et al., 2012); [h]: (Milovanović et al., 2015).

production mediated by these marine morphotypes might be connected to BBD in corals (Myers et al., 2007). Yet, the production of bioactive exometabolites has been reported from the marine *Geitlerinema* sp. So-11 (Caicedo, Kumirska, Neumann, Stolte, & Thöming, 2012). Additionally, the production of water-soluble toxins was confirmed for the freshwater *Geitlerinema splendidum* CCIBt3223, analysed in a toxicological and histopathological study (Rangel et al., 2014). These studies indicated the potential of different ecological morphotypes of *Geitlerinema* to produce a wide range of substances with diverse actions.

Chemical analyses

In the present study, relevant information concerning natural nucleosides is reported for the very first time for marine cyanobacterial strains isolated from Brazilian coastal areas. These results confirm previously reported data which mention the cytotoxic and fungicidal nucleosides tubercidin, toyocamycin, as well as their derivatives 5′-α-D-glucopyranosides isolated from cyanobacteria (Stewart et al., 1988). It is important to mention that marine sponges have been the best source of nucleosides in free form, which are involved in vital components of all living cells and in several biological processes (Huang et al., 2014). Bergmann and Stempien (1957) suggested that nucleosides are products of the autolysis of more complex units such as nucleotides and nucleic acids. These authors also associated the presence of unusual nucleosides in the sponge *Cryptotethya* with its very low RNA content, differently from what was observed for species of algae investigated. Another important factor to be considered regarding the presence of nucleosides in free form concerns the growth conditions. It has been observed that certain algae of the same species collected in distant regions had different nucleoside contents (Duan et al., 2011).

The CENA556 strain (*Geitlerinema*-like) proved to be a new prolific source of nucleosides due to the diversity and reasonable quantities obtained of these glycosylamines. Marine nucleosides have been targeted as new "lead compounds" for drug design, particularly in the area of viral and parasitic infections and cancer. Several analogues of bioactive marine nucleosides have been synthesized and evaluated for biological activities (Huang et al., 2014). Studies regarding the chemical composition of cyanobacteria from high mountain habitats have led to the quantification of large amounts of primary metabolites such as nucleosides, amino acids and nucleic bases (Hartmann, Albert, & Ganzera, 2015). The cited study also reported an increased synthesis of these compounds after cyanobacterial exposure to UV radiation (Hartmann et al., 2015). Three of the compounds detected by these authors match those found in our study: thymine, adenosine and phenylalanine. Therefore, we could suggest that the substances produced by CENA556 have been produced as major constituents for the same reason, since this strain was isolated from rocks in a coastal area, a natural environment directly exposed to sunlight. More importantly, this natural potential seems to be stable under laboratory conditions, given the culture conditions applied to cyanobacterial cultivation.

The synthesis of pyomelanin, which belongs to the allomelanins, a category of melanin, requires large amounts of the amino acids tyrosine and phenylalanine and this compound is recognized as a potent agent against oxidative stress in bacteria (Hartmann et al., 2015; Schmaler-Ripcke et al., 2009; Turick, Knox, Becnel, Ekechukwu, & Millike, 2010). Furthermore, adenosine has been used in cosmetology as an anti-wrinkle agent (Jeon et al., 2015), which could be

associated with the antioxidant properties of nucleosides. In addition, the rare amino acid *D*-leucine can be used as a precursor of different compounds by binding to other structures and to produce standards or opening the possibility to synthesize new substances (Choi, Mevers, Byrum, Valeriote, & Gerwick, 2012). As a consequence, the CENA556 strain is a potential target for antioxidant and UV-protection use and should be considered as a promising source of these primary polar metabolite class of bioactive compounds.

Different classes of compounds were identified by GC-MS analysis, including alcohol, aldehydes, esters and aliphatic hydrocarbons, as well as other unidentified compounds (Table 2). The major constituents observed in the present study were octadecane (24.5%) and neophytadiene (21.2%) from the CENA556 strain, followed by 2-hexyl-1-decanol (15.7%) from CENA552. The prevalent classes in CENA552 are the alkenes, whereas those prevailing in CENA556 are aldehydes, ester and alkanes. The unknown compounds of the fractions obtained from the CENA552 and CENA556 strains corresponded to a total of 35.3 and 70%, respectively.

The primary alcohol 2-hexyl-1-decanol, observed in the CENA552 strain, was the major compound (15.7%). Fatty alcohols are component of biofuels, and their production by genetically engineered cyanobacteria has been already described (Singh et al., 2017). The potential for genetic manipulation coupled with a high-producing strain shed light on the use of cyanobacteria as important sources of biofuel production (Machado & Atsumi, 2012).

The aldehyde group was represented by hexadecanal and dodecanal, which were found in the CENA556 strain. Dodecanal was also recently observed in the marine Brazilian cyanobacterium *Cyanobium* sp. CENA181 (Armstrong et al., 2019) isolated from a mangrove (Silva et al., 2014). It has been reported that metabolic engineering can be used to convert a single intermediate derived from lipid biosynthesis into a variety of biofuel precursors including alkanes, free fatty acids and wax esters (Kaiser et al., 2013). It is worth mentioning that the cyanobacterial aldehyde-deformylating oxygenase is a key enzyme involved in the conversion of fatty-aldehydes into alkanes, highlighting their application for biofuel production (Schirmer, Rude, Li, Popova, & Del Cardayre, 2010; Zargar et al., 2017; Zhang, Lu, & Li, 2013).

The ester class showed a relatively low abundance (Table 2), and was represented by methyl-(*Z*)-9-hexadecenoate (methyl-palmitoleate), methyl palmitate, ethyl hexadecanoate, and *cis*-9,10-epoxy stearic acid methyl ester. Methyl palmitate was produced by both *Geitlerinema*-like strains (CENA552 and CENA556), and has been previously shown to have anti-inflammatory, antioxidant and anti-fibrotic activities (El-Demerdash, 2011; Sharawy, El-Agamy, Shalaby, & Ammar, 2013). This compound was also previously reported to be present in *Nostoc muscorum* and *Oscillatoria* sp. (Abdel-Hafez, Abo-Elyousr, & Abdel-Rahim, 2015). The Methyl-palmitoleate was also recently detected in the cyanobacterial strain *Cyanobium* sp. CENA178 (Armstrong et al., 2019), which was also isolated from the coastal area of São Paulo state. Thus, some chemical similarity can be observed for distinct species isolated from this coastal area.

Considering the alkenes, the major compound was the diterpenoid neophytadiene, which was found in both *Geitlerinema*-like strains. Some reports have described the presence of neophytadiene in cultures of the freshwater species *Aphanizomenon ovalisporum* and *Cylindrospermopsis raciborskii* (Ríos et al., 2014). In addition, branched chain hydrocarbons have been described in filamentous cyanobacteria, which can provide a promising and innovative strategy for the development of biofuels with advanced fuel properties and solar-driven energy input (Liu, Zhu, Lu, & Song, 2013).

Conclusions

Morphotypes resembling the genera *Leptolyngbya* and *Geitlerinema* were isolated from periphytic samples collected along the Brazilian seashore. These new strains correspond to phylogenetic lineages differing from the typical and reference clusters of the above-mentioned genera. *Geitlerinema*-like strains are known to be able to produce several substances with important biological activities such as cytotoxic, antiviral and cardiovascular function regulator properties. Here, it was observed that the *Geitlerinema*-like strains were able to produce compounds of distinct classes, such as nucleosides, amino acids, hydrocarbons, aldehydes, alcohols, and esters, among others. Some important substances such as methyl palmitate, neophytadiene, hexadecanal and dodecanal plus those mentioned here have been previously reported in the literature. Many compounds identified in the present study had all been previously characterized as important resources for pharmaceutical purposes, providing the basis for antimicrobial, cytotoxic, and antioxidant agents as well as biofuels. Other unidentified metabolites did not match the databases after calculating the Kovats retention index, demonstrating the potential for discovery of novel compounds. The results presented here also contribute new chemical information about the underexplored diversity of cultivable cyanobacterial strains from the Atlantic coast of Brazil, and further stimulate new biological and biotechnological studies.

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Disclosure statement

The authors declare that they have no conflict of interest.

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